Comprehensive Bioactive Natural Products

Vol 1 Potential & Challenges

V K Gupta

Studium Press LLC

Comprehensive
Bioactive Natural Products

Volume 1
Potential & Challenges

V.K. GUPTA

Indian Institute of Integrative Medicine
(Council of Scientific & Industrial Research)
Canal Road, Jammu (J&K State)- 180 001, India

2010

Studium Press LLC, U.S.A.

"This page is Intentionally Left Blank"

Comprehensive
Bioactive Natural Products

Vol. 1: Potential & Challenges

ISBN : 1-933699-51-5
SERIES ISBN : 1–933699–50–7

Published by:

STUDIUM PRESS, LLC
P.O. Box-722200, Houston, Texas-77072, USA
Tel. 713-541-9400; Fax:713-541-9401
E-mail: studiumpress@studiumpress.com

Printed at:

Thomson Press (India) Ltd.

"This page is Intentionally Left Blank"

Comprehensive
Bioactive Natural Products:
(*Multi-Volume Set*)

Series Editor* : *V.K. Gupta
E-mail: vgupta_rrl@yahoo.com; vguptaiiim@gmail.com

Volumes Published (2010)

Vol. 1* : *Potential & Challenges
Ed. V.K. Gupta

Vol. 2* : *Efficacy, Safety & Clinical Evaluation I
Ed. V.K. Gupta

Vol. 3* : *Efficacy, Safety & Clinical Evaluation II
Ed. V.K. Gupta

Vol. 4* : *Antioxidants & Nutraceuticals
Eds. V.K. Gupta & Anil K. Verma

Vol. 5* : *Immune-modulation & Vaccine Adjuvants
Ed. V.K. Gupta

Vol. 6* : *Extraction, Isolation & Characterization
Eds. V.K. Gupta, S.C. Taneja & B.D. Gupta

Vol. 7* : *Structural Modifications & Drug Development
Eds. V.K. Gupta, S.C. Taneja & B.D. Gupta

Vol. 8* : *Quality Control & Standardization
Eds. V.K. Gupta, S.C. Taneja & B.D. Gupta

MEMBERS

Dr. A. Panossian: Swedish Herbal Institute Research and Development, Kovlingevagen 21, Vallberga, SE-312 50, Sweden;
E-mail: alexander.panossian@shi.se

Prof. Yu Zhao: Department of TCM & Natural Drug Research, College of Pharmaceutical Sciences, Room 513, Zhejiang University, Zijingang Campus, 388 Yuhangtang Rd., Hangzhou 310058, China;
Email: dryuzhao@zju.edu.cn; dryuzhao@126.com

Prof. A. Evidente: Department of Organic Chemistry, Dipartimento di Scienze del Suolo, della Pianta, dell'Ambiente e delle Produzioni Animali, Università di Napoli Federico II, Portici, Italy;
E-mail: evidente@unina.it

Prof. Mirian Salvador: Instituto de Biotecnologia, Universidade de Caxias do Sul, Rua Francisco Getúlio Vargas, 1130, CEP 95070-560, Caxias do Sul, Rio Grande do Sul, Brazil;
E-mail: msalvado@ucs.br

SERIES ISBN : 1–933699–50–7

About the Series

Nature abounds with a rich potential heritage of bioactive natural products as that have been exploited for effective and beneficial use either as a therapeutic resource against a human disease or as a chemopreventive, antioxidant, nutraceutical or diet supplement. A natural product is a substance or a chemical compound produced by a living organism found in nature that usually has a pharmacological or biological activity. Some of these agents are derived from terrestrial plants, whereas, others are obtained from microorganisms, marine or freshwater organisms and even the higher animals.

Past few decades have witnessed an unprecedented worldwide growth in the interest of bioactive natural products based medicines, both in developed and developing countries, and offer a resource-pool for lead identification in drug discovery owing to the reason that isolation of a novel molecule is easier than *de novo* synthesis, especially when the molecular structure of the compound is very complex. On account of such advantages, pharmaceutical companies are opting for bioactive natural products based drugs. More than 20 new drugs, launched world over between 2000 and 2005, originate from natural products. Scrutiny of medical indications by source of compounds has demonstrated that natural products and related drugs are used to treat 87% of all categorized human diseases (infectious and non-infectious). The development of new bioassay techniques, biotechnology methods, screening and high performance analytical methods have introduced new concepts and possibilities of rational drug design and drug discovery.

Interest in bioactive natural products research is growing more strongly and can be attributed to several factors, including remarkable diversity of natural products, unmet therapeutic needs, the development of novel and sensitive techniques to detect, isolate, purify and structurally characterize these active constituents. This has opened opportunities for multidisciplinary research that joins the forces of natural product chemistry, molecular and cellular biology, synthetic and analytical chemistry, bio-chemistry, bio-technology, general biology, and pharmacology to exploit the vast diversity of bioactive natural products.

Taking cognizance of these facts, we have initiated efforts in editing the present series "**Comprehensive Bioactive Natural Products**" and brought eight volumes (1 to 8) providing edited information from over 139 original research and review articles by eminent scientists and researchers

from India and abroad, representing 40 countries of the world on wide range of topics in the area of natural products categorized under the themes *viz.*,

1. *Potential & Challenges*
2. *Efficacy, Safety & Clinical Evaluation I*
3. *Efficacy, Safety & Clinical Evaluation II*
4. *Antioxidants & Nutraceuticals*
5. *Immune-modulation & Vaccine Adjuvants*
6. *Extraction, Isolation & Characterization*
7. *Structural Modifications & Drug Development*
8. *Quality Control & Standardization*

These volumes critically evaluate the present state-of-art, current status and future prospects in a well-illustrated manner. It is hoped that this series, which reflects the contributor's research results as well as world wide reviews will be widely read and used by all interested in these fields and shall open new vistas of research and academic pursuits for those engaged in the fields of bioactive natural products.

I would like to take this opportunity to thank each of the authors who have contributed in the preparation of this book series.

Jammu, India

V.K. Gupta
Series Editor

AMITY INSTITUTE FOR HERBAL
AND BIOTECH PRODUCTS DEVELOPMENT

- An Institution of Ritnand Balved Education Foundation - Thiruvananthapuram

Prof. (Dr.) P. PUSHPANGADAN,
M.Sc. M.Phil. Ph.D., FBRS FES. FNRS, FNSE, FNESA, FNAASc, FNASc.,
(UN Equator Initiative Laureate)
Director General & Senior Vice President, RBEF
(Former Director, NBRI, Lucknow)

08-06-2009

Foreword to the Series

Medicine among the ancient civilizations was a blend of Religion and Science. Natural products played an important role throughout the world in the prevention and treatment of diseases. These natural products originated from varied sources such as plants, animals, micro-organisms, marine organisms, terrestrial vertebrates and invertebrates. In man's quest for discovering new and potent remedies for diseases, he keenly observed his immediate neighbourhood and experimented not only with organic substances but also with in-organic materials such as metals, minerals, ores etc. Over the years, the accumulated medical wisdom was compiled into excellent treatises and these have later developed into the various classical systems of medicine associated with the ancient civilizations and cultures of the world. Apart from the classical systems of medicines, which by its nature and practice had universal applications, there existed another stream of medicine in the countryside, among the less privileged class of people, among the forest dwellers and among the nomadic tribes which were location specific, confined to small communities, tribes and families. These medical systems were mostly perpetuated by oral traditions. Nevertheless, the value of the oral traditions cannot be overlooked as these localized knowledge systems are veritable treasures of invaluable information on the properties and uses of hundreds of plant, animal and mineral products.

Ethno medical information is now increasingly being utilized in the search for novel bioactive molecules from natural products. Several well known plants such as Licorice (***Glycyrrhiza glabra***) myrrh (***Commiphora sps***) and poppy capsule latex (***Papaver somniferum***) were mentioned in the clay tablets dating back to 2600 B.C, obtained from Mesopotamia. These plants and their derivatives are still used in various forms as herbal drugs as well as pure isolates in different systems of medicine. For instance,

codeine, papaverine, morphine etc isolated from ***P. somniferum*** are still used in modern medicine. Similarly hemisuccinate carbenoxolone sodium, a semi synthetic derivative of glycyrrhetic acid isolated from licorice is used for the treatment of gastric and duodenal ulcers. Plants, especially those with ethno medical history have been the major source of bio active molecules. According to a study by Fabricant and Famsworth (2001), out of 122 plant derived drugs, 80% had their origin from plants with ethno medical use. Since secondary metabolites from plants are products of biosynthetic reactions occurring in living systems, bioactive molecules from natural sources are excellent drug candidates as they are perceived to show more biological friendliness than synthetic drugs.

The ever increasing popularity and acceptance of natural products have evinced an extra ordinary interest and scope in the subject in recent years. Compendium of Bioactive Natural Products- a series of eight books edited by Dr. V.K. Gupta (Volumes 1,2,3 and 5) and Dr. V.K. Gupta, Dr. S.C. Taneja, Dr. B.D. Gupta and Dr. A.K.Verma (Volumes 4,6,7 and 8), published by M/S. Studium press LLC, U.S.A are an excellent compilation, giving comprehensive and reliable data on the current development in the field of bioactive natural products. This series contain 139 original research and review articles contributed by a galaxy of eminent scientists and academicians from 40 countries around the world. The editors have taken care to invite eminent scholars in the relevant areas to contribute each chapter in the series. The series deals with wide ranging topics related to natural products such as extraction, isolation and characterization, structural modifications and drug development, quality control and standardization and, immune modulation and vaccine adjuvants, antioxidants and nutraceuticals, efficacy, safety and clinical evaluation (Parts-1 and 2) and potentials and challenges. The editors of this series Dr. V.K. Gupta, Dr. S.C. Taneja, Dr. B.D. Gupta and Dr. Anil K. Verma are known to me for more than three decades and they are all stalwarts in natural products and related areas.

It is gratifying to know that this series is dedicated to the memory of Col. Sir Ram Nath Chopra, the Founder Director of Regional Research Laboratory, now known as Indian Institute of Integrative Medicine (IIIM), Jammu.

Col. Sir R.N. Chopra (1882-1973) was a pioneer of systematic studies of indigenous drugs, promoter of Indian Systems of Medicine and Patron of Pharmacy in India. Most of the medicinal plants, he studied were in use, in Ayurveda for thousands of years. The major fields of Cot. Chopra's research were general pharmacology, chemotherapy, indigenous drugs, drug addiction and drug assays. At the Calcutta school of Tropical Medicine, he developed a pattern of research in studying the action of medicinal plants which influenced an entire generation of pharmacologists. Pharmacists and physiologists in India. In the words of Dr. G.V. Satyawati, Former Director General of Indian Council of Medical Research, the credit of kindling the interest of Indian Chemists and Pharmacologists in Medicinal Plants should rightfully go to

Sir Ram Nath Chopra who, has been claimed as the Father of Indian Pharmacology'.

I had the privilege and good fortune of meeting Col, Sir. Ram Nath Chopra along with Prof. Dr. E.K. Janaky Ammal at his residence in 1969 immediately after my joining Regional Research Laboratory as a Scientific Assistant. My meeting with Col. Sir R.N. Chopra had left a lasting impression of a great scientist in my memory.

It is a fitting tribute to Col. Sir. R.N. Chopra that the editors have decided to dedicate these eight volumes to his memory. I congratulate the editors for bringing out this series which is timely, relevant and important to all those who are engaged in the study of natural products and development of" therapeutically useful bioactive molecules. I am sanguine that these volumes will receive wider acceptance among the scientific community, especially among those engaged in Natural Product research.

(P. Pushpangadan)

About the Editor

Dr. Vijay Kumar Gupta, Ph.D., FLS, London (born 1953-) Deputy Director & Head, Animal House, Indian Institute of Integrative Medicine (CSIR), Jammu, India. He did his M.Sc. (1975) and Ph.D. (1979) in Zoology both from University of Jammu, Jammu-India. His research capabilities are substantiated by his excellent work on histopathology, ecology and reproductive biology of fishes, turtles, birds and mammals, which has already got recognition in India and abroad. Dr. Gupta has to his credit more than 75 scientific publications and review articles which have appeared in internationally recognized Indian and foreign journals. Founder fellow, life member and office bearer of many national societies, academies and associations. He has successfully completed a number of research/consultancy projects funded by various governments, private and multinational agencies. His current areas of interest are histopathology, toxicology, pre-clinical safety pharmacology and reproductive efficacy studies of laboratory animals. He is also Editor-in-chief of the books 1) Utilisation and Management of Medicinal Plants 2) Medicinal Plants: Phytochemistry, Pharmacology and Therapeutics 3) Perspectives in Animal Ecology and Reproduction (Vols.1-6). The Editor-in-chief of the American Biographical Institute, USA, has appointed him as *Consulting Editor* of *The Contemporary Who's Who*. Dr. Gupta also appointed as Nominee for the *Committee for the Purpose of Control and Supervision of Experiments on Animals* (CPCSEA, Govt. of India). Recently the *Linnaean Society of London, U.K.* has awarded fellowship to him in November 2009 in recognition of his contribution towards the cultivation of knowledge in Science of Natural History.

Preface

Ancient civilization greatly depended on local flora and fauna for their survival and experimented with various berries, roots, leaves, minerals or animal parts to find out what effects they had and as a result, many crude drugs were observed by the local healer to have some medical use. It is a well established fact now that many of these aqueous, ethanolic, distilled, condensed or dried extracts do indeed have a real and beneficial effect and a study of ethnobotany can give us clues as to which plant might be worth studying in more detail. Nature produces a large number of chemical compounds whose structure and properties are of great interest and fascination for the majority. These are organic compounds formed by living system and are emerging from the cocoon of organic chemistry. The term "natural products" is applied to materials derived from plants, microorganisms, invertebrates and vertebrates, which are the fine biochemical factories for biosynthesis of these metabolites and over 40% of medicines, have origin from these compounds.

Nature abounds with a rich potential heritage of these therapeutics resources that have been exploited for effective and beneficial uses against many human diseases, either in prevention strategy or as therapeutics armamentaria. The vast majority of them have been the plant-derived extracts. This has resulted in an inherited pool of information of the healing potential of the plant species, thus making them important source of starting material for drug discovery and development. A different set of metabolites is usually produced in the different anatomical parts of the plant and botanical knowledge is crucial also for correct taxonomical determination of the identified bioactive plant. The selection of starting material may be done by collecting knowledge on use of plants and other natural products as herbal medicines and thereby get an idea of potential biological activities.

Plants especially, provide a large bank of rich, complex and highly varied structures which are likely to be synthesized in laboratories. Furthermore, evolution has already carried out a screening process itself whereby plants are more likely to survive if they contain potent compounds which deter the predators and even today, the number of plants that have been extensively studied is relatively very few and vast majority have not been studied at all. Likewise animals both terrestrial as well as aquatic do have wealth of biologically potent chemicals for instance, venoms and toxins which are extremely potent because of their target specific features. As a result, they have proved to be the important tools, in studying receptors, ion channels and enzymes, and many of these toxins are extremely potent and have been used as lead compounds in the development of novel drugs. The neurotoxins can prevent cholinergic transmission and could well prove a lead for

development of novel anti-cholinergic drug. It can be a challenging task to obtain information from practitioners of traditional medicine under a genuine long term relationship is made. The investigation of the chemistry of terrestrial and marine organisms has also led to a host of novel bioactive compounds many of which now find use in medicine. Such materials offer exciting challenges to the scientists and a variety of brilliant approaches and strategies are still needed in this regard.

Despite the great successes already achieved, we have barely begun to tap the potential of our molecular diversity and just 5-15% of the 2,50,000 species of higher terrestrial plants in existence have been pharmacologically investigated in systematic fashion. The percentage of insects, marine organisms and microbes investigated is far lower still. In the case of microbes, it is estimated that 95 to 99% of existing species are currently not even known, never mind analyzed.

Even with all the challenges facing drug discovery, natural products isolated from natural sources can be predicted to remain an essential component in the research for new medicines. It was with this approach that this volume entitled, "**Comprehensive Bioactive Natural Products – *Potential & Challenges***" has been compiled. Some of the interesting studies included in the volume are: Biological activity of peptides derived from marine organisms; Bioactive natural products as anti-staphylococcal infections; Pharmacological activities, phytochemical investigations and *in vitro* studies of *Gymnema sylvester* R.Br: - a historical review; Andirobas of the state of Acre (Brazil): chemical and biological aspects associated with use and management; Natural bioresource of the North Himalayan region as a source of promising radiation countermeasure agents: lessons from *Podophyllum hexandrum*; Red ginseng as a potential anti-obesity agent; Carotenoids in commercially important crustaceans from Indian waters; Therapeutic uses of venoms; Potentials of bryophytes for biotechnological use; Review: molecular biology approach to viper venoms; Bee-pollen therapeutical value; Herbal products in healthcare: challenges and potential; Anticancer properties of plant-derived food, phytochemicals and plant–expressed recombinant pharmaceuticals; Global medicinal plants with anti-HIV activities; New fungal metabolites, as antifungal, herbicides and insecticides for biocontrol of agrarian plant pests; On the potential of some natural colouring agents: an overview.

The editor express his gratefulness to the contributors who have shared valuable thoughts through their scholarly contributions and timely submission of manuscripts for this volume and also thanks to the publisher, Studium Press, LLC, Texas, USA and their staff for timely and expeditious job rendering the manuscript press-ready.

Jammu, India **V.K.Gupta**

ISBN : 1-933699-51-5

Table of Contents

1

Biological Activity of Peptides Derived from Marine Organisms

SE-KWON KIM[1,2*] AND Y. DOMINIC RAVICHANDRAN[1,3]

ABSTRACT

A variety of novel and active metabolites containing simple linear peptides and complex macrocyclic polymers have been found from marine organisms. Thus great interest has been taken in the structure, composition and sequential properties of bioactive peptides. Peptides derived from marine organisms reveal a variety of biological activities such as antioxidative, antihypertensive, anticancer, anticoagulant, immune-stimulatory, anti-inflammatory and antimicrobial. Recently marine peptides have created a new perspective for pharmaceutical developments. Hence, marine peptides may be a new choice in the development of lead compounds for biomedical research. This review describes the recent and novel information regarding activities of peptides derived from marine organisms.

Key words : Peptides, marine organisms, biological activities

INTRODUCTION

Marine source has been reported as the largest reservoir of biologically active molecules and marine organisms have been a rich source of structurally novel and biologically active metabolites. Currently, there is a very big competition between pharmaceutical companies to develop drugs

1. Marine Bioprocess Research Center, Pukyong National University, Busan - 608 737, Republic of Korea.
2. Department of Chemistry, Pukyong National University, Busan - 608 737, Republic of Korea.
3. Department of Science and Humanities, Sri Krishna College of Engineering and Technology, Coimbatore - 641 008, India.
* *Corresponding author* : E-mail : sknkim@pknu.ac.kr

derived from marine organism in order to obtain large profits (De Vries & Beart, 1995). Many unique, structurally novel and biologically active metabolites have been found from marine organisms because of their living in a very different, competitive and aggressive surrounding from that of the terrestrial environment (Aneiros & Garateix, 2004). Up to now, some of them are under investigation in pre-clinical or clinical trials to be as new pharmaceuticals (Rawat *et al.*, 2006; Faulkner, 2000a; Faulkner, 2000b; Da Rocha *et al.*, 2001; Schwartsmann *et al.*, 2001). Among them, peptides from marine organisms have received attention in recent years.

Recently marine peptides have created a new perspective for pharmaceutical developments (Aneiros & Garateix, 2004). The discovery of various peptides from marine organisms and understanding of the molecular mechanisms have increased our knowledge about peptides with specific actions such as antioxidant, antimicrobial, anticancer, anti Alzheimer's, etc. (Kim & Mendis, 2006; Subramanian *et al.*, 1998). Hence, marine peptides are a new choice in the development of lead compounds for biomedical research.

ANTIOXIDANT ACTIVITY

Antioxidant is a substance that delays or inhibits oxidation of substrate. An antioxidant can act at different levels in an oxidation sequence such as preventive antioxidants or chain breaking antioxidants. These compounds play an important role as a health-protecting component. Most of the research suggests that antioxidants reduce the risk of chronic diseases including cancer, heart disease and Alzheimer's (Subramanian *et al.*, 1998). Even though plants serve as a main source of antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens, the marine organisms have attracted special interest for their potential use in drug and value added food production.

Reactive oxygen species (ROS) are oxygen containing free radicals (which contain an unpaired electron) or non-radical molecules (H_2O_2) with reactive chemical properties. Formation of free radicals such as superoxide anion and hydroxyl radical is unavoidable in aerobic organisms during respiration. These radicals are very unstable and react rapidly with other groups or substances in the body, leading to cell or tissue injury. These species are constantly generated through a variety of pathways in biological systems including both enzyme-catalyzed reactions and non-enzyme reactions (Pelicano *et al.*, 2004). Furthermore these species have been reported to play a major role in many diseases such as cancer (Bae *et al.*, 1995; Leanderson *et al.*, 1997; Muramatsu *et al.*, 1995), gastric ulcers (Debashis *et al.*, 1997; Sussman & Bulkley, 1990), Alzheimer's, arthritis and ischemic reperfusion (Vajragupta *et al.*, 2000).

Lipid peroxidation occurring in food products is one of the main reasons for the deterioration of food quality during processing and storage. Consuming oxidative foods is thought to cause serious diseases such as hepatomegaly or necrosis of epithelial tissues (Poling & Rice, 1962). The lipid peroxides and compounds with low molecular weight produced during the later stage of the oxidative reaction are the factors involved in these diseases (Kanazawa *et al.*, 1985). The secondary breakdown product of lipid peroxides such as 4-hydroxynonenal (4-HNE) can react with proteins, phospholipids and nucleic acids to produce modifications and cross-linking of proteins or peptide molecules (Uchida & Stadman, 1992). Hence it is very important to inhibit lipid peroxidation occurring in foodstuffs and thereby provide protection against serious diseases (Halliwell *et al.*, 1995).

Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroxyquinone (TBHQ) and *n*-propyl gallate (PG) exhibit strong antioxidant activity against several oxidation systems. However, these synthetic antioxidants are suspected to pose potential risks and their use in foodstuffs is discouraged. Therefore the search for safer antioxidants from natural sources is of great interest and marine organisms serve as one of the major source for many natural antioxidants. Recently, many peptides from marine organisms have been reported to exhibit antioxidant activity (Qian *et al.*, 2008; Je *et al.*, 2007; Kim *et al.*, 2007; Jung *et al.*, 2007; Qian *et al.*, 2007a; Je *et al.*, 2005; Mendis *et al.*, 2005; Rajapakse *et al.*, 2005; Jeon & Kim, 2002; Kim *et al.*, 2001). The mechanisms of antioxidative action of peptides include radical scavenging, adduct formation, substrate shielding, metal chelating and enzyme inhibition.

The antioxidant peptides could be purified by combining membrane filtration and consecutive chromatography (Kim *et al.*, 2001 and Guerard *et al.*, 2005). The peptides isolated from the skin of various fishes have been tested for their antioxidant activity in number of studies. Two peptides with strong antioxidative activity were isolated from Alaska Pollack skin with 13 and 16 amino acid residues respectively and both peptides contained a glycine residue at the C-terminus and the repeating motif of Gly-Pro-Hyp. Among these, the peptide with 16 amino acid with the sequence of Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly was more effective in inhibiting the formation of TBARS in linolenic acid compared to the other peptide with 13 amino acid with the sequence of Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly (Kim *et al.*, 2001).

Peptides Leu-Glu-Glu-Leu-Glu-Glu-Glu-Leu-Glu-Gly-Cys-Glu (1487 Da) from the skin protein of bullfrog *Rana catesbeiana* Shaw (Qian *et al.*, 2008), His-Gly-Pro-Leu-Gly-Pro-Leu (797 Da) from tryptic hydrolysate of the Hoki *Johnius belengerii* skin gelatine (Mendis *et al.*, 2005a), Phe-

Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu (880.18 Da) and Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg (1241.59 Da) from tryptic hydrolysate of jumbo squid *Dosidicus gigas* skin gelatine (Mendis *et al.*, 2005b) have been isolated and were found to exhibit strong inhibition against lipid peroxidation much higher than that of natural antioxidant, α-tocopherol. These peptides were also found to scavenge DPPH, hydroxyl, superoxide and peroxyl radicals. The hydrophobic amino acids present in the peptide sequences was attributed to the observed antioxidant activity.

Marine animals and their processing by-products are rich in protein. Hydrolysis of protein leads to the generation of protein hydrolysates, which have been found to show inhibitory effect on lipid oxidation. Capelin protein hydrolysate inhibited the formation of thio-barbituric acid reactive substances (TBARS) by 17.7–60.4% (Shahidi *et al.*, 1995). Mackerel, *Scomber austriasicus* hydrolysates were found to possess different antioxidant activities including the inhibition of linoleic acid autoxidation, the scavenging effect on α,α-diphenyl-β-picrylhydrazyl free radical, and the reducing power. It was also found that the peptide with molecular weight of approximately 1400 Da possessed stronger *in vitro* antioxidant activity than that with smaller molecular weight (Wu *et al.*, 2003).

Peptides Leu-Lys-Gln-Glu-Leu-Glu-Asp-Leu-Leu-Glu-Lys-Gln-Glu (1.60 kDa) from Oyster, (*Crassostrea gias*) protein hydrolysate (Qian *et al.*, 2007a) and Leu-Gly-Leu-Asn-Gly-Asp-Asp-Val-Asn (928 Da) from conger eel protein hydrolysates (Ranathunga *et al.*, 2005), were isolated and these peptides were found to show higher activity against polyunsaturated fatty acids (PUFA) peroxidation than that of native antioxidant, α-tocopherol and the free radical scavenging activity of hydroxyl and superoxide radicals. Moreover, it was found that these peptides could scavenge the cellular radicals and protect the DNA from the damage caused by the free radicals. The peptic hydrolysate of the backbone protein of tuna fish (a fish processing waste) was purified using consecutive chromatographic methods to yield an antioxidant peptide Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Gln-Gln-Leu-Ser (1519 Da). This antioxidant peptide significantly inhibited lipid peroxidation in linoleic acid emulsion system and also quenched free radicals (DPPH, hydroxyl and superoxide) in a dose-dependent manner (Je *et al.*, 2007b).

The fish frames and cut offs that result from mechanically deboned fish contain considerable amounts of muscle proteins. These muscle proteins are considered to be rich in protein with well-balanced amino acid composition (Venugopal *et al.*, 1996). Protein hydrolysates from these by-products have been analyzed for their antioxidant activity. Peptides Leu-Pro-His-Ser-Gly-Tyr (672 Da) from the Alaska pollack frame protein (Je *et al.*, 2005b) and Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn (1801 Da) from hoki frame protein (Kim *et al.*, 2007) have

been isolated and were found to show inhibitory effect on lipid oxidation like that of α-tocopherol. Furthermore these peptides were also observed to exhibit radical scavenging activity.

Lipid oxidation is a natural metabolic process under normal conditions. PUFA, the main components of membrane lipids are susceptible to oxidation. Lipid peroxide (LP) is one of the most investigated consequences of ROS action on the membrane structure and function. Peptides Leu-Val-Gly-Asp-Glu-Gln-Ala-Val-Pro-Ala-Val-Cys-Val-Pro (1.59 kDa) from *Mytilus coruscus* muscle protein using an *in vitro* gastrointestinal digestion system (Jung *et al.*, 2007), Leu-Glu-Gln-Gln-Val-Asp-Asp-Leu-Glu-Gly-Ser-Leu-Glu-Gln-Glu-Lys-Lys (1.99 kDa) from bullfrog muscle protein (Je *et al.*, 2007a), Asn-Ala-Asp-Phe-Gly-Leu-Asn-Gly-Leu-Glu-Gly-Leu-Ala (1307 Da) and Asn-Gly-Leu-Glu-Gly-Leu-Lys (747 Da) from giant squid *Dosidicus gigas* muscle protein by ultrafiltration (Rajapakse *et al.*, 2005c) have been isolated and these peptides exhibited higher protective activity against PUFA peroxidation than the native antioxidants, ascorbic acid and α-tocopherol. In addition these peptides were found to show free radical scavenging activity and to protect DNA from the damage caused by hydroxyl radical dose-dependently.

The muscle of the prawn *Penaeus japonicus* was hydrolyzed by various proteases, and antioxidant activity of the hydrolysates was examined. Among the digests, pepsin digest was found to have the most potent antioxidant activity and three peptides Ile-Lys-Lys, Phe-Lys-Lys, and Phe-Ile-Lys-Lys were isolated and purified by chromatography from the active peptidic fraction (Suetsuna, 2000).

According to Rajapakse *et al.* (2005a) peptide with the amino acid sequence His-Phe-Gly-Asx-Pro-Phe-His (962 kDa) was derived from fermented marine blue mussel *Mytilus edulis*. They could scavenge superoxide (98%), hydroxyl (96%), carbon-centered (91%) and DPPH radicals (72%) at 200 μg/ml concentration. In addition, they exhibited a strong inhibition to lipid peroxidation at 54 μM concentration and 95% higher Fe^{2+} chelating effect when compared with α-tocopherol and citrate respectively.

The peptide Ser-Asn-Pro-Glu-Trp-Ser-Trp-Asn (1 kDa) from the enzyme hydrolysate of cord teiset protein was also found to show antioxidant activity 10% higher than that of α-tocopherol (Kim *et al.*, 2000).

ANTIHYPERTENSIVE ACTIVITY

Hypertension is a risk factor for cardiovascular disease and stroke. The consumption of higher doses of antihypertensive peptides has shown to considerably reduce the blood pressure of moderately hypertensive subjects in divergence with antihypertensive drugs. The bioactive peptides with

hormone or drug-like activity eventually modulate physiological function through binding interactions to specific receptors on target cells leading to induction of physiological responses (FitzGerald & Murray, 2006). Among these bioactive peptides, angiotensin converting enzyme (ACE)-inhibitory peptides have been extensively studied because of their ability to control hypertension (Fandino *et al.*, 2006). ACE plays an important physiological role in regulating blood pressure (Skeggs *et al.*, 1957). It is a class of zinc proteases located in the vascular endothelial lining of the lungs and acts as an exopeptidase that cleaves dipeptides from the C-terminus of various oligopeptides (Curtiss *et al.*, 1978; Yang *et al.*, 1971). It also converts an inactive form of the decapeptide, angiotensin I, to the octapeptide angiotensin II, a potent vasoconstrictor, and inactivates bradykinin, which has a depressor action. The search for ACE inhibitory activity is the most common strategy followed in the selection of antihypertensive hydrolysates and/or peptides. The classical approaches involve the *in vitro* determination of the ACE inhibitory activity of protein hydrolysates, followed by the separation, purification, identification of peptide structures and to confirm their activity.

Recently, utilization of fish by-products for the production of high value bioactive hydrolysates/peptides has risen rapidly. Peptides derived from fish skin were found to show (ACE) inhibitory activity. Two peptides composed of Gly-Pro-Leu and Gly-Pro-Met have been isolated from the gelatin extract of Alaska Pollack *Theragra chalcogramma* skin with high ACE inhibitory activity with the IC_{50} values 2.6 μM and 17.13 μM respectively (Byun & Kim, 2001).

The peptides derived from fish muscle were also reported as antihypertensive agents. The peptides Gly-Ala-Ala-Glu-Leu-Pro-Cys-Ser-Ala-Asp-Trp-Trp (1.3 kDa) from the aclase hydrolysate of *R. catesbeiana* muscle (Qian *et al.*, 2007b), Trp-Pro-Glu-Ala-Ala-Glu-Leu-Met-Met-Glu-Val-Asp-Pro (1.581 kDa) from the pepsin hydrolysate of *Thunnus obesus* dark muscle (Qian *et al.*, 2007c), Met-Ile-Phe-Pro-Gly-Ala-Gly-Gly-Pro-Glu-Leu (1.3 kDa) from the frame protein hydrolysates of Yellow fin sole *Limanda aspera* (Jung *et al.*, 2005), Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala from the frame protein hydrolysates of Alaska Pollack *Theragra chalcogramma* (Je *et al.*, 2004) were isolated and found to act as a non-competitive inhibitors against ACE I with IC_{50} values of 0.95 μM, 21.6 μM, 28.7 μM and 14.7 μM respectively. These peptides also exhibited *in vitro* activities by lowering blood pressure in spontaneously hypertensive rats.

The angiotensin I converting enzyme (ACE) inhibitory activity of fermented blue mussel sauce (FBMS) was investigated. According to Je *et al.* (2005a) blue mussels were fermented with 25% NaCl (w/w) at 20 degrees C for 6 months and the resultant mixture was passed through a 40-mesh sieve, desalted using an electrodialyzer and then lyophilized. An ACE inhibitory peptide Glu-Val-Met-Ala-Gly-Asn-Leu-Tyr-Pro-Gly from

FBMS was purified using Sephadex G-75 gel chromatography, SP-Sephadex C-25 ion exchange chromatography and reversed-phase high-performance liquid chromatography on a C-18 column. The IC_{50} value of purified ACE inhibitory peptide was 19.34 μg/ml and further it was also found to exhibit *in vitro* activity by lowering blood pressure in spontaneously hypertensive rats.

Catfish protein hydrolysates were also found to show ACE inhibition and among the isolated peptides, the smallest peptide showed high ACE inhibition activity (Theodore & Kristinsson, 2007). Two ACE inhibitory peptides Met-Ile-Pro-Pro-Tyr-Tyr and Gly-Leu-Arg-Asn-Gly-Ile from cod liver protein with IC_{50} 10.9 μM and 35.0 μM have also been isolated (Choi *et al.*, 2000).

ANTICANCER ACTIVITY

Cancer is the second major cause of death in the world resulting in one out of five deaths. Although important advances have been made in anticancer therapies, unfortunately some forms of cancer are unresponsive or become resistant to conventional treatments. This implies the need to develop novel therapeutic agents with innovative mechanisms of action. Recently, number of marine peptides with promising anticancer activity has been isolated. Didemnin was the first marine peptide that entered in human clinical trials for treatment of cancer, and other anticancer peptides such as aplidine, kahalalide F, dolastatins and hemiasterlin have also entered in the clinical trials.

Didemnins are cyclic depsipeptides isolated from the Caribbean tunicate *Tridemnun solidum*. Among them didemnin B (chemical structure is shown in figure and numbered: (**1**) was found to be the most potent anticancer agent which inhibits the synthesis of RNA and DNA. It also binds noncompetitively to palmitoyl protein thioesterase (Vera & Joullié, 2002) and hence has been selected for clinical development. Didemnin B showed antitumor activity against a variety of tumor cells such as breast, cervical, myeloma, glioblastoma, astrocytoma, lung and ovarian. Even though this compound has exhibited potent activity against different type of cancers, the clinical trials were terminated because of its high toxicity and side effects (Nuijen *et al.*, 2000).

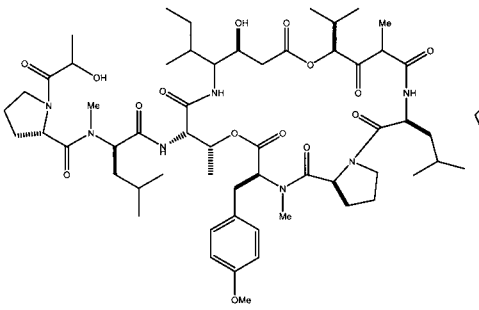
Aplidin (**2**), dehydrodidemnin B is a marine depsipeptide isolated from Mediterranean tunicate, *Aplidium albicans*, shows strong antitumor activity against different human cancer cells growing *in vitro* and *in vivo*. Mechanistic study revealed that it interferes with the synthesis of DNA, proteins and induces G1–G2 cell cycle arrest. Aplidin exhibits cytotoxicity since it inhibits the ornithine decarboxylase, an enzyme that is critical in the process of tumor formation, growth and angiogenesis. It was found that aplidin also inhibits the expression of the vascular endothelial growth factor

gene having antiangiogenic effects. Aplidin was found to be more active than didemnins in the preclinical studies, and exhibited substantial activity against a variety of solid tumor models including tumors noted to be resistant to didemnins. Generally, the treatment with aplidin has been well tolerated and the most common side effects were asthenia, nausea, vomiting, transient transaminitis and hypersensitivity reactions. Interestingly, aplidine does not induce hematological toxicity, mucositis or alopecia. aplidin was found to selectively target and preferentially kill human leukemic cells in blood samples taken from children and adults. Furthermore, the activity of Aplidin was found to be independent of other anticancer drugs commonly used for leukaemia and lymphoma. Recently, aplidin has entered Phase II clinical trials covering renal, head and neck, and medullary thyroid (Rawat *et al.*, 2006).

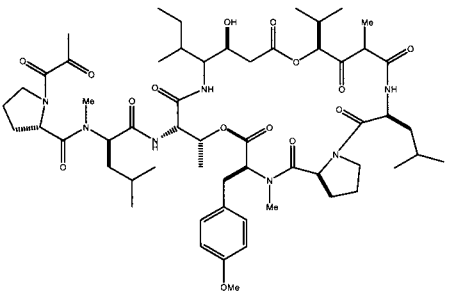
Kahalalide F (**3**) is a depsipeptide isolated from the sacoglossan mollusk *Elysia rufescens* (Hamann & Scheuer, 1993). It exhibits potent cytotoxic activity *in vitro* against various cell lines such as prostate, breast, colon carcinomas, neuroblastoma, chondrosarcoma, and osteosarcoma (Sewell *et al.*, 2005). Kahalalide F alters the function of the lysosomal membranes inducing cell death by oncosis and hence, is currently under Phase II clinical trials for hepatocellular carcinoma, non-small cell lung cancer (NSCLC) and melanoma.

The dolastatins are a group of peptides with unusual amino acids that were isolated from the Indian ocean sea hare *Dolabella auricularia* (Pettit *et al.*, 1987, 1989; Kamano *et al.*, 1989). The potent antitumor agent, dolastatin 10 (**4**), was originally isolated from the sea hare, *Dolabella auricularia*, of the Indian ocean (Pettit *et al.*, 1993) and from the marine cyanobacterium *Symploca* sp. VP642 of Palau (Luesch *et al.*, 2001) which inhibits cell growth in human ovarian and colon-carcinoma cell lines (Aherne *et al.*, 1996). It has also demonstrated *in vitro* and *in vivo* efficacy in the DU-145 human prostate cancer model. Therefore a Phase II clinical trial was designed in patients with hormone-refractory prostate cancer (Vaishampayan *et al.*, 2000).

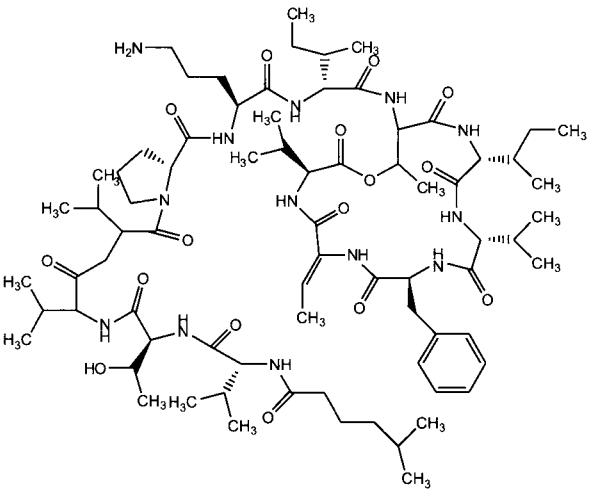
Cytotoxic peptides such as geodiamolides A to F, hemiasterlin and its derivatives A and B were isolated from marine sponges *Hemiasterella minor* (Talpir *et al.*, 1994) and *Cymbastela* (Jimeno, 2002). Hemiasterlin (**5**) and its derivatives A and B were found to interact with tubulin and gives microtubule depolymerisation in an identical manner to that reported for other anticancer compounds such as vinblastine and nocodazole. Hemiasterlin compounds have very unique property as these compounds can overcome the Pglycoprotein mediated chemo-resistance (Krishnamurthy *et al.*, 2003).



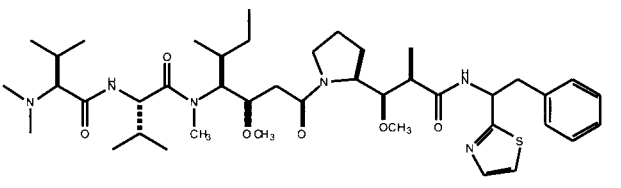
Didemnin B (1)



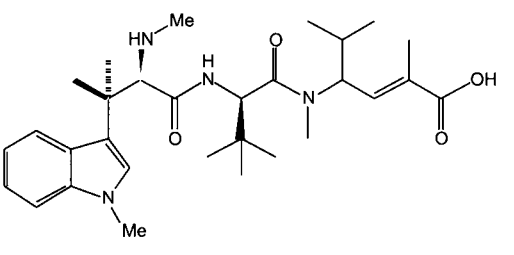
Aplidine (2)



Kahalalide (3)



Dolastatin 10 (4)



Hemiasterlin (5)

Fig 1. Chemical structures of peptides with anticancer activity

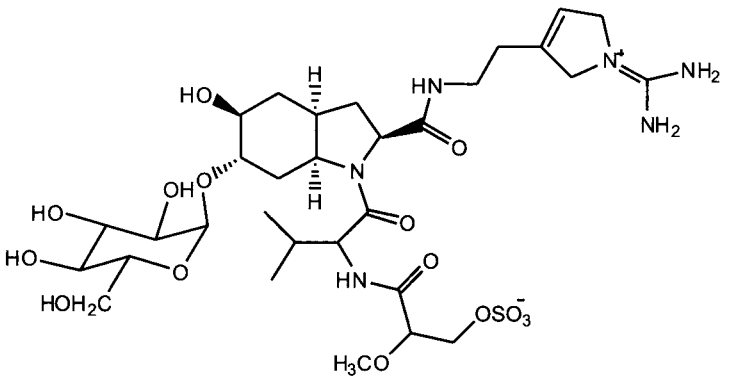
ANTICOAGULANT ACTIVITY

Blood coagulation and platelet-mediated primary haemostasis have evolved as important defence mechanism against bleeding. The coagulation system is triggered in response to rupture of endothelium, which allows exposure of blood to the extra vascular tissue and regulated by the coagulation factors as zymogens and cofactors (Davie *et al.*, 1991). Blood clotting is processed by various coagulation factors in order to stop the blood flow through the injured vessel wall whenever an abnormal vascular condition and exposure to non-endothelial surfaces at sites of vascular injury occurs.

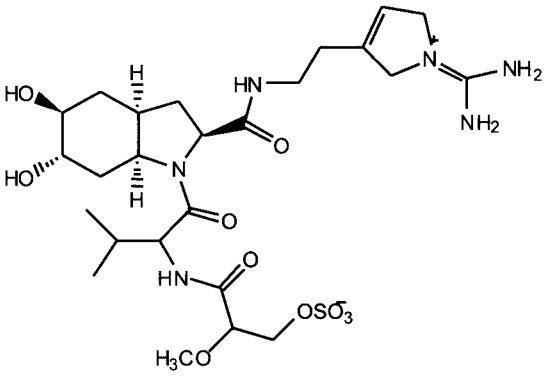
Blood coagulation can be prolonged or stopped with the interference of exogenous or endogenous anticoagulants. These anticoagulants have been used as convenient tools for exploration of the complex mechanisms of coagulation cascade, and coincidentally, an importance of research for anticoagulants has come up with therapeutic purposes like cure of haemophilia (Jung *et al.*, 2002). The importance of anticoagulants as treatments in patients with cardiovascular diseases is highly recognized. Other than the natural anticoagulants found in the coagulation cascade, a number of anticoagulant have also been identified from diverse natural sources (Urata *et al.*, 2003; Tanaka-Azevedo *et al.*, 2003). Recently, peptides from marine organisms have also been identified as natural anticoagulants.

Jung *et al.* (2002) purified an anticoagulant protein from the edible portion of a blood ark shell, *Scapharca broughtonii,* by ammonium sulphate precipitation and column chromatography on DEAE-sephadex A-50, Sephadex G-75, DEAE- shephacel and Biogel P-100, which prolonged the activated partial thromboplastin time (APTT) and inhibited the factor IX in the intrinsic pathway of the blood coagulatin cascade with human plasma. A novel anticoagulant peptide (UAP), Gly-Glu-Leu-Thr-Pro-Glu-Ser-Gly-Pro-Asp-Leu-Phe-Val-His-Phe-Leu-Asp-Gly-Asn-Pro-Ser-Tyr-Ser-Leu-Tyr-Ala-Asp-Ala-Val-Pro-Arg (3344 Da) isolated from the marine echiuroid worm *Urechis unicinctus* (Jo *et al.*, 2008), potently prolonged the activated partial thromboplastin time (APTT), corresponding to inhibition of an endogenous blood coagulation factor in the intrinsic pathway. It was also observed that the addition of UAP significantly decreased FIXa activity in normal plasma with IC_{50} 42.6 mg/ml, indicating that UAP bound to FIXa prolongs blood clotting time by inhibiting the conversion of FX to FXa in the intrinsic tenase complex.

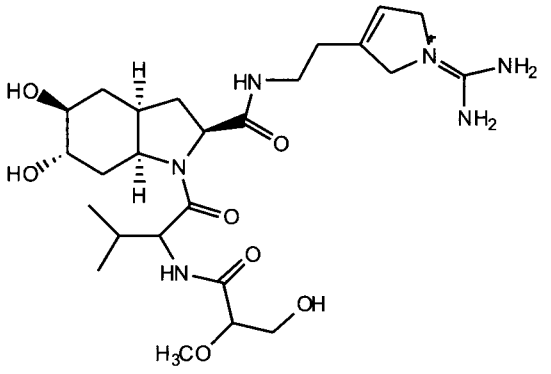
Three new peptides, dysinosins B, C and D, (**6–8**) isolated from the sponge *Lamellodysidea chlorea* were found to inhibit the blood coagulation cascade serine proteases factor VIIa and thrombin (Carroll *et al.*, 2004). Furthermore, the study revealed that two structural motifs of the dysinosins contributed to the binding of these compounds to factor VIIa and thrombin proteases. A novel fish protein was enzymatically extracted from the marine



Dysinosins B (6)



Dysinosins C (7)



Dysinosins D (8)

Fig 2. Chemical structures of peptides with anticoagulant activity

fish, yellowfin sole *Limanda aspera* and purified to single-chain monomeric protein (12.01 kDa). This yellow fin sole anticoagulant protein (YAP) was found to bind with FXIIa and platelet membrane integrins to inhibit thrombosis *in vitro* (Rajapakse *et al.*, 2005b).

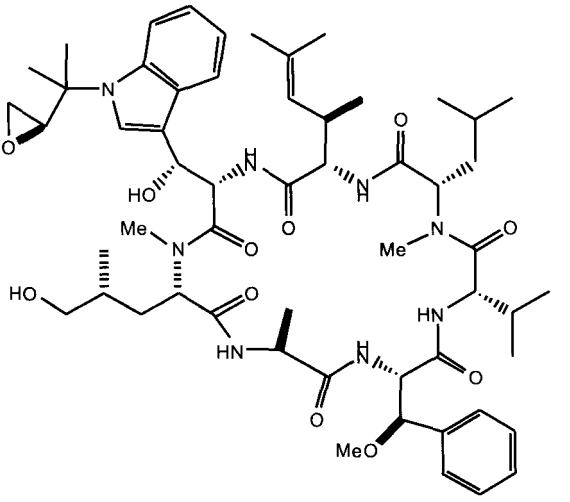
IMMUNOSTIMULATORY EFFECTS

Immune-stimulants are chemical compounds that activate white blood cells (leukocytes) and hence may render animals more resistant to infections by viruses, bacteria, fungi and parasites. Immune-stimulants may also be active against human cancer because they activate the white blood cells which recognise and destroy tumor cells (Raa, 2000). Nutritional factors such as vitamins B and C, growth hormone and prolactin have also been reported to be Immune-stimulants. These immune-stimulants mainly facilitate the function of phagocytic cells and increase their bactericidal activities. Several immune-stimulants also stimulate the natural killer cells, complement lysozyme and antibody responses. The activation of these immunological functions is associated with increased protection against infectious diseases. Resistance to bacterial pathogens such as *Vibrio anguillarum*, *V. salmonicida*, *Aeromonas salmonicida*, *Yersinia rukeri* and *Streptococcus* spp. and to parasitic infections such as white spot disease can be increased by administration of immune-stimulants, but not to intracellular pathogens such as *Renibacterium salmoninarum* and *Pasteurella piscicida* (Sakai, 1999).

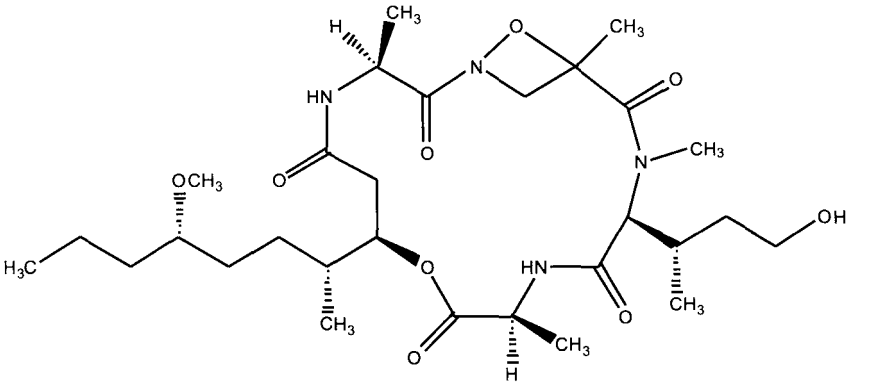
According to Gildberg *et al.* (1996) four medium size (500–3000 Da) acid peptide fractions were separated from a emptied stomach hydrolysates of Atlantic cod *Gadus morhua* and fractionated on a S-Sepharose cation exchange chromatography column. These peptide fractions were found to show better effects in stimulatory experiments with head kidney leucocytes from Atlantic salmon *Salmo salar*. Furthermore, the stimulation was observed to be good and in most cases better than the stimulation achieved with similar concentrations of lipopolysaccharides from the fish pathogen *Aeromonas salmonicida*.

ANTI-INFLAMMATORY ACTIVITY

Prostaglandins and nitric oxide (NO) are ubiquitous mediator systems which have numerous vascular and inflammatory effects. Production of prostaglandins or NO by the constitutive isoenzymes cyclo-oxygenase-1 (COX-1) or endothelial NO synthase, are implicated in the physological regulation of vascular tone and homeostatic functions. COX-2 and inducible NO synthase are not generally expressed in resting cells, but are induced following appropriate stimulation with



Cyclomarin A (9)



Halipeptin A (10)

Fig 3. Chemical structures of peptides with anti-inflammatory activity

proinflammatory agents such as cytokine, lipopolysaccharide and zymosan in numerous cell types including macrophages. The induction of COX-2 and NO synthase results in the increased synthesis of prostaglandins and NO, which play a key role in the pathophysiology of inflammatory conditions.

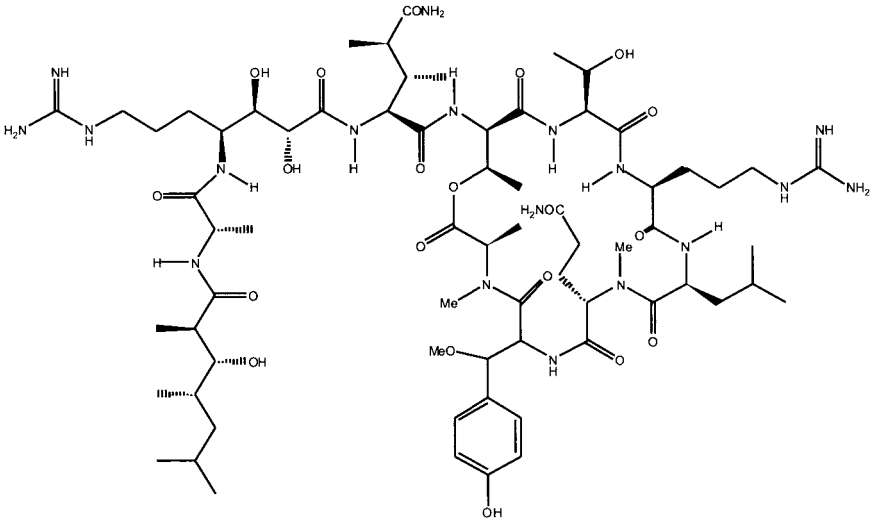
Renner *et al.* (1999) isolated three new cyclic heptapeptides, cyclomarins A–C from the extracts of a cultured marine bacterium collected in the vicinity of San Diego, CA. The major metabolite, cyclomarin A (**9**), made of three common and four unusual amino acids was found to display significant antiinflammatory activity in both *in vivo* and *in vitro* assays.

Two anti-inflammatory 17-membered cyclic depsipeptides, halipeptins A and B were isolated from the marine sponge *Haliclona* sp. Among these halipeptin A (**10**) was found to possess very potent anti-inflammatory activity *in vivo*, causing about 60% inhibition of edema in mice at the dose of 300 mg/kg (*i.p.*), suggesting that halipeptin A was more potent than indomethacin and naproxen (Randazzo *et al.*, 2001).

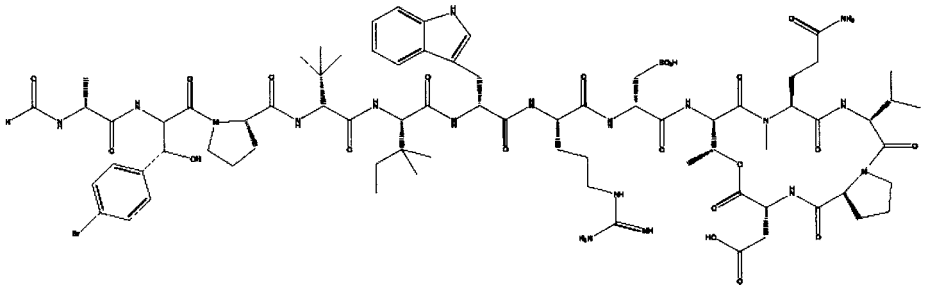
ANTIMICROBIAL ACTIVITY

Antimicrobial peptides are one of the major components of the natural immune defence system in marine organisms and are widespread in invertebrates, especially in tissues such as the gut and respiratory organs. Normally peptides with molecular mass of 10 kDa or less show antimicrobial properties (Boman, 1995) and provide an immediate and rapid response to invading microorganisms (Bartlett *et al.*, 2002). The major classes of antimicrobial peptides include helices, sheet and small proteins, peptides with thio-ether rings, peptides with an overrepresentation of one or two amino acids, lipopeptides and macrocyclic cystine knot peptides (Epand & Vogel, 1999). The majority of antimicrobial peptides are amphiphilic, displaying both hydrophilic and hydrophobic surfaces. These peptides generally act by forming pores in microbial membranes or disrupting membrane integrity (Tam *et al.*, 2000), which is made possible by their amphiphilic structure. Cationic antimicrobial peptides have been found to exhibit host defences, associated with acute inflammation (Hancock & Diamond, 2000). The value of antimicrobial peptides is their natural immunity and small size, which makes them easily synthesizable (Relf *et al.*, 1999). Furthermore, many antibacterial peptides exhibit very high specificity for prokaryotes with low toxicity for eukaryotic cells, which has created interests in exploring peptides as potential antibiotics (Zasloff, 1992). Recently, it has been found that there are a growing number of bacteria resistant to conventional antibiotics. Hence, the development of antibiotics with novel mechanisms of action is required (Lohner & Staudegger, 2001). Endogenous peptides are exciting candidates as new antibacterial agents due to their broad antimicrobial spectra, highly selective toxicities, and the difficulty for bacteria to develop resistance to these peptides (Boman, 1998; Hancock & Scott, 2000; Lehrer & Ganz, 1999).

Tachyplesin a cyclic broad-spectrum antimicrobial peptide forming a rigid, antiparallel β-sheet because of two intramolecular S–S linkages was isolated from acid extracts of horseshoe crab *Tachypleus tridentatus* hemocyte debris. It consists of 17 amino acid residues Lys-Trp-Cys-Phe-Arg-Val-Cys-Tyr-Arg-Gly-Ile-Cys-Tyr-Arg-Arg-Cys-Arg and arginine alpha-amide as the carboxyl-terminal (2.26 kDa). Tachyplesin inhibits the growth of both Gram-negative and -positive bacteria at low concentrations and formed a complex with bacterial lipopolysaccharide (Nakamura *et al.*, 1988).



Callipeltin A (11)



Microspinosamide (12)

Fig 4. Chemical structures of peptides with anti-microbial activity

It also reduced the viabilities of *B. ostreae* and *P. marinus* in a dose-dependent manner and displayed a potent activity against marine vibrios, with a MIC of 0.4–0.8 μg/ml against *Vibrio* P1 (Morvan *et al.*, 1997). Furthermore, it causes a rapid K^+ efflux from *Escherichia coli* cells concurrent with a reduced cell viability (Matsuzaki *et al.*, 1991) by permeabilizing both bacterial and artificial lipid membranes (Laederach *et al.*, 2002; Matsuzaki *et al.*, 1997).

Furthermore, tachyplesin suppresses the development of cytopathic effects of human immunodeficiency virus by 70% when added during the adsorption period of the virus and has been shown to inactivate vesicular stomatitis virus and slightly inactivate influenza A virus (Morimoto *et al.*, 1991; Murakami *et al.*, 1991).

Cyclic depsipeptide from marine sponges have been found to exhibit anti-HIV activity. A cyclic depsidecapeptide, callipeltin A (**11**) was isolated

from a shallow water sponge of the genus *Callipelta*, collected in the waters of New Caledonia. Callipeltin A has been found to protect cells infected by human immunodeficiency (HIV) virus at CD_{50} of 0.29 mg/ml and ED_{50} of 0.01 mg/ml (Zampella *et al.*, 1996). Microspinosamide (**12**), a new cyclic depsipeptide incorporating 13 amino acid residues, was isolated from extracts of an Indonesian collection of the marine sponge *Sidonops microspinosa*. This cyclic depsipeptide incorporates numerous uncommon amino acids, and it is the first naturally occurring peptide to contain a β-hydroxy-*p*-bromophenylalanine residue. It inhibited the cytopathic effect of HIV-1 infection in an XTT-based *in vitro* assay with an EC_{50} value of approximately 0.2 μg/mL (Rashid *et al.* 2001).

NEUROPROTECTIVE ACTIVITY

Neurodegeneration induced by excitatory neurotransmitter glutamate is considered to be of particular relevance in several types of acute and chronic neurological impairments ranging from cerebral ischaemia to neuropathological conditions such as motor neuron disease, Alzheimer's, Parkinson's disease and epilepsy. The hyperexcitation of glutamate receptors coupled with calcium overload can be prevented or modulated by using well-established competitive and non-competitive antagonists targeting ion/receptor channels. The exponentially increasing body of pharmacological evidence over the years indicates potential applications of peptides, due to their exquisite subtype selectivity on ion channels and receptors, as lead structures for the development of drugs for the treatment of wide variety of neurological disorders (Rajendra *et al.*, 2004).

According to Williams *et al.* (2000) Conantokin G (Con G), a 17-amino-acid peptide H-Gly-Glu-Gla-Gla-Leu-Gln-Gla-Asn-Gln-Gla-Leu-Ile-Arg-Gla-Lys-Ser-Asn-NH2 from the venom of the marine cone snail, *Conus geographus* was shown to decrease excitotoxic calcium responses to NMDA and to exhibit differential neuroprotection potencies against hypoxia/hypoglycemia-, NMDA-, glutamate-, or veratridine-induced injury, using the intraluminal filament method of middle cerebral artery occlusion as an *in vivo* rat model of transient focal brain.

REFERENCES

Aherne, G.W., Hardcastle, A., Valenti, M., Bryant, A., Rogers, P., George, R., Pettit, G.R., Srirangam, J.K. and Kelland, L.R. (1996). Antitumour evaluation of dolastatins 10 and 15 and their measurement in plasma by radioimmunoassay. *Cancer Chemotherapy and Pharmacology,* **38**: 225-232.

Aneiros, A. and Garateix, A. (2004). Bioactive peptides from marine sources: pharmacological properties and isolation procedures. *Journal of Chromatography B*, **803**: 41-53.

Ariyoshi, Y. (1993). Angiotensin-converting enzyme inhibitors derived from food proteins. *Trends in Food Science and Technology,* **4**: 139-144.

Atkinson, A.B. and Robertson, J.I.S. (1979). Captopril in the treatment of clinical hypertension and cardiac failure. *Lancet,* **2**: 836-839.

Bae, I., Fan, S., Bhatia, K., Kohn, K.W., Fornace, A.J. and O'Connor, P.M. (1995).

Relationships between G_1 arrest and stability of the p. 53 and p. $21^{CiP1/Waf1}$ proteins following γ-irradiation of human lymphoma cells. *Cancer Research,* **55**: 2387-2393.

Bartlett, T., Cuthbertson, B.J., Shepard, E., Chapman, R., Gross, P. and Warr, G. (2002). Crustins, homologues of an 11.5-kDa antibacterial peptide, from two species of penaeid shrimp, *Litopenaeus vannamei* and *Litopenaeus setiferus. Mar. Biotechnol,* **4**: 278-293.

Beak, H.H., and Cadwallader, K.R. (1995). Enzymatic hydrolysis of crayfish processing by-products. *J. Food Sci.*, **60**: 929-935.

Boman, H. (1998). Gene-encoded peptide antibiotics and the concept of innate immunity: an update review. *Scand. J. Immunol,* **48**: 15-25.

Boman, H.G. (1995). Peptide antibiotics and their role in innate immunity. *Annu. Rev. Immunol.,* **13**: 61-92.

Byun, H.G. and Kim, S.K. (2001). Purification and characterization of angiotensin I converting enzyme inhibitory peptides from Alaska pollack skin. *Process Biochemistry,* **36**: 1155-1162.

Carroll, A.R, Buchanan, M.S., Edser, A., Hyde, E., Simpson, M. and Quinn, R.J. (2004). Dysinosins B–D, inhibitors of factor VIIa and thrombin from the Australian sponge. *Lamellodysidea chlorea. J. Nat Prod.*, **67**: 1291-1294.

Cheftel, C.C., Ahern, M., Wang, D.I C. and Tannenbaum, S. R. (1971). Enzymatic solubilization of fish protein concentrate: Batch studies applicable to continuous enzyme recycling processes. *J. Agric. Food Chem.*, **19**: 155-161.

Chen, H.M., Muramoto, K., and Yamauchi, F. (1995). Structural analysis of antioxidative peptides from soybean β-conglycinin. *J. Agric. Food Chem.*, **43**: 574-578.

Chen, H.M., Muramoto, K., Yamauchi, F. and Nokihara, K. (1996). Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. *J. Agric. Food Chem.*, **44**: 2619-2623.

Chen, H.M., Muramoto, K., Yamauchi, F., Fujimoto, K. and Nokihara, K. (1998). Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digested of a soybean protein. *J. Agric. Food Chem.*, **46**: 49-53.

Choi, Y.R., Park, P.J., Choi, J.H., Byun, H.G., Jeong, I.C., Moon, S.H., and Kim, S.K. (2000). Purification and characterization of angiotensin I converting enzyme inhibitory peptides from enzymatic hydrolysate of cod liver protein. *Korean J. Life Sci.,* **10(2)**: 140-149.

Curtiss, C., Chon, J.N., Vrobel, T. and Francious, J.A. (1978). Role of the rennin-angiotensin system in the systemic vasoconstriction of chronic congestive heart failure. *Circulation,* **58**: 763-770.

Da Rocha, A.B., Lopes, R.M. and Schwartsmann, G. (2001). Natural products in anticancer therapy. *Curr. Opin. Pharmacol.,* **1**: 364-369.

Davie, E.W., Fujikawa, K. and Kisel, W. (1991). The coagulation cascade: Initiation, maintenance and regulation. *Biochemistry,* **30**: 10363-10370.

De Vries, D.J. and Beart, P.M. (1995). Fishing for drugs from the sea: status and strategies. *Trends in Pharmacological Sciences*, **16**: 275-279.

Debashis, D.D., Bhattacharjee, B.M. and Banerjee, R.K. (1997). Hydroxyl radicals is the major causative factor in stress-induced gastric ulceration. *Free Radical Biology and Medicine,* **23**: 8-18.

Epand, R. and Vogel, H. (1999). Diversity of antimicrobial peptides and their mechanisms of action. *Biochim. Biophys. Acta*, **1462**: 11-28.

Fandino, R.L., Otte, J. and Camp, J.V. (2006). Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. *International Dairy Journal*, **16**: 1277-1293.

Faulkner, D.J. (2000a). Highlights of marine natural products chemistry (1972-1999). *Nat. Prod. Rep.,* **17**: 1-6.

Faulkner, D.J. (2000b). *In*: Marine Pharmacology Vol. 77, Antonie Van Leeuwenhoek, pp. 135-145.

Fitzgerald, R.J. and Murray, B.A. (2006). Bioactive peptides and lactic fermentations. *International Journal of Dairy Technology*, **59**: 118-125.

Gildberg, A., Bogwald, J., Audny Johansen, A. and Stenberg, E. (1996). Isolation of acid

peptide fractions from a fish protein hydrolysate with strong stimulatory effect on atlantic salmon (*Salmo salar*) Head kidney leucocytes. Comp. *Biochem. Physiol.,* **114B**: 97-101.

Guerard, F., Sumaya-Martinez, M.T., Linard, B. and Duffosse, L. (2005). Marine protein hydrolysate with antioxidant properties. *Agro. Food Ind. Hi. Tech.*, **16**: 16-18.

Halliwell, B., Murcia, M.A., Chirico, S. and Arumoma, O.I. (1995). Free radicals and antioxidants in food and *in vivo*: what they do and how they work. *Crit. Rev. Food Sci. Nutr.*, **35**: 7-20.

Hamann, M.T., and Scheuer, P.J. (1993). Kahalalide F.A. Bioactive depsipeptide from the Sacoglossaa mollusk *Elysia rufescens* and the green alga *Bryopsis* sp. *J. Am. Chem. Soc.*, **115**: 5825.

Hancock, R.E.W. and Diamond, G. (2000). The role of cationic antimicrobial peptides in innate host defenses. *Trends Microbiol.,* **8**: 402-410.

Hancock, R.E.W. and Scott, M. (2000). The role of antimicrobial peptides in animal defenses. *Proc. Natl. Acad. Sci. U.S.A.,* **97**: 8856-8861.

Hattori, M., Yamaji-Tukamoto, K., Kumagai, H., Feng, Y. and Takahashi, K. (1998). Antioxidative activity of soluble elastin peptides. *J. Agric. Food Chem.*, **46**: 2167-2170.

Hoyle, N.T. and Merritt, J.H. (1994). Quality of fish hydrolysates from herring (*Clupea harengus*). *J. Food Sci.*, **59**: 76-79.

Je, J.Y., Park, P.J., Byun, H.G., Jung, W.K. and Kim, S.K. (2005a). Angiotensin I converting enzyme inhibitory peptide derived from fermented mussel, *Mytilus edulis*. *Bioresource Technology.*, **96**: 1624-1629.

Je, J.Y., Park, P.J. and Kim, S.K. (2005b). Antioxidant activity of a peptide isolated from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. *Food Research International*, **38**: 45-50.

Je, J.Y., Park, P.J., Kwon, J.Y. and Kim, S.K. (2004). A novel angiotensin I converting enzyme (ACE) inhibitory peptide from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. *J. Agric. Food Chem.*, **52**: 7842-7845.

Je, J.Y., Qian, Z.J. and Kim, S.K. (2007a). Antioxidant peptide isolated from muscle protein of bullfrog, *Rana catesbeiana* Shaw. *Journal of Medicinal Food*, **10(3)**: 401-407.

Je, J.Y., Qian, Z.J., Byun, H.G. and Kim, S.K. (2007b). Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis. *Process Biochemistry*, **42**: 840-846.

Jimeno, J.M. (2002). A clinical armamentarium of marine-derived anti-cancer compounds. *Anti-Cancer Drugs*, **13(Suppl 1)**: S15.

Jo, H.Y., Jung, W.K. and Kim, S.K. (2008). Purification and characterisation of a novel anticoagulant peptide from marine chiuroid worm, *Urechis unicinctus*. *Process Biochemistry,* **43**: 179-184.

Jordan, M.A., Walker, D., de Arruda, M., Barlozzari, T. and Panda, D. (1998). Suppression of microtubule dynamics by binding of cemadotin to tubulin: Possible mechanisms for its antitumor action. *Biochemistry*, **37**: 17571-17578.

Jung, W.K., Je, J.Y., Kim, H.J. and Kim, S.K. (2002). A novel anticoagulant protein from *Scapharca broughtonii*. *J Biochem Mol Biol.*, **35**: 199-205.

Jung, W.K., Qian, Z.J., Lee, S.H., Choi, S.Y., Sung, N.J., Byun, H.G. and Kim, S.K. (2007). Free radical scavenging activity of a novel antioxidative peptide isolated from *in vitro* gastrointestinal digests of *Mytilus coruscus*. *Journal of Medicinal Food*, **10(1)**: 197-202.

Jung, W.K., Mendis, E., Je, J.Y., Park, P.J., Son, B.W., Kim, H.C., Choi, Y.K. and Kim, S.K. (2005). Angiotensin I-converting enzyme inhibitory peptide from yellowfin sole (*Limanda aspera*) frame protein and its antihypertensive effect in spontaneously hypertensive rats. *Food Chemistry*, **94**: 26-32.

Kamano, Y., Herald, C.L. and Pettit, G.R. (1989). Cell growth inhibitory substance. United States patent, US 4816444.

Kanazawa, K., Kanazawa, E. and Nakane, M. (1985). Uptake of secondary autoxidation products of linoleic acid by rat. *Lipids*, **20**: 412-419.

Kim, S.K., Choi, Y.R., Park, P.J., Choi, J.H. and Moon, S.H. (2000). Purification and characterization of antioxidative peptides from enzymatic hydrolysate of cod teiset protein. *Korean Fisher. Sci.*, **33**: 198-204.

Kim, S.K., Kim, Y.T., Byun, H.G., Nam, K.S., Joo, D.S. and Shahidi, F. (2001). Isolation and characterization of antioxidative peptides from gelatin hydrolysate of alaska pollack skin. *J. Agric. Food Chem.*, **49**: 1984-1989.

Kim, S.K. and Mendis, E. (2006). Bioactive compounds from marine processing by-products – A review. *Food Research International,* **39**: 383-393.

Kim, S.K., Byun, H.G., Park, P.J. and Shahidi, F. (2001). Angiotensin I converting enzyme inhibitory peptides from bovine skin gelatine. *J. Agric. Food Chem.,* **49**: 2992-2997.

Kim, S.Y., Jae-Young Je, J.Y. and Kim, S.K. (2007). Purification and characterization of antioxidant peptide from hoki (*Johnius belengerii*) frame protein by gastrointestinal digestion. *The Journal of Nutritional Biochemistry*, **18**: 31-38.

Krishnamurthy, G., Cheng, W., Mei-Chu, L., Aulabaugh, A., Razinkov, V., Ding, W., Loganzo, F., Zask, A., and Ellestad, G. (2003). Biophysical characterization of the interactions of HTI-286 with tubulin heterodimer and microtubules. *Biochemistry,* **42**: 13484.

Laederach, A., Andreotti, A., and Fulton. D. (2002). Solution and micelle-bound structures of tachyplesin I and its active aromatic linear derivatives. *Biochemistry,* **41**: 12359-12368.

Leanderson, P., Faresjo, A.O. and Tagesson, C. (1997). Green tea polyphenols inhibits oxidant-induced DNA strand breakage in cultured lung cells. *Free Radical Biology and Medicine,* **23**: 235-242.

Lehrer, R. and Ganz. T. (1999). Antimicrobial peptides in mammalian and insect host defense. *Curr. Opin. Immunol.,* **11**: 23-27.

Lohner, K. and Staudegger, E. (2001). Development of novel antimicrobial agents: emerging strategies. Horizon Scientific Press, Wymondham, United Kingdom.

Luesch, H., Moore, R.E., Paul, V.J., Mooberry, S.L., and Corbett, T.H. (2001). Isolation of dolastatin 10 from the marine cyanobacterium *Symploca* species VP642 and total stereochemistry and biological evaluation of its analogue symplostatin 1. *J. Nat. Prod.,* **64**: 907-910.

Matsuzaki, K., Fukui, M., Fujii, N. and Miyajima. K. (1991). Interactions of an antimicrobial peptide, tachyplesin I, with lipid membranes. *Biochim. Biophys. Acta,* **1070**: 259-264.

Matsuzaki, K., Yoneyama, S., Fujii, N., Miyajima, K., Yamada, K., Kirino, Y. and Anzai. K. (1997). Membrane permeabilization mechanisms of a cyclic antimicrobial peptide, tachyplesin I, and its linear analog. *Biochemistry*, **36**: 9799-9806.

Meisel, H. (1997). Biochemical properties of bioactive peptides derived from milk proteins: potential nutraceuticals for food and pharmaceutical applications. *Livestock Production Science,* **50**: 125-138.

Mendis, E., Rajapakse, N. and Kim, S.K. (2005a). Antioxidant properties of a radical scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *J. Agri. Food Chem.*, **253**: 581-587,

Mendis, E., Rajapakse, N., Byun, H.G. and Kim, S.K. (2005b). Investigation of jumbo squid (*Dosidicus gigas*) skin gelatin peptides for their *in vitro* antioxidant effects. *Life sciences*, **77**: 2166-2178.

Morimoto, M., Mori, H., Otake, T., Ueba, N., Kunita, M.N., Niwa, Murakami, T. and Iwanaga, S. (1991). Inhibitory effect of tachyplesin I on the proliferation of human immunodeficiency virus *in vitro*. *Exp. Chemother.*, **37**: 206-211.

Morvan, A., Iwanaga, S., Comps, M. and Bachere. E. (1997). *In vitro* activity of the *Limulus* antimicrobial peptide tachyplesin I on marine bivalve pathogens. *J. Invertebr. Pathol.* **69**: 177-182.

Murakami, M.T., Niwa, Tokunaga, F., Miyata, T. and Iwanaga, S. (1991). Direct virus inactivation of tachyplesin I and its isopeptides from horseshoe crab hemocytes. *Chemotherapy,* **37**: 327-334.

Muramatsu, H., Kogawa, K., Tanaka, M., Okumura, K., Nishihori, Y., Koike K. *et al.* (1995). Superoxide anion dismutase in SAS human tongue carcinoma cell line is a factor defining invasiveness and cell motility. *Cancer Research*, **55**: 6210-6214.

Nakamura, T., Furunaka, H., Miyata, T., Tokunaga, F., Muta, T. and Iwanaga. S. (1988). Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab *Tachypleus tridentatus*. *J. Biol. Chem.*, **263**: 16709-16713.

Nuijen, B., Bouma, M., Manada, C., Jimeno, J.M., Schellens, J.H.M., Bult, A. and Beijnen, J.H. (2000). Pharmaceutical development of anticancer agents derived from marine sources. *Anti-Cancer Drugs,* **11**: 793.

Ondetti, M.A. (1977). Design of specific inhibitors of angiotensin-converting enzyme: New class of orally active antihypertensive agents. *Science,* **196**: 441-444.

Onodenalore, A.C. and Shahidi, F. (1996). Protein dispersions and hydrolysates from shark (*Isurus oxyrinchus*). *J. Aquat. Food Prod. Technol.*, **5**: 43-59.

Patchett, A.A., Harris, E., Tristram, E.W., Wyvratt, M.J., Wu, M.T., Taub, D. *et al.* (1980). A new class of angiotensin-converting enzyme inhibitors. *Nature,* **298**: 280-283.

Pelicano, H., Carney, D. and Huang, P. (2004). ROS stress in cancer cells and therapeutic implications. *Drug Resistance Updates*, **7**: 97-110.

Pettit, G.R., Kamano, Y., Herald, C.L., Fujii, Y., Kizu, H., Boyd, M.R., Boettner, F.E., Doubek, D.L., Schmidt, J.M., Chapuis, J.C. and Michel, C. (1993). Isolation of dolastatins 10-15 from the marine mollusc *Dolabella auricularia*. *Tetrahedron*, **49**: 9151-9170.

Pettit, G.R. and Kamano, Y. (1989). Isolation and structural elucidation of the cytostatic linear depsipeptide dolastatin 15. United States patent, US 4879278.

Pettit, G.R., Kamano, Y., Dufresne, C., Cerny, R.L., Herald, C.L. and Schmidt J.M. (1989). Isolation and structure of the cytostatic linear depsipeptide dolastatin 15. *J. Org. Chem.*, **54**: 6005-6006.

Pettit, G.R., Kamano, Y., Herald, C.L. *et al.* (1987). The isolation and structure of a remarkable marine animal constituent: dolastatin 10. *J. Am. Chem. Soc.,* **109**: 6883-6885.

Pihlanto-Leppälä, A. (2001). Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. *Trends in Food Science and Technology,* **11**: 347-356.

Poling, C.E. and Rice, E.E. (1962). The influence of temperature, heating time and aeration upon the nutritive value of fats. *J. Am. Oil Chem. Soc.*, **39**: 315-320.

Qian, Z.J., Jung, W.K., Byun, H.G., Kim, S.K. (2007a). Protective effect of an antioxidative peptide purified from gastrointestinal digests of oyster, *Crassostrea gigas* against free radical induced DNA damage. *Bioresource Technology*, **99**: 3365-3337.

Qian, Z.J., Jung, W.K. and Kim, S.K. (2008). Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin, *Rana catesbeiana* Shaw. *Bioresource Technology*, **99**: 1690-1698.

Qian, Z.J., Je, J.Y. and Kim, S.K. (2007b). Antihypertensive effect of angiotensin 1 converting enzyme-inhibitory peptide from hydrolysates of Bigeye Tuna Dark Muscle, *Thunnys obesus*. *Journal of Agricultural and Food Chemistry*, **55**: 8398-8403.

Qian, Z.J., Jung, W.K., Lee, S.H., Byun, H.G. and Kim, S.K. (2007c). Antihypertensive effect of an angiotensin I converting enzyme inhibitory peptide purified from bullfrog muscle (*Rana catesbeiana* Shaw) protein in spontaneously. *Process Biochemistry*, **42**: 1443-1448.

Quaglia, G. and Orban, E. (1987). Enzymic solubilization of proteins of sardine (*Sardina pilchardus*) by commercial proteases. *J. Sci. Food Agric.*, **38**: 263-269.

Raa, J. (2000). The use of immune-stimulants in fish and shellfish feeds. *In*: Avances en Nutrición Acuícola V. Memorias del V Simposium Internacional de Nutrición Acuícola. *Ed. by* Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Olvera-Novoa, M.A. and Civera-Cerecedo, R.Y., Mérida, Yucatán, Mexico, pp. 47-56.

Rajapakse, N., Jung, W.K., Mendis, E., Moon, S.H. and Kim, S.K. (2005b). A novel anticoagulant purified from protein hydrolysate inhibits factor XIIa and platelet aggregation. *Life Sciences*, **76**: 2607-2619.

Rajapakse, N., Mendis, E., Byun, H.G. and Kim, S.K. (2005c). Purification and *in vitro* antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *The Journal of Nutritional Biochemistry*, **16**: 562-569.

Rajapakse, N., Mendis, E., Jung, W.K., Je, J.Y. and Kim, S.K. (2005a). Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Research International*, **38**: 175-182.

Rajendra, W., Armugam, A. and Jeyaseelan, K. (2004). Neuroprotection and peptide toxins. *Brain Research Reviews*, **45**: 125-141.

Ranathunga, S., Rajapakse, N. and Kim, S.K. (2006). Purification and characterization of antioxidant peptide derived from muscle of conger eel (*Conger myriaster*). *Eur. Food Res. Technol.*, **222**: 310-315.

Randazzo, A., Bifulco, G., Giannini, C., Bucci, M., Debitus, C., Cirino, G. and Gomez-Paloma, L. (2001). Halipeptins A and B: Two novel potent anti-inflammatory cyclic depsipeptides from the *Vanuatu marine* sponge *Haliclona* species. *J. Am. Chem. Soc.*, **123**: 10870-10876.

Rashid, M.A., Gustafson, K.R., Laura K. Cartner, L.K., Shigematsu, N., Pannell, L.K. and Boyd, M.R. (2001). Microspinosamide, a new HIV-inhibitory cyclic depsipeptide from the marine sponge *Sidonops microspinosa*. *J. Nat. Prod.*, **64**: 117-121.

Rawat, D.S., Joshi, M.C., Joshi, P. and Atheaya, H. (2006). Marine peptides and related compounds in clinical trial. *Anti-Cancer Agents - Med. Chem.*, **6**: 1-8.

Rebeca, B.D., Pena-Vera, M.T. and Diaz-Castaneda, M. (1991). Production of fish protein hydrolysates with bacterial proteases; yield and nutritional value. *J. Food Sci.*, **56**: 309-314.

Relf, J., Chisholm, J., Kemp, G. and Smith, V. (1999). Purification and characterization of a cysteine-rich 11.5-kDa antibacterial protein from the granular haemocytes of the shore crab, *Carcinus maenas*. *Eur. J. Biochem.*, **264**: 350-357.

Renner, M.K., Shen, Y.C., Cheng, X.C., Jensen, P.R., Frankmoelle, W., Kauffman, C.A., William Fenical, W., Lobkovsky, E. and Clardy, J. (1999). Cyclomarins A-C, new antiinflammatory cyclic peptides produced by a marine bacterium (*Streptomyces* sp.) *J. Am. Chem. Soc.*, **121**: 11273-11276.

Sakai, M. (1999). Current research status of fish immunostimulants. *Aquaculture*, **172**: 63-92.

Schwartsmann, G., da Rocha, A.B., Berlinck, J.G.S. and Jimeno, J. (2001). Marine organisms as a source of new anticancer agents. *Lancet Oncol.*, **2**: 221-225.

Sewell, J.M., Mayer, I., Langdon, S. P., Smyth, J. F., Jodrell, D.I. and Guichard, S.M. (2005). The mechanism of action of Kahalalide F: Variable cell permeability in human hepatoma cell lines. *Eur. J. Cancer*, **41**: 1637.

Shahidi, F., Han, X.Q. and Synowiecki, J. (1995). Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). *Food Chem.*, **53**: 285-293.

Shahidi, F., Synowiecki, J. and Balejko, J. (1994). Proteolytic hydrolysis of muscle proteins of harp seal (*Phoca groenlandica*). *J. Agric. Food Chem.*, **42**: 2634-2638.

Skeggs, L.T., Kahn, J.E. and Shumway, N.P. (1957). The preparation and function of the angiotensin-converting enzyme. *Journal of Experimental Medicine*, **103**: 295-299.

Subramanian, R., Tanuja Koppal, T., Michael Green, M., Servet Yatin, S., Jordan, B., Drake, J. and Butterfield, D.A. (1998). The free radical antioxidant vitamin E protects cortical synaptosomal membranes from amyloid B-peptide (25-35) toxicity, but not from hydroxynonenal toxicity: relevance to the free radical hypothesis of alzheimer's disease. *Neurochemical Research*, **23**: 1403-1410.

Suetsuna, K. (2000). Antioxidant peptides from the protease digest of prawn (*Penaeus japonicus*) muscle. *Marine Biotechnology*, **2**: 5-10.

Sussman, M.S. and Bulkley, G.B. (1990). Oxygen-derived free radicals in reperfusion injury. *Methods Enzymology*, **186**: 711-723.

Talpir, R., Benayahu, Y., Kashman, Y., Pannell, L., and Schleyer, M. (1994). Hemiasterlin and geodiamolide TA; two new cytotoxic peptides from the marine sponge *Hemiasterella minor* (Kirkpatrick). *Tetrahedron Lett.*, **35**: 4453.

Tam, J., Lu, Y. and Yang. J. (2000). Marked increase in membranolytic selectivity of novel cyclic tachyplesins constrained with an antiparallel two-B strand cysteine knot framework. *Biochem. Biophys. Res. Commun.*, **267**: 783-790.

Tanaka-Azevedo, A.M., Tanaka, A.S. and Sano-Martins, I.S. (2003). A new blood coagulation inhibitor from the snake *Bothrops jararaka* plasma: Isolation and characterization. *Biochemical and Biophysical Research Communications*, **308**: 706-712.

Theodore, A.E. and Kristinsson, H.G. (2007). Angiotensin converting enzyme inhibition of fish protein hydrolysates prepared from alkaline-aided channel catfish protein isolate. *Journal of the Science of Food and Agriculture,* **87**: 2353-2357.

Uchida, K. and Stadman, E.R. (1992). Modification of histidine residues in proteins by reaction with 4-hydroxynonenal. *Proc. Natl. Acad. Sci. U.S.A.*, **89**: 4544-4548.

Urata, J., Shojo, H. and Kaneko, Y. (2003). Inhibition mechanisms of hematophagous invertebrate compounds acting on the host blood coagulation and platelet aggregation pathways. *Biochimie,* **85**: 493-500.

Vaishampayan, U., Glode, M., Du, W., Kraft, A., Hudes, G., Wright, J. and Hussain, M. (2000). Phase II study of dolastatin-10 in patients with hormone-refractory metastatic prostate adenocarcinoma. *Clin. Cancer Res.,* **6**: 4205-4208.

Vajragupta, O., Boonchoong, P. and Wongkrajang, Y. (2000). Comparative quantitative structure–activity study of radical scavengers. *Bioorganic & Medicinal Chemistry,* **8**: 2617-2628.

Venugopal, V., Chawala, S.P. and Nair, P.M. (1996). Spray dried protein powder from thread fin beam: preparation properties and comparision with the FPC type-B. *Journal of Muscle Foods*, **7**: 55-71.

Vera, M.D. and Joullié, M.M. (2002). Natural products as probes of cell biology: 20 years of didemnin research. *Med. Res. Rev.*, **22**: 102.

Williams, A.J., Dave, J.R., Phillips, J.B., Lin,Y., McCabe, R.T. and Tortella, F.C. (2000). Neuroprotective efficacy and therapeutic window of the high-affinity *N*-methyl-D-aspartate antagonist conantokin-G: *In vitro* (primary cerebellar neurons) and *in vivo* (rat model of transient focal brain ischemia) studies. *The Journal of Pharmacology and Experimental Therapeutics,* **294**: 378-386.

Wu, H.C., Chen, H.M. and Shiau, C.Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Research International*, **36**: 949-957.

Yamamoto, N. (1997). Antihypertensive peptides derived from food proteins. *Biopolymers,* **43**: 129-134.

Yang, H.Y.T., Erdös, E.G. and Levin, Y. (1971). Characterization of a dipeptide hydrolase (Kininase II: angiotensin I converting enzyme). *Journal of Pharmacology and Experimental Therapeutics*, **177**: 291-300.

Zampella, A., D'Auria, M.V., Paloma, L.G., Casapullo, A., Minale, L., Debitus, C. and Henin, Y. (1996). Callipeltin A, an Anti-HIV Cyclic Depsipeptide from the New Caledonian Lithistida Sponge *Callipelta* sp. *J. Am. Chem. Soc.*, **118**: 6202-6209.

Zasloff, M. (1992). Antibiotic peptides as mediators of innate immunity. *Curr. Opin. Immunol.,* **4**: 3-7.

2

Bioactive Natural Products as Anti-Staphylococcal Infections

SUPAYANG PIYAWAN VORAVUTHIKUNCHAI[1*], BEATRICE OLAWUMI IFESAN[2], SASITORN CHUSRI[2] AND JONGKON SAISING[2]

ABSTRACT

Staphylococcus aureus is a versatile pathogen of human and animals, because of its intrinsic virulence factors, its ability to cause an array of life-threatening conditions, and its capacity to adapt to different environmental conditions. It can cause a wide variety of diseases ranging from superficial infections to more severe diseases such as pneumonia, endocarditis, septicaemia, and a variety of toxin-mediated diseases including gastroenteritis, staphylococcal scalded-skin syndrome, and toxic shock syndrome. Plants are one of the most important resources of human foods and medicines. Rapidly increasing knowledge on nutrition and medicine has dramatically changed the concepts about food and health, and brought in a revolution on them. Phytotherapy has emerged as new concepts and widely spread during the past decades. The use of phytotherapy has become progressively popular to improve health, and to prevent and treat diseases. With these trends, bioactive components in medicinal plants have become targets of complimentary medicines both for noninfectious and infectious diseases. As a part of continuing research work in our laboratory, we have attempted to search for an effective plant to prevent and cure infections caused by S. aureus. The present communication discusses data from important findings. The following aspects are covered: (i) an overview of staphylococcal infections, (ii) problems encountered with antibiotic treatment, (iii) prevalence of methicillin-resistant S. aureus

1. Department of Microbiology and Director of Natural Products Research Center, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.
2. Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.
* *Corresponding author* : E-mail : supayang.v@psu.ac.th

infections, (iv) antibacterial mechanisms of medicinal plants, (v) applications of natural products in staphylococcal infections, (vi) detailed studies on some selected plant species with high potency for further development, and (vii) current and future developments.

Key words : Antibacterial activity, antibacterial mechanism, bioactive compounds, complimentary medicine, *Eleutherine americana*, food poisoning, herb, medicinal plant, methicillin-resistant *Staphylococcus aureus*, natural products, *Quercus infectoria*, *Rhodomyrtus tomentosa*, *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus is an important pathogen of public health concern. Its severely antimicrobial resistance has been considered as a major problem. The organism can cause a wide variety of diseases ranging from superficial infection to more severe diseases such as pneumonia, endocarditis, septicaemia, and a variety of toxin-mediated diseases including staphylococcal scalded-skin syndrome and toxic shock syndrome (Fidalgo *et al.*, 1990; Roberts *et al.*, 1991; Lowry, 1998). In other context, staphylococcal food poisoning, caused by ingestion of food containing *S. aureus* cells or preformed staphylococcal enterotoxin, has been increasingly reported. In processed foods in which *S. aureus* should have been destroyed by processing, the enterotoxin is not destroyed. This bacterium is present on the skin and mucosa of food-producing animal reservoirs, such as ruminants and it is frequently associated to subclinical mastitis leading to contamination of milk and dairy products (Jablonski & Bohach, 2001). As a consequence, food products may originally become contaminated during or after processing either from the hands and body of the processors, the equipments and the processing plants.

Problems arising due to antibiotic-resistant organisms as well as consumer demand for partial or complete removal of chemically synthesized preservatives from foods have led to renewed interest in natural drugs and preservatives for maintaining safety of foods. Although the antibacterial properties of many herbs and spices have been reported, their potentials as drugs and natural food additives have not been fully exploited.

Plants are not only important to the million of people to whom traditional medicine serves as the only opportunity for health care and to those who use plants for various purposes in their daily lives, but also as a source of new pharmaceuticals. To overcome the problem of antibiotic resistance, medicinal plants have been extensively studied as alternative treatments for infectious diseases. *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) are major causes of hospital- and community-acquired infections, and can result in serious consequence as well as high healthcare costs (Shorr, 2007). In recent year, increasing attention has

been focused on phytochemicals as medicinal decolonization agents. As part of continuing research work in our laboratory, we have attempted to search for an effective plant to prevent and cure infections caused by *S. aureus*. The present document discusses data from important findings. Aspects with an overview of staphylococcal infections, problems encountered with antibiotic treatment, prevalence of methicillin-resistant *S. aureus* infections, antibacterial mechanisms of medicinal plants, and applications of natural products in the infections are included. Detailed studies on some selected plant species with high potency for further development are highlighted with some comments on current and future developments.

AN OVERVIEW OF STAPHYLOCOCCAL INFECTIONS

General Characteristics of Staphylococci

Staphylococci are Gram-positive, catalase positive cocci, 0.5-1.0 μm in diameter, occurring singly, in pairs, and irregular clusters. They are non motile, nonendospore forming, and facultative anaerobes (Holt *et al.*, 1994). *Staphylococcus aureus* is present on the skin and mucosa of animals, and the environment (Jay, 2000). The bacteria readily colonize the skin or mucosal surfaces and produce numerous virulence factors that promote their survival and subsequent dissemination. It is well recognized that *S. aureus* is the main species of clinical importance in the genus *Staphylococcus*. *Staphylococcus epidermidis*, a nonpathogenic member of normal cutaneous microbial microbiota can express few virulence factors under normal conditions. *Staphylococcus aureus* has an ability to survive in the host by forming biofilms on the surface of horny layers and indwelling medical devices. It may be found in many parts of our environment including dust, water, air, faeces, clothing, and utensils (Bremer *et al.*, 2004). The organism can be differentiated from other species on the basis of the golden pigmentation of colonies and positive results of coagulase, mannitol fermentation, and deoxyribonuclease test (Betley *et al.*, 1992; Wilkinson, 1997). Most pathogenic strains ferment mannitol, in addition to producing yellow pigment, haemolysin, and coagulase. Colonies have buttery to gummy consistency when touched with inoculating loop (Bennett & Lancette, 1998). On Baird-Parker medium, their colonies are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowded plates, grey to jet-black, frequently with light-coloured (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone.

Growth Requirements

The nutritional requirements of *S. aureus* are complex and vary from strain to strain. The conditions under which this bacterium grows also depend on the composition of nutrients. In general, *S. aureus* grows between 7-47°C, with an optimum temperature of 30-37°C. Enterotoxins by enterotoxin producing strains are produced between 10-46°C, with an optimum

temperature of 35-45°C. Enterotoxin production is substantially reduced at 20-25°C. It is generally accepted that the enterotoxin production is unlikely to occur at temperatures below 10°C. Optimum growth and toxin production occur at water activity level less than 0.99. Reduced level of water activity may inhibit toxin synthesis more than growth. However, toxin production has been reported to occur at as low as water activity 0.86 (Nottermans & Heuvelman, 1983; Miller *et al.*, 1997; Jay, 2000). Optimum enterotoxin production occurs at pH 6-7, and it is influenced by atmospheric conditions, carbon, nitrogen, and salt level.

Resistance of the Organism

Staphylococcus aureus is a hardy organism that can withstand dessication and can survive in dust and on dry metal, glass or porcelain surfaces for a long period of time. In aerobic cultures, hydrogen peroxide does not accumulate because the organisms elaborate catalase. Staphylococci possess lipolytic enzymes which render them resistant to the bacteriocidal lipids of human skin. This organism has high heat resistance for a nonendospore-forming bacteria with D-value of 60°C being reported to range from 2-50 minutes. Most chemical sanitizers used routinely in food industry including chlorine, other halogens, and quartenary ammonium compounds will destroy *S. aureus* on surfaces only when applied correctly. In addition, cells that recovered after exposure to sanitizers were subsequently more resistant to them (Bolton *et al.*, 1988; Kusumaningrum *et al.*, 2003).

Infections Caused by Staphylococci

Although *S. aureus* is an important pathogen, many healthy people carry it as part of the normal population of microorganism associated with the nose, throat, perineum or skin. It is important to know that *S. aureus* is not an important resident on human skin. It is usually regarded as a transient, pathogenic organism in the skin (Peacock *et al.*, 2001). The organism can be found in certain intertriginous areas such as the perineum of healthy people without any pathogenic event. The carrier rate varies in different populations and the nasal passages are reported to harbour *S. aureus* in 10-50% of the healthy people (Bremer *et al.*, 2004). It is a major cause of serious hospital-acquired infections. In the past decade, the prevalence of MRSA strains has increased worldwide and they now represent a serious cause of nosocomial infections in many countries. For the majority of diseases caused by *S. aureus*, pathogenesis depends on the ability of the strain to survive, multiply under a number of conditions, and produce various extracellular compounds such as haemolysins, nuclease, protease, coagulase, lipase, and enterotoxins.

Skin Infection

Skin and soft-tissue infections are among the most common infections which may lead to serious local and systemic complications. Human skin is colonized by large numbers of microorganisms. Many skin and wound infections that

complicate skin lesions are caused by mixed bacterial microbiota (Oumeish *et al.*, 2000; Brook, 2002). Hence, under normal circumstance, human skin has a high degree of natural resistance to colonization by *S. aureus* as well as other pathogenic microorganisms. Skin and soft tissue infections in hospitalized patients cause considerable morbidity with significant attributable mortality (Shmuely *et al.*, 2000). They contribute to longer hospital stays, significantly increase the cost of medical care, and likely have an important role in the development of antimicrobial resistance. Rennie *et al.* (2003) found that *S. aureus* and *Pseudomonas aeruginosa* were the most common isolates from skin and soft tissue infections in the United States and Canada.

Cellulitis

Cellulitis is a bacterial infection of the skin and subcutaneous tissues that may or may not be preceded by a traumatic break in the skin such as laceration, puncture, or varicella lesion. It presents as a localized area of tenderness, warmth, edema, and erythema of the skin, which may advance rapidly. It may involve any site on the body but is most common on the extremities. More severe or advanced disease may include fever, chills, and malaise. The most common bacterial causes are *S. aureus* and group A β-haemolytic streptococcus (Tack *et al.*, 1997; Rhody, 2000).

Cutaneous abscesses

Cutaneous abscesses begin as obstructed hair follicles that become infected, appearing as a small pustule called 'folliculis'. This lesion may expand to become a well defined, circular, tender, erythematous mass called 'furuncle' or 'boil'. *Staphylococcus aureus* is a pathogen typically recovered from cutaneous abscesses.

Impetigo

The most common skin infection in children occurs in two clinical forms; bullous (blistering) and nonbullous (crusting). Both forms involve only the most superficial layers of the skin. The nonbullous is primarily caused by *S. aureus* and group A β-haemolytic streptococcus. Bullous impetigo, also highly contagious, is caused universally by *S. aureus*.

Food Poisoning

Foodborne disease has been defined by the World Health Organization (WHO) as a disease of an infectious or toxic nature caused by the consumption of food or water (Adams & Moses, 1995). Food poisoning is a common, usually mild, but sometimes deadly illness. The Centers for Disease Control and Prevention (CDC) estimates that in the United States alone, food poisoning causes about 76 million illnesses, 325,000 hospitalizations, and up to 5,000 deaths each year (Centers for Disease Control and Prevention, 2000). In most cases of foodborne illness, we consider the pathogenic effect occurs in

the alimentary tract giving rise to symptoms such as diarrhoea and vomiting. Food poisoning can be caused by foodborne bacterial, viral, or protozoal pathogens that produce toxins and/or invade tissue of the gastrointestinal tract. Important foodborne pathogens commonly encountered in our daily life are listed in Table 1.

Table 1. Important food poisoning organisms

Organisms	Infective dose	Incubation time	Symptoms	Reference(s)
Aeromonas spp.	10^6-10^8	6-48 h	D, A, DH	Kirov, 1993
Bacillus cereus (diarrhoeal)	10^5-10^7	1-6 h	D, A, DH	Kramer & Gilbert, 1989
Bacillus cereus (emetic)	$\geq 10^5$	6-12 h	V, A	Kramer & Gilbert, 1989
Campylobacter spp.	10^3-10^5	2-5 days	F, BD	Butzle & Oosterom, 1991
Clostridium botulinum	Toxins	12-36 h	V, A, D, ND	Hauschild, 1989
Clostridium perfringens	10^8	6-16 h	D, A, DH	Granum, 1990
Escherichia coli (LT)	10^5-10^8	16-18 h	BD, A, DH	Doyle & Padhye, 1989
Escherichia coli (ST)	10^5-10^8	4-6 h	D, F	Doyle & Padhye, 1989
Listeria monocytogenes	10^7-10^8	1-90 days	I, M	Schuchat *et al.*, 1991
Salmonella spp.	10-10^6	7 h-21 days	V, D, F, A, DH	D'Aoust, 1989
Shigella spp.	10^2-10^5	1-7 days	D, F, A, BD, DH	Wachsmuth & Morris, 1989
Staphylococcus aureus	Toxins	1-6 h	V, A, D	Bergdoll, 1989
Vibrio cholerae	10^8	2-5 days	D, A, DH	Wachsmuth *et al.*, 1994
Vibrio parahaemolyticus	10^3-10^4	9-24 h	A, V, F	Adams & Moses, 1995
Yersinia enterocolitica	10^7	2-7 days	V, D, A, DH	Kapperud, 1991

A = abdominal pain; BD = bloody diarrhoea; D = diarrhoea; DH = dehydration; F = fever; I = influenza-like; LT = heat-labile toxin; M = meningitis; ND = neurological disturbances; ST = heat-stable toxin; V = vomiting.

Staphylococcal food poisoning is a prevalent cause of foodborne disease worldwide (Shimizu *et al.*, 2000; Jablonski & Bohach, 2001). The organism is an important foodborne pathogen due to its ability to produce enterotoxins. When large numbers of enterotoxigenic staphylococci grow in foods, they elaborate enough toxin to cause food poisoning after the foods are ingested.

An outbreak of acute gastroenteritis in an Australian boarding school was reported where *S. aureus* was implicated. About 101 out of 113 cases were hospitalized, while the causative agent was isolated from stool specimens and swab of palmar skin lesion of one of the healthy kitchen worker (Schmid *et al.*, 2007). In a study of food poisoning in England, the most prevalent contaminated foods (75%) were meat (ham), poultry or their products. Other contaminated food products included fish and shell-fish and milk products. Most contamination took place in the home followed by restaurants and food stores (Wieneke *et al.*, 1993). Minor outbreaks of staphylococcal food poisoning are not usually reported and true incidence is probably underestimated. Nevertheless, staphylococcal food poisoning represents a considerable social burden in terms of hospital expenses, loss of patients' working days and productivity, together with the cost of disposing the contaminated food (Normanno *et al.*, 2005).

Types of staphylococcal enterotoxin

Staphylococcal enterotoxin (SE) functions both as potent gastrointestinal toxins as well as superantigens that stimulate non-specific T-cell proliferation (Balaban & Rasooly, 2000). When grown in artificial media, pathogenic staphylococci release a number of different exotoxins whose production is stimulated in an atmosphere of 30% carbon dioxide. The enterotoxins are thermostable protein, resistant to proteolytic enzymes such as rennin, papain, and chymotrypsin but sensitive to pepsin at pH 2 (Balaban & Rasooly, 2000; Jay, 2000), forming a single chain with a molecular weight ranging from 26,000-29,600 Da. They are produced during phases of growth, but mainly during the middle and at the end of the exponential phase. Eighteen types of SEs, SEA-SEU, are currently known (Su & Wong, 1995; Munson *et al.*, 1998; Fitzgerald *et al.*, 2001; Letertre *et al.*, 2003; Blaiotta *et al.*, 2004). Five major antigenic types of SE (SEA-SEE) (Dinges *et al.*, 2000) and four additional SEs (SEG-SEJ) have been reported, and their corresponding genes have been described (Ren *et al.*, 1994; Munson *et al.*, 1998). The amount of enterotoxin necessary to cause intoxication is very small. Ingestion of 100-200 ng of enterotoxin can induce symptoms of food poisoning (Bergdoll, 1988; Evenson *et al.*, 1988). In an outbreak of gastroenteritis in United States due to chocolate milk containing SEA, the mean amount of SEA in the 400 ml container was 0.5 ng/ml and a total dose of about 200 ng (Evenson *et al.*, 1988). The symptoms of staphylococcal food poisoning usually develop within 4 h after the ingestion of contaminated food, although a range of 1-6 h has been reported (Dinges *et al.*, 2000). The symptoms may include nausea, vomiting, abdominal cramps (which are usually severe), diarrhoea, sweating, headache, prostration, and sometimes a fall in body temperature. The intoxication is not lethal, mortality rate is very low or nil (Dinges *et al.*, 2000; Jay, 2000; Le Loir *et al.*, 2003). The elderly are more susceptible to morbidity and mortality from foodborne induced gastroenteritis than younger individuals (Balaban & Rasooly, 2000).

Systemic Infections

The virulence of *S. aureus* infection is remarkable, given that the organism is a commensal that colonizes the nares, maxillae, vagina, pharynx, or damaged skin surfaces (Hoeger *et al.*, 1992; van Belkum, 2006). Infections are initiated when a breach of the skin or mucosal barrier allows staphylococci access to adjoining tissues or the bloodstream. Systemic infections caused by *S. aureus* include the blood stream, bone, heart, lung, and meninges (Drinka *et al.*, 2001). The organism has been reported as an active agent of nosocomial bacterial meningitis and associated with a high mortality rate (Arda *et al.*, 2005). The most frequently seen nosocomial Gram-positive bacterial infections were primary bloodstream infections (Celebi *et al.*, 2007). Bacteraemia caused by *S. aureus* continues to be a common problem worldwide. Coagulase-negative staphylococci are the most common nosocomial Gram-positive bacteria isolated, followed by *S. aureus*. Rates of *S. aureus* bacteraemia are published performance indicators for hospital-acquired infection. It was reported that *S. aureus* bacteraemia in adults was often associated with central venous catheters, mortality was high and up to 40%, whilst both neonates and children had a lower mortality and a lower incidence of MRSA (Denniston & Riordan, 2006). There was no significant difference between rates of disseminated infection for MRSA-infected patients and methicillin-sensitive *Staphylococcus aureus* (MSSA) infected patients, though the rate of death due to disseminated infection was significantly higher than death due to uncomplicated infections (Melzer *et al.*, 2003). The proportion of patients whose death was attributable to MRSA was significantly higher than that for MSSA (Wang *et al.*, 2008).

The bacterium has a diverse arsenal of components and products that contribute to the pathogenesis of infection. These components and products have overlapping roles and can act either in concert or alone. A great deal is known about the contribution of these bacterial factors to the development of infection (Rott & Fleischer, 1994; De Kimpe *et al.*, 1995; Jonsson *et al.*, 2002; Iwatsuki *et al.*, 2006; Clarke *et al.*, 2007). Staphylococcal bacteraemia may be complicated by endocarditis, metastatic infection, or the sepsis syndrome (Willcox *et al.*, 1998; Uckay *et al.*, 2007). The endothelial cell is central to these pathogenic processes. Not only is it a potential target for injury, but also its activation contributes to the progression of endovascular disease (Kerdudou *et al.*, 2006; Heying *et al.*, 2007). Staphylococci adhere to endothelial cells and bind through adhesion-receptor interactions (Peacock *et al.*, 1999; Soderquist *et al.*, 1999). *In vitro* studies demonstrate that after adherence, staphylococci are phagocytized by endothelial cells (Vann & Proctor, 1987; 1988). The intracellular environment protects staphylococci from host defense mechanisms as well as the bactericidal effects of antibiotics. Intraendothelial-cell milieu fostered the formation of small-colony variants. These factors might enhance bacterial survival and contribute to the development of persistent or recurrent

infections (von Eiff *et al.*, 2000). Staphylococcal strains that cause endocarditis are resistant to serum adhere to both damaged and undamaged native valvular surfaces, platelet microbicidal proteins, and elaborate proteolytic enzymes that facilitate spread to adjacent tissues (Wu *et al.*, 1994; Fowler *et al.*, 2000). The adherence of staphylococci to the platelet-fibrin thrombus that forms on damaged valvular surfaces may involve the adherence of microbial surface components recognizing adhesive matrix molecule (Wann *et al.*, 2000; Rivas *et al.*, 2004). The invasion of endothelial cells by *S. aureus* may initiate the cellular alterations, including the expression of tissue factor, that promote the formation of vegetations (Vercellotti *et al.*, 1984; Menzies & Kourteva, 2000; Park *et al.*, 2007). The capacity to invade endovascular tissue also favours spread to other tissues. The tissue tropism of *S. aureus* cannot be explained solely on the basis of patterns of blood flow. Microbial surface components recognizing adhesive matrix molecule may mediate the adherence of staphylococci to exposed matrix molecules in the presence of endovascular injury, as a means of tissue invasion. In staphylococcal infection, monocytes and macrophages have a central role, although polymorphonuclear leukocytes, endothelial cells, and platelets also play a part. The monocytes release tumor necrosis factor α and interleukin-1, interleukin-6, and interleukin-8 after contact with intact staphylococci, peptidoglycan, or lipoteichoic acid (Heumann *et al.*, 1994; Verdrengh & Tarkowski, 2000). In contrast, the expression of interleukin-1 and interleukin-6 by endothelial cells requires bacterial phagocytosis. As a result of cytokine and cellular activation, the complement and coagulation pathways are activated, arachidonic acid is metabolized, and platelet activating factor is released. Peptidoglycan, especially when combined with lipoteichoic acid, reproduces many of the physiologic responses of endotoxin in animal models of sepsis (Spika *et al.*, 1982). In animal models, α-toxin alone reproduces many of the findings of sepsis, including hypotension, thrombocytopenia, and reduced oxygenation (Buerke *et al.*, 2002).

PROBLEMS ENCOUNTERED WITH ANTIBIOTIC TREATMENT

Antimicrobial resistance has been considered as a major problem in public health. Until recently, staphylococci were generally MSSA strains and therefore susceptible to β-lactam antibiotics. However, in the late 1990s, reports of a wide spectrum of infections in children caused by community-acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) have been frequently documented. Since then, CA-MRSA has replaced MSSA as the predominant pathogen.

The mechanisms of acquisition of resistance in *S. aureus* are classified into two main categories: (i) mutation of a bacterial gene on the chromosome and (ii) acquisition of a resistance gene from other organisms by some form of genetic exchange (conjugation, transduction, or transformation) (Ito &

Hiramastu, 2003). Beta-lactams inhibit bacterial growth by interfering with cell wall assembly. They bind to the active site of a series of membrane-bound enzymes, penicillin binding proteins (PBPs), responsible for inserting the peptidoglycan precursors into the nascent cell wall (Ghuysen, 1994; Goffin & Ghuysen, 1998). Several of these PBPs are bifunctional and retain both a transglycosidase and a transpeptidase activity. The organism carries one bifunctional PBP (PBP2) and three monofunctional transpeptidases (PBP1, PBP3, and PBP4). Transpeptidation takes place at the D-ala-D-ala terminal of the precursor. Beta-lactams are mechanism-based inhibitors of this transpeptidation step. They compete with the wall precursor for binding to the active site of the enzyme and undergo nucleophilic attack at their C=O residue in a similar manner to the PBP natural D-ala-D-ala substrate. However, unlike natural D-ala-D-ala, β-lactam-PBP acyl adduct is stable, resulting in irreversible blockage of PBP function.

The most common mechanism of resistance of *S. aureus* to β-lactams is mediated by penicillinase, which hydrolyses penicillinase-susceptible compounds and is encoded by the *blaZ* gene which is usually carried on a plasmid (Ghuysen, 1994). Because of this, penicillin is no longer effective for the treatment of most *S. aureus* infections. Beta-lactamase-resistant penicillins, such as oxacillin and methicillin, are the drugs of choice against *S. aureus* that produce β-lactamase.

Methicillin is a semisynthetic β-lactamase-resistant penicillin. Introduced clinically in 1959, the first strain of MRSA was identified in 1961 (Jevons *et al.*, 1963), and is now endemic around the world. MRSA produce newly acquired PBP2A which is a wall-building transpeptidases that resists blockage by β-lactams. PBP2A is an absolute requirement for high-level β-lactam-resistance in MRSA (Chambers *et al.*, 1985). Blocking its activity in isolation, as in PBP2A negative mutants, restores susceptibility to β-lactams. However, inhibition of such mutants still requires β-lactams in order to block their remaining native PBPs. Conversely, the few staphylococci expressing borderline methicillin-resistance by over expression of penicillinase are not clinically relevant.

Methicillin-resistant *S. aureus* strains have been reported in major food animals and this was attributed to the extended use and misuse of antibiotics in animal husbandry (Lee, 2003; Kitai *et al.*, 2005; Pesavento *et al.*, 2007). In the analysis of 1,913 specimens from milk and meat of beef, pork and chicken meat, 15 strains of *S. aureus* harbouring the *mecA* gene were isolated (Lee, 2003). A community-acquired MRSA infection was reported when a family was involved in an outbreak after eating baked pork meat, contaminated from the food handler (Jones *et al.*, 2002). When a few cells of *S. aureus* enter an immunocompetent host, they are destroyed by gastric juices, but when an immunocompromised patient's food contains cells of *S. aureus*, they can reach the circulatory system and cause infections that may evolve to septicaemia (Pesavento *et al.*, 2007).

Vancomycin is a useful antibiotic against Gram-positive pathogens. In 1996, the first *S. aureus* strain with reduced susceptibility to vancomycin, designated VRSA for vancomycin-resistant *S. aureus* (Hiramatsu *et al.*, 1997) was reported while glycopeptide-intermediate *S. aureus* (GISA) was isolated from a Japanese patient who contracted vancomycin-refractory surgical incision site infection (Centers for Disease Control and Prevention, 1997; Tenover *et al.*, 1998). Chang *et al.* (2003) described a patient infected with fully VRSA that contained vancomycin-resistance gene (*vanA*). Although the acquired vancomycin-resistance genes including *vanA*, *vanB*, *vanD*, *vanE*, *vanF*, and *vanG* have been reported in vancomycin-resistant enterococci, these genes have not previously been identified in any clinical isolates of *S. aureus*. However, conjugative transfer of the *vanA* gene from enterococci to *S. aureus* has been demonstrated *in vitro* (Noble *et al.*, 1992). They suspected the *vanA* detected in the current patient's VRSA isolate probably originated in vancomycin-resistant *Enterococcus faecalis*, which was also isolated from the patient.

PREVALENCE OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* INFECTIONS

It is obvious that the increasing prevalence of MRSA is a worldwide problem, affecting both affluent and poor countries. Infections caused by multiple-antibiotic-resistant *S. aureus* strains including MRSA are particularly difficult to treat. Infections with MRSA are often associated with higher mortality and increased healthcare costs compared to methicillin-sensitive strain infections (Cosgrove *et al.*, 2003). Numerous surveillance studies confirm that methicillin resistance is common among *S. aureus* isolates in Africa, Asia, Australia, Europe, and the USA, in both hospital- and community-acquired infections (Table 2). In the USA, approximately 60% of staphylococcal infections in the intensive care unit are now caused by MRSA (Rice, 2006). In Europe, MRSA prevalence rates vary between countries, from less than 1% in northern Europe to greater than 40% in Southern and Western Europe (Tiemersma *et al.*, 2004). In Asia, several research teams collected data on MRSA reported that the prevalence of MRSA in hospital-acquired infections varied from less than 10% to greater than 60%.

Table 2. Studies on the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospital-and community-acquired infections

Nation	Percentage of MRSA (Year)	Reference(s)
Australia	24% (2005); 22.5-43.4% (2005)	Nimmo *et al.*, 2007; Turnidge *et al.*, 2007
Austria	8.2-15.8% (1994-1998)	Assadian *et al.*, 2003
Belgium	42.9% (2002-2004)	Libert *et al.*, 2008
Cameroon	21.3% (1996-1997)	Kesah *et al.*, 2003

Table 2. *Contd.*

Nation	Percentage of MRSA (Year)	Reference(s)
China	27.8% (1998-1999)	Bell & Turnidge, 2002
Czech Republic	5.9% (2000-2002)	Tiemersma *et al.*, 2004
Denmark	0.6% (2004)	Tiemersma *et al.*, 2004
Finland	<1.0-2.8% (1997-2004)	Kerttula *et al.*, 2007
France	33.1% (2004)	Tiemersma *et al.*, 2004
Germany	13.8% (2004)	Tiemersma *et al.*, 2004
Greece	44.4% (2004)	Tiemersma *et al.*, 2004
Hong Kong	69.8% (1998-1999); 1.9-4.2% (2005)	Bell & Turnidge, 2002; Ho *et al.*, 2007
India	54.85% (2003); 40-50% (2003-2004); 1.4% (2006)	Anupurba *et al.*, 2003; Arakere *et al.*, 2005; Patil *et al.*, 2006
Ireland	41.2% (2004)	Tiemersma *et al.*, 2004
Israel	38.4% (2004)	Tiemersma *et al.*, 2004
Italy	40.9% (2004)	Tiemersma *et al.*, 2004
Japan	69.5% (1998-1999); 4.3% (2001)	Bell & Turnidge, 2002; Hisata *et al.*, 2005
Kenya	27.7% (1996-1997)	Kesah *et al.*, 2003
Korea	58.4% (2005)	Kim *et al.*, 2007
Malaysia	35.4% (1990-1991)	Cheong *et al.*, 1994
Nigeria	29.6% (1996-1997)	Kesah *et al.*, 2003
Philippines	5% (1998-1999)	Bell & Turnidge, 2002
Singapore	62.3% (1998-1999); 43.14% (2004)	Bell & Turnidge, 2002; Hsu *et al.*, 2005
Spain	24.8% (2004)	Tiemersma *et al.*, 2004
Taiwan	39-75%	Hsueh *et al.*, 2005
Thailand	68.8% (2000-2002)	Thongpiyapoom *et al.*, 2004
Turkey	32.8% (2000-2002); 0.3	Ciftci *et al.*, 2007; Savas *et al.*, 2007
USA	43-58%	Klein *et al.*, 2007
United Kingdom	41.5% (2004)	Tiemersma *et al.*, 2004

Human isolates of *S. aureus*, unlike animal isolates, are frequently resistant to the penicillinase-resistant penicillins (Kloss & Bannerman, 1995; Tenover & Gaynes, 2000). They become methicillin resistant by the acquisition of the *mecA* gene which encodes a penicillin binding protein (PBP2a) with a low affinity for β-lactams. The strains producing PBP2a are resistant to all β-lactams (Chambers, 1997). Such organisms are also frequently resistant to most of the commonly used antimicrobial agents including the aminoglycosides, macrolides, chloramphenicol, tetracycline, and fluoroquinolones (Mandell *et al.*, 1995). Normanno *et al.* (2007) reported presence of *mecA* in 6 out of 160 *S. aureus* strains analyzed and all of the MRSA strains were able to synthesize enterotoxins.

ANTIBACTERIAL MECHANISMS OF MEDICINAL PLANTS

Although medicinal plants have been historically used for infectious diseases throughout the world, very few of them have been validated by scientific data (Table 3). Very limited reports have been focused on the antibacterial properties and the mechanisms of action of certain plant species and plant-derived compounds (Table 4).

Metabolic profiling (metabolomics/metabonomics) is the measurement in biological systems of the complement of low-molecular-weight metabolites and their intermediates that reflects the dynamic response to genetic modification and physiological, pathophysiological, and developmental stimuli. Nowadays, this high-throughput method is introduced to provide some useful information on metabolism and mechanisms of action of some plants (Yi *et al.*, 2007; Yu *et al.*, 2007a; 2007b). Main active antimicrobial components, palmatine and jatrorrhizine, isolated from *Radix tinosporae* have been reported to be responsible for anti-staphylococcal mechanisms and the antibacterial mode of action is similar to those of rifampicin and norfloxacin, which act on nucleic acid (Yu *et al.*, 2007a; 2007b).

The cell wall of staphylococci represents a surface organelle which is composed of a primary polymer, peptidoglycan, and secondary polymers such as protein, carbohydrates, and teichoic acids that are immobilized in the peptidoglycan scaffold. Teichoic acids are essential polyanionic polymers of the cell wall of Gram-positive bacteria, which appear to extend to the surface of the peptidoglycan layer. They can be either covalently linked to N-acetylmuramic acid of the peptidoglycan layer, wall teichoic acids, or anchored into the outer leaflet of the cytoplasmic membrane via a glycolipid, lipoteichoic acids. Penicillin-binding proteins are a set of enzymes catalyzing terminal reactions of bacterial peptidoglycan biosynthesis (Roychoudhury *et al.*, 1994; Wada & Watanabe, 1998; Leski & Tomasz, 2005). The 30-100 nm thick cell wall in *S. aureus* is composed of the repeating disaccharide N-acetylmuramic acid-(β1-4)-N-acetylglucosamine (MurNAc-GlcNAc). MurNAc is amide-linked to alanine of the cell wall tetrapeptide, L-Ala-D-isoGln-L-Lys(NH2-Gly5)-D-Ala, which is linked to adjacent strands of tetrapeptide through a pentaglycine cross-bridge (Thiemermann, 2002).

Epicatechin gallate may interfere with peptidoglycan synthesis according to the appearance of thickened wall when bacteria were grown in the presence of epicatechin gallate (Stapleton *et al.*, 2004b). Pseudomulticellular aggregates with thickened internal cell walls have also been detected in MRSA treated with an active principle of crude tea extract (*Camellia sinensis*) (Hamilton-Miller & Shah, 1999). In addition, epigallocatechin gallate, mixed with *S. aureus* cells at 4MIC, gave rise to a moderate increase in permeability of the staphylococcal cytoplasmic membrane (Stapleton *et al.*, 2004b). Several studies have further indicated that epicatechin gallate and epigallocatechin gallate have the capacity to

Table 3. Comparisons of anti-staphylococcal activity of medicinal plants

Plant species	Family	Plant part	MIC (μg/ml)	Active constituents	Reference(s)
Achillea clavennae	Asteraceae	Aerial parts	14.3[a]	Apigenin, centaureidin	Stojanovic *et al.*, 2005
Alchornea cordifolia	Euphorbiaceae	Leaves/stem bark	1600-3200	Gallic acid, protocatechuic acid, triisopentenyl guanidine	Pesewu *et al.*, 2008 Igbeneghu *et al.*, 2007
Allium cepa	Allium	Essential oils	<50	Allicin-derived organo-sulphur compounds	Corzo-Martinez *et al.*, 2007 Benkeblia *et al.*, 2004
Allium sativum	Allium	Essential oils	<50	Allicin-derived organo-sulphur compounds	Corzo-Martinez *et al.*, 2007 Benkeblia *et al.*, 2004
Aloe barbadensis	Liliacae	Leaves	2500	Anthraquinones	Habeeb *et al.*, 2007
Alpinia galangal	Zingiberaceae	Rhizomes	320-780	Phenolic compounds	Norajit *et al.*, 2007 Oonmetta-aree *et al.*, 2006
Aralia continentalis	Araliaceae	Root	8-16	Continentalic acid	Jeong *et al.*, 2006
Arnebia euchroma	Boraginaceae	Root	1.56-6.25	Naphthazarins	Shen *et al.*, 2002
Atuna racemosa	Chrysobalanaceae	Kernel	16-32	Not reported	Buenz *et al.*, 2007
Caesalpinia paraguariensis	Fabaceae	Legume	64	Oleanolic acid	Woldemichael *et al.*, 2003a
Caesalpinia sappan	Leguminosae	Heartwood	156-2500	Flavonoids	Kim *et al.*, 2004
Caiophora coronata	Loasaceae	Aerial parts	4	Triterpene	Khera *et al.*, 2003
Calceolaria pinifolia	Scrophulariaceae	Aerial parts	2-8	Diterpenes	Woldemichael *et al.*, 2003b
Camellia japonica	Theaceae	Petals	14.0[a]	Fumaric acid	Kim *et al.*, 2001
Camellia sinensis	Theaceae	Leaves	280	Epigallocatechin gallate epigallocatechin catechin gallate	Hamilton-Miller & Shah, 2000
Citrus bergamia	Rutaceae	Peel	1.0 [b]	Citral, limonene, linalool	Fisher & Phillips, 2006
Citrus limon	Rutaceae	Peel	>4.0 [b]	Citral, limonene, linalool	Fisher & Phillips, 2006
Citrus paradisi	Rutaceae	Peel	17.5-100	Bergamottin epoxide grapefruit oil	Abulrob *et al.*, 2004 Williams *et al.*, 2007
Cordia gilletii	Boraginaceae	Root/stem bark	125-250	Alkaloids, saponins, terpenoids, tannins	Okusa *et al.*, 2007

Table 3. *Contd.*

Plant species	Family	Plant part	MIC (μg/ml)	Active constituents	Reference(s)
Cornus officinalis	Cornaceae	Fruits	18.0 [a]	Not reported	Mau *et al.*, 2001
Curcuma longa	Zingiberaceae	Rhizome	>200	Bisdemethoxycurcumin, curcumin demethoxycurcumin	Kim *et al.*, 2005 Park *et al.*, 2005
Dodonaea angustifolia	Sapindaceae	Leaves/twigs	391-1560	Flavonoidvan	van Heerden *et al.*, 2000 Thring *et al.*, 2007
Dryopteris crassirhizoma	Aspidiaceae	Rhizomes	7.8-31.25	Phenolic compounds	Kwon *et al.*, 2007
Eremophila serrulata	Myoporaceae	Leaves	62-250	*O*-naphthoquinone serrulatane diterpenoid	Ndi *et al.*, 2007a Ndi *et al.*, 2007b
Erythrina subumbrans	Leguminosae	Stems	0.38-12.5	Pterocarpans	Rukachaisirikul *et al.*, 2007
Garcinia nigrolineata	Guttiferae	Twigs/stem bark	2	Brasilixanthone, latisxanthone D, nigrolineaxanthone F	Rukachaisirikul *et al.*, 2005
Garcinia scortechinii	Guttiferae	Latex/stem bark	2	Caged-polyprenylated xanthones	Sukpondma *et al.*, 2005
Guatteria multivenia	Annonaceae	Root	3.13	Sesquiterpenoids	Zhang *et al.*, 2002
Helichrysum italicum	Compositae	Flowers	125-500	Flavonoids	Nostro *et al.*, 2001a
Hypericum beanii	Clusiaceae	Aerial parts	16-32	Acylphloroglucinol	Shiu & Gibbons, 2006
Hypericum perforatum	Clusiaceae	Aerial parts	1	Hyperforin	Reichling *et al.*, 2001
Iostephane heterophylla	Asteraceae	Root	16-32	Xanthorrizol	Mata *et al.*, 2001
Lippia alba	Verbenaceae	Root	200-500	Essential oil (37% citral, 15% myrcene, 5% geraniol, 5% germacrene)	Oliveira *et al.*, 2006 Hennebelle *et al.*, 2008
Magnolia officinalis	Magnoliaceae	Stem bark	6.25-25	Neolignans	Syu *et al.*, 2004
Melaleuca alternifolia	Myrtaceae	Leaves	0.25 [b]	Linalool, terpinen-4-ol, α-terpineol	Cox *et al.*, 2001; Carson *et al.*, 2006

Table 3. *Contd.*

Plant species	Family	Plant part	MIC (µg/ml)	Active constituents	Reference(s)
Nepeta cataria	Labiatae	Leaves	0.1	Alkaloids, flavonoids, terpenes	Nostro *et al.*, 2001b
Phrygilanthus acutifolius	Loranthaceae	Flowers	625	Not reported	Daud *et al.*, 2005
Pinus nigra	Pinaceae	Immature cones	32-64	Diterpene isopimaric acid	Smith *et al.*, 2005
Piper longicaudatum	Piperaceae	Leaves/twigs	4.5	Asebogenin	Joshi *et al.*, 2001
Planchonia careya	Lecythidaceae	Leaves	0.8-9	Gallocatechins α-dimorphecolic acid hyptatic acid-A	McRae *et al.*, 2008
Plectranthus cylindraceus	Labiatae	Aerial parts	7.8-6205	Carvacrol/ α-terinolene	Marwah *et al.*, 2007
Plectranthus grandidentatus	Labiatae	Aerial parts/root	0.98-15.63	Abietanes (diterpenes)	Gaspar-Marques *et al.*, 2006
Punica granatum	Punicaceae	Rind	1 [b]	Ellagic acid, gallagic acid, punicalins, punicalagins	Reddy *et al.*, 2007
Quercus infectoria	Fagaceae	Nutgall	125	Gallic acid, tannic acid, ellagitannin	Voravuthikunchai & Kitpipit, 2005 Chusri & Voravuthikunchai, 2008, 2009
Rhodomyrtus tomentosa	Myrtaceae	Leaves	0.5	Rhodomyrtone	Saising *et al.*, 2008
Salvia officinalis	Lamiaceae	Leaves	4-100	Oleanolic acid, ursolic acid	Horiuchi *et al.*, 2007
Sesuvium portulacastrum	Alzoaceae	Leaves	12.1 [a]	Monoterpene	Magwa *et al.*, 2006
Turraeanthus africanus	Meliaceae	Stem bark	not reported	Diterpenoids	Tatsimo *et al.*, 2005
Zataria multiflora	Lamiaceae	Epicarps	0.1-0.4 [b]	Not reported	Fazeli *et al.*, 2007

[a] Mean values of inhibition zones (mm)

[b] Minimum inhibitory concentration (%, v/v)

Table 4. The effects of selected medicinal plant on *Staphylococcus aureus* cells

Biological effects	Medicinal plants	Reference(s)
Nucleic acid	*Berberis* spp.	Yi *et al.*, 2007
	Tinospora capillipes	Yu *et al.*, 2007a
Cytoplasm	*Alpinia galanga*	Oonmetta-aree *et al.*, 2006
Cytoplasmic membrane	*Alpinia galanga*	Oonmetta-aree *et al.*, 2006
	Camellia sinensis	Stapleton *et al.*, 2006
	Helichrysum aureonitens	Cushnie & Lamb, 2005a, 2005b
		Cushnie *et al.*, 2007
	Hypoxis rooperi	Laporta *et al.*, 2007
	Melaleuca alternifolia	Carson *et al.*, 2002
	Origanum vulgare	Lambert *et al.*, 2001
	Podocarpus spp.	Micol *et al.*, 2001
	Quercus infectoria	Chusri & Voravuthikunchai, 2009
Cell wall	*Camellia sinensis*	Hamilton-Miller & Shah, 1999
	Hemsleya pengxianensis	Biao-Yi *et al.*, 2008
	Melaleuca alternifolia	Carson *et al.*, 2002
Protein synthesis	*Aquilegia oxysepala*	Yu *et al.*, 2007b
	Tabebuia avellanedae	Pereira *et al.*, 2006
Virulence factors	*Helichrysum italicum*	Alonzo, 2001; Nostro *et al.*, 2001a; Nostro *et al.*, 2002
(adhesion/enzymes/toxins)	*Caesalpinia sappan*	Kim *et al.*, 2004
	Camellia sinensis	Lee *et al.*, 2006
	Curcuma longa	Kim *et al.*, 2005
	Nepeta cataria	Nostro *et al.*, 2001b
	Rhodomyrtus tomentosa	Limsuwan & Voravuthikunchai, 2008

reduce oxacillin-resistance in *S. aureus* (Shiota *et al.*, 1999; Hamilton-Miller & Shah, 2000; Stapleton & Taylor, 2002; Stapleton *et al.*, 2004b). The peptidoglycan binding capacity of epigallocatechin gallate was discussed as being the reason for its synergistic effect with oxacillin. However, Zhao *et al.* (2001) have mentioned that the binding of epigallocatechin to penicillin-binding protein 2′ was not relevant to any synergy with β-lactam antibiotics. In addition, the observed restoration of β-lactam antibiotic activity might be explained by the presence of hydrolysable tannins, corilagin and tellimagrandin I, and a polymeric proanthocyanidin, ZP-CT-A that suppressed the activity of β-lactamase (Shiota *et al.*, 2004; Kusuda *et al.*, 2006). We are now closely investigating the anti-MRSA mechanism of *Quercus infectoria* (nutgalls) extract as well as its effective components. The appearance of pseudomulticellular bacteria in the nutgall ethanol extract treated cells and the synergistic effect of the plant extract with β-lactamase susceptible penicillins indicated that the extract may interfere with staphylococcal enzymes including autolysins and β-lactamase (Chusri & Voravuthikunchai, 2009).

The adherence of microorganisms is mediated by a variety of specific factors such as bacterial adhesins (Liang & Ji, 2007) or nonspecific factors such as hydrophobicity and electrostatic charge (Lerebour *et al.*, 2004). Bacterial cell surface hydrophobicity (CSH) is one of the most important factors that govern initial bacterial adhesion to various surfaces such as medical devices (Hanlon *et al.,* 2004), teeth (Prabu *et al.,* 2006), contact lenses (Bruinsma *et al.,* 2001), and glass surface (Nostro *et al.,* 2004).

Many reports confirmed that the influence of anti-infective agents on CSH would be important for anti-adhesion and anti-biofilm formation of treated organisms (Nostro *et al.,* 2004; Prabu *et al.,* 2006; Walencka *et al.,* 2007; Wojnicz & Jankowski, 2007). There was a statistical correlation between CSH of microorganisms and their capability to adhere to human cell or biological surfaces (Grivet *et al.*, 2000; Malm *et al.*, 2005; Pan *et al.*, 2006). Several researchers have reported that sub-MICs of some antibiotics normally decrease the surface hydrophobicity and the ability of bacteria to adhere to epithelial (Furneri *et al.*, 2003; Kustos *et al.*, 2003; Wojnicz & Jankowski, 2007). Recently, the influence of the ethanolic extract of bearberry on the CSH of 25 food-related bacteria was determined by bacterial adhesion to hydrocarbon (BATH) assay. The presence of this extract caused a significant increase in hydrophobicity index (HPBI) of most test bacteria (14/25), while a significant decrease in HPBI was found in four bacteria (Dykes *et al.*, 2003). Puupponen-Pimia *et al.* (2005) hypothesized that antimicrobial activity of berries may also be related to antiadherence of bacteria to epithelial cells, which is a prerequisite for colonisation and infection of many pathogens. We have reported a number of plant extracts with the ability to increase cell aggregation in *E. coli* O157: H7 (Voravuthikunchai & Limsuwan, 2006). Closer investigations on certain plant species including *Quercus infectoria* and *Punica granatum* have demonstrated the effects of both plant species on cell surface hydrophobicity of clinical isolates of *Helicobacter pylori*, irrespective of their antibiotic resistance patterns (Voravuthikunchai *et al.*, 2006b). The adherence characteristic of *S. aureus* is necessary for the strains that attached to superficial skin tissue, but not essential for the strains that had infiltrated the deep skin tissues (Akiyama *et al.*, 2000). A limited study on plant extracts revealed both enhancing and lowering of the CSH of bacteria (Annuk *et al.*, 1999; Dykes *et al.*, 2003; Nostro *et al.*, 2004; Ishida *et al.*, 2006; Prabu *et al.*, 2006; Voravuthikunchai & Limsuwan, 2006). Contrastly, an enhancing of cell aggregation of all test strains of *H. pylori* by salt aggregation test was observed in the presence of bearberry and cowberry extracts, the plant extracts with a high amount of tannins (Annuk *et al.*, 1999).

The cytoplasmic membrane of microorganisms is composed essentially of a phospholipid bilayer with embedded proteins. It is semipermeable and regulates the transfer of solutes and metabolites in and out of the cell

cytoplasm. It is also associated with several enzymes involve in various metabolic functions as well as is often considered as the major target site for biocides (Maillard, 2002). The disruption of bacterial cytoplasmic membrane has been discussed as a mode of actions of the plant compounds including epigallocatechin gallate (Ikigai *et al.*, 1993), epicatechin gallate (Stapleton *et al.*, 2006), galangin (Cushnie & Lamb, 2005), galangal (Oonmetta-aree *et al.*, 2006), and essential oils (Carson *et al.*, 2002; Trombetta *et al.*, 2005). Membrane damages can take several forms such as total disruption causing cell lysis, dissipation of the proton motive force, inhibition of membrane-associated enzyme activity, and leakage of the intracellular constituents (Russell & Chopra, 1996). Some plant-derived agents caused cytoplasmic membrane damage and provokes whole-cell lysis (Carson *et al.*, 2002). Previous studies have shown the leakage of 5, 6-carboxyfluorescein from epigallocatechin gallate treated liposomes (Ikigai *et al.*, 1993). The killing effect resulted from bacterial membrane damage possibly through the interaction of the catechin with phosphatidylethanolamine (Caturla *et al.*, 2003). Catechins are capable of binding to lipid bilayer and binding affinity appears to correlate with its activity (Caturla *et al.*, 2003). Anti-staphylococcal activity of the catechins could be improved by increasing their capacity to interact with the cytoplasmic membrane (Stapleton *et al.*, 2004b). Previous work has shown that epicatechin gallate and epigallocatechin gallate, the main constituents of catechins, could reduce the tolerance of β-lactam resistant and susceptible strains of *S. aureus* to high ionic strength and low osmotic potential (Zhao *et al.*, 2001; Stapleton *et al.*, 2006). Flavonol galangin, an antimicrobial constituent of the traditional medicines propolis and *Helichrysum aureonitens*, causes staphylococcal cells to clump together may implicate the cytoplasmic membrane as a target site for the antibacterial activity of this compound (Cushnie *et al.*, 2007).

DETAILED STUDIES ON SOME SELECTED PLANT SPECIES WITH HIGH POTENCY FOR FURTHER DEVELOPMENT

Due to increasing problems with infections caused by antibiotic-resistant organisms, many researchers have investigated alternative approaches to treat staphylococcal infections. However, there have been very limited reports on the investigation of medicinal plants with antibiotic-resistant *S. aureus* in relation to its antibacterial mechanisms and its applications. Natural products including plant extracts have been extensively studied during the past decades. Herbs have been found to have antimicrobial properties for over 4,500 years. A number of plant compounds are directly antibacterial agents either producing cidal or static effects. Series of work from our laboratory have demonstrated three effective plant species against bacterial infections which are worth studying for further drug and antimicrobial agent development.

Rhodomyrtus tomentosa (Aiton) Hassk.

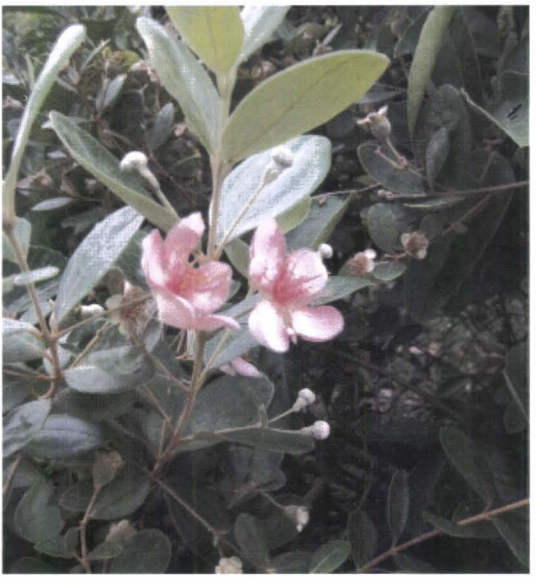


Fig 1. *Rhodomyrtus tomentosa* (Aiton) Hassk.

Family: Myrtaceae

Common names: Downy rose myrtle, Downy myrtle, Hill gooseberry, Hill guava

Botanical description

Evergreen shrub or small tree, up to four metre tall with dense, short, soft hairs on young stems. Simple, opposite, entire, elliptic-oval leaves, glossy green above, densely soft-hairy below with three conspicuous longitudinal veins. Rose-pink flowers with five hairy sepals and petals, many stamens with pink filaments, solitary or in three-flowered dichasia in upper axils. A globose, dark purple berry, with sweet, aromatic flesh fruit with many seeds.

Habitat description

Downy rose myrtle, *Rhodomyrtus tomentosa*, is an evergreen shrub native to Southeast Asia (Latiff, 1992). The plant is growing wild and cultivated in South-East Asia, India, Sri Lanka, and Southern China. However, it has been reported as serious invader of native plant communities in Florida (Winotai *et al.*, 2005). It thrives in open, often in degraded sandy sites, along the shore and on river banks. It tolerates full sun and flooding.

Traditional uses

In Malaysia, the fruits have been employed as a cure for dysentery and diarrheoa. A decoction of its roots or leaves is used for diarrhoea and stomach-ache and as a prospective medicine after birth. In Indonesia, the crushed leaves are used to dress wounds.

Chemical components

Triterpenoids, steroids, tannins, flavone glycosides, acylphloroglucinols.

Research findings

Hui *et al.* (1975) reported triterpenoids and steroids from *Rhodomyrtus tomentosa* containing lupeol, β-amyrin, β-amyrenonol, betulin, friedelin, α-amyrin, taraxerol, and two unidentified compounds. The ethanol extract of the leaves contained betulinic, ursolic, and aliphitolic acids. Two triterpenoids, hopenediol, and oleananolides were isolated (Hui & Li, 1976). Tomentosin, C-glycosidic hydrolysable tannin, was isolated from the leaves of *Rhodomyrtus tomentosa* (Liu *et al.*, 1997). Four hydrolysable tannins were isolated from the leaves and roots of *Rhodomyrtus tomentosa* and three of them were C-glycosidic hydrolysable tannins. Their structures were detected by chemical methods and spectroscopic analysis as pedunculagin, casuariin, castalagin, and tomentosin (Liu *et al.*, 1998). Three flavone glycosides and ellagitannin were isolated from the leaves including myricetin-3-O-α-L-rhamnoside, myricetin-3-O-α-L-furanoarabinoside, myricetin-3-O-α-D-glucoside, and 2,3-hexahydroxydiphenyl-D-glucose (Hou *et al.*, 1999). The ethyl acetate extract of the leaves yielded rhodomyrtone which is closely biogenetically related to acylphloroglucinols, antibiotics isolated from fern (Dachryanus *et al.*, 2002).

Although many studies in chemistry have been reported on *Rhodomyrtus tomentosa*, there are still very limited investigations relating to its antimicrobial properties. The methanol extract of the leaves of *Rhodomyrtus tomentosa* has been claimed to exert antibacterial activity against *E. coli* and *S. aureus* without supporting data (Dachryanus *et al.*, 2002). Contrastly, the ethanol extract demonstrated good activity against many Gram-positive bacteria but not Gram-negative bacteria (Voravuthikunchai *et al.*, 2007a). The good antibacterial activities of ethanol extract of *Rhodomyrtus tomentosa* leaves and twigs against Gram-positive bacteria including *B. cereus*, *E. faecalis*, *L. monocytogenes*, *Streptococcus pyogenes*, and *S. aureus* have been documented (Voravuthikunchai *et al.*, 2007a; Limsuwan & Voravuthikunchai, 2008; Voravuthikunchai *et al.*, 2008c; Limsuwan & Voravuthikunchai, 2009).

We have carried out extensive studies on the effect of *Rhodomyrtus tomentosa* on *S. aureus*. The ethanol extract of this plant species possesses

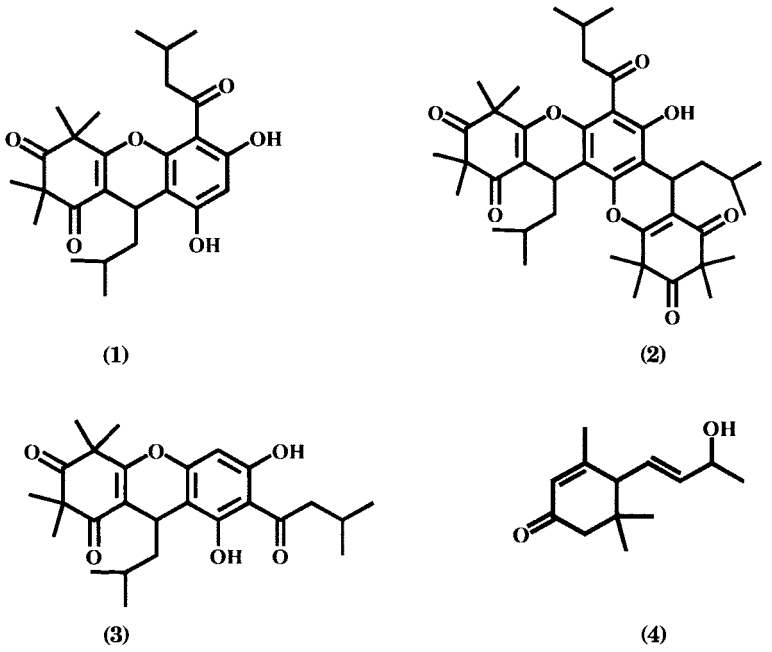


Fig 2. Two new acylphloroglucinol derivatives **(1-2)**, rhodomyrtone **(3)**, and *trans*-3-oxo-α-ionol **(4)** isolated from the leaf of *Rhodomyrtus tomentosa*

remarkable activity on coagulase-positive and coagulase-negative staphylococci isolated from acne lesions. The average inhibition zones of coagulase-positive and coagulase-negative staphylococci were 12 and 14 mm, respectively. The MICs were from 32-1024 mg/ml with similar values of the MBCs. The numbers of viable cells of the test staphylococci after exposure to 4 MIC of the extract decreased at least 3 log-fold within 6-8 hours (Saising *et al.*, 2008). The extract showed antibacterial properties against *B. cereus* isolated from contaminated food. Inhibition zones ranged from 10.90-15.45 mm. MICs and MBCs were 15.6-62.5 mg/ml. The results demonstrated that the extract killed endospores and vegetative cells within 105 minutes and 18 hours, respectively (Dola *et al.*, 2007).

Antimicrobial researches with this plant species have not been well-established and its antibacterial mechanisms have not yet published. Dachryanus *et al.* (2002) isolated an acylphloroglucinol derivative and suggested the trivial name as rhodomyrtone. We tried to isolate active compounds from this plant species (Fig 2). The results from our study further demonstrated that rhodomyrtone and two new acylphloroglucinol derivatives from the leaves exhibited strong antibacterial activity against *S. pyogenes* (Voravuthikunchai *et al.*, 2007a; Hiranrat & Mahabusarakam, 2008; Limsuwan *et al.*, 2009). The compound was very effective against *S.*

aureus with the MIC value at 0.5 μg/ml which is very close to that of vancomycin (Saising *et al.*, 2008). These compounds have similar structure to an antibiotic compound, myrtucommulone B obtained from *Myrtus communis* (Hui *et al.*, 1975).

Detailed studies on the antibacterial mechanisms of the extract of *Rhodomyrtus tomentosa* established that it produced strong inhibition on quorum sensing as well as biofilm formation in *S. pyogenes* (Limsuwan & Voravuthikunchai, 2008b). Protein expression in *S. aureus* and MRSA after treated with subinhibitory concentration of the ethanol extract of *Rhodomyrtus tomentosa* has been altered (Visutthi *et al.*, 2008). Our research is ongoing to investigate other active compounds in this plant as well as its antibacterial mechanisms. Testing on their antibacterial mechanisms and their toxicity on humans are warranted for further studies. This finding challenges further investigation of rhodomyrtone in order to develop an alternative agent for staphylococcal infections.

Quercus infectoria G. Olivier

Fig 3. *Quercus infectoria* G. Olivier

Family: Fagaceae

Common names: Downy oak, Gall nut, Oak gall tree

Botanical description

Quercus infectoria G. Olivier (Fagaceae) is a small tree or a shrub from four to six feet tall, crooked and shrubby-looking, with smooth and bright-green leaves. This tree is valued for its excrescences which are formed upon the young branches, and known in market under the names of galls or nut galls. The tree capitulate galls that emerge on its shoots as a consequence of assault of an insect, gall wasp (*Diplolepis gallae tinctoriae*, or *Cynips quercufolii*, or *Cypnis gallae tincotoriae*) for the purpose of depositing its egg (Samuelsson, 1999). They are the result of a puncture made in the bark. A small tumour soon follows the puncture, and forms a very dense mass about the egg. The egg hatches into the fly while in these tumours, eating its way by a small opening. The excrescences vary from the size of a

large pea to that of a small hickory nut, are nearly round, hard, and quite smooth with the exception of small tubercles scattered over the surface. Those in which the egg has not yet turned into larva are most compact and heavy, of a dark blue or bluish-green colour externally, grayish-brown internally, and of an almost flinty fracture. When the larva has been developed, the external colour lightens; and those of large size and grayish appearance are more or less fed upon internally by the grub, and depreciate in value. The flowers are monoecious (individual flowers are either male or female, but both sexes can be found on the same plant) and are pollinated by wind.

Habitat description

This species is most abundant in Greece, Asia Minor, and extends to middle Asia.

Traditional uses

Bark: The bark and acorns are astringent. They are used in the treatment of intertrigo, impetigo and eczema.

Nut galls: In Asia, the galls of *Quercus infectoria* have been used for centuries in oriental traditional medicines for treating inflammatory diseases (Chopra *et al.*, 1956).

The tannic and gallic acids extracted from the galls are used as dental powder and in the treatment of toothache and gingivitis. They may be used as a wash and gargle in aphthous sores and putrid sore throat, and as an injection in bad leucorrhoea; in which cases they arrest putrefactive tendencies, and may be combined with suitable stimulants. By coagulating the blood, they frequently arrest haemorrhage from small vessels; and sometimes are used for bleeding piles, both as ointment and suppository, but are inadmissible when the tumours are sensitive. They are often used in dysentery and diarrhoea. A tincture is made by macerating four ounces of galls in diluted alcohol for two days, and then treating them by percolation till a quart has passed.

Chemical components

The galls contain tannic acid (gallo-tannic acid) as the principal constituent.

Research findings

The components in *Quercus infectoria* have been studied since 1977. The main constituents found in the galls of *Quercus infectoria* are tannin (50-70%) and small amount of free gallic acid and ellagic acid (Ikram & Nowshad, 1977; Evans, 1996; Wiart & Kumar, 2001). Ikram and Nowshad (1977) isolated syringic, gallic and ellagic acid from ethanol extract from the galls. The galls of *Quercus infectoria* possess a great medicinal value and have pharmacologically been deciphered since 1976. Two fractions were prepared, including a dried acetone-treated methanol extract dissolved in water

(Fraction A) and a chloroform-methanol extraction (Fraction B). Fraction A was active as an analgesic in rats and significantly reduced blood sugar levels in rabbits. Fraction B had central nervous system depressant activity (Dar *et al.*, 1976). In 1977, the syringic acid was isolated from the galls and showed the central nervous system depressant activity (Ikram & Nowshad, 1977). Its anti-inflammatory has been documented (Kaur *et al.*, 2004). In 1983, two structural isomer of pentagalloylglucose, four isomers of hexagalloylglucose, and tetragalloylglucose were isolated from *Quercus infectoria* (Nishisawa *et al.*, 1983). Hexagalloylglucose, which was isolated from methanol extract of this medicinal plant, significantly inhibited α-glycosidase such as sucrose, maltase, and isomaltase (Hwang *et al.*, 2000). Regerat *et al.* (1989) hydrolyzed tannin extract and reported high yield of gallic acid. We have recently isolated a new compound in the group of ellagitannin from the nut galls (Fig 4).

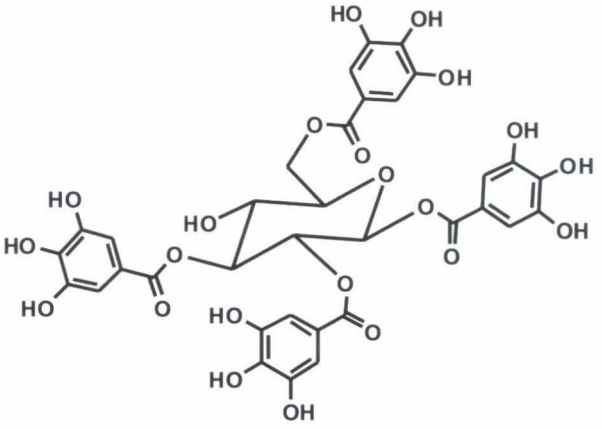


Fig 4. Ellagitannin, a new compound isolated from the nut galls of *Quercus infectoria*

Anti-amoebic (Sawangjaroen *et al.*, 2004) and anti-cariogenic (Hwang *et al.*, 2004) activities have been reported. There have been a number of reports on antibacterial activity of *Quercus infectoria* galls. Alkofahi *et al.* (1996) found that ethanol extract from this medicinal plant showed the strongest activity against *Pseudomonas mirabilis*, *Pseudomonas aeruginosa*, and *S. aureus*. Natural dye prepared from *Quercus infectoria* showed the maximum inhibition zone, thereby indicating best antimicrobial activity against *E. coli*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *P. aeruginosa* (Singh *et al.*, 2005). *Quercus infectoria* was used in different tooth powder (Farooqi *et al.*, 2001). Hwang *et al.* (2004) reported this plant showed the anticariogenic activity against *Streptococcus mutans*. We have demonstrated antibacterial activity of *Quercus infectoria* against a wide range of both Gram-positive and Gram-negative bacteria (Voravuthikunchai *et al.*, 2004a, 2004b, 2006a, 2006b, 2006c; Voravuthikunchai & Mitchell,

2008; Voravuthikunchai & Suwalak, 2008; Suwalak & Voravuthikunchai, 2009; Voravuthikunchai & Suwalak, 2009). An ethanol extract of the galls presented the antibacterial activity against all 35 clinical isolates of MRSA with MIC and MBC at 0.2-0.4 and 0.4-1.6 mg/ml, respectively (Voravuthikunchai & Kitpipit, 2005). With Gram-negative pathogenic bacteria, both aqueous and ethanol extracts of *Quercus infectoria* were highly effective against *E. coli* O157: H7 with MIC and MBC values at 0.09, 0.78, and 0.19, 0.39 mg/ml, respectively (Voravuthikunchai *et al.*, 2004b).

***Eleutherine americana* Merr.**

Fig 5. *Eleutherine americana* Merr.

Family: Iridaceae

Common names: Not known

Botanical description

Herbs perennial (or shrubs or annuals), with rhizomes, bulbs, or corms. Alternate leaves, often two-ranked, often oriented edgewise to aerial stem, usually sword-shaped to linear, parallel veined, base sheathing. The red bulb is the part used as a folk medicine and flavouring agent.

Habitat description

Eleutherine americana originates from tropical America where its elongated red tubers have been used by local inhabitants to treat numerous diseases. It has been cultivated in Javanese gardens and now can be found worldwide particularly in South Africa and rubber plantations in Java.

Traditional uses

Bulb: Dysentery, haematochezia, jaundice, prostitis, cardiac diseases especially coronary disorders.

Chemical components

Naphthoquinone, elecanacin, anthraquinones, bi-eleutherol isoeleutherine, eleutherinone, and naphthalene derivatives.

Research findings

Several compounds, including elecanacin, isoelecanacin, isoeleutherol, isoeleutherin, eleutherin, β-sitosterol, 8-hydroxy-3,4-dimethoxy-1-methylanthraquinone-2-carboxylic acid methyl ester, 4-hydroxyeleutherin, hongconin, 4,8-dihydroxy-3-methoxy-1-methyl-anthraquinone-2-carboxylic acid methyl ester, dihydroeleutherinol, and 1,3,6-trihydroxy-8-methylanthraquinone have already been isolated and identified (Hara *et al.*, 1997; Nielsen & Wege, 2006; Xu *et al.*, 2006).

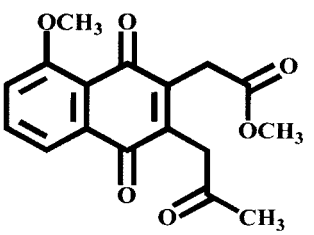
Active compounds from *Eleutherine americana* has been reported to display important biological activities. The antifungal, antiviral, and anticancer activities of some of these compounds have been studied (Hara *et al.*, 1997; Xu *et al.*, 2006). Eleutherin was described as forming a type of noncleavable complex with topoisomerase II with stereospecific and selective inhibitory activity (Hara *et al.*, 1997). Eleutherinone, eleutherine, and isoeleutherine isolated from another plant species within the same genus, *Eleutherine bulbosa*, exhibited strong antifungal activity against *Cladosporium sphaerospermum* (Alves *et al.*, 2003). Three compounds, described as, brown needle, which has a structure similar to eleutherol, yellow needle, closely related to anthraquinones, and the third compound, described as orange slice, and elucidated as anthrax-quinone were obtained from this plant. These bioactive constituents produced weak activities against *Pyricularia oryzea*, but the second compound inhibited the proliferation of human erythroleukemia cancer cell line (Xu *et al.*, 2006).

The antibacterial activities of this plant have been previously reported from our laboratory (Voravuthikunchai *et al.*, 2007a; Ifesan & Voravuthikunchai, 2009a, 2009b; Ifesan *et al.*, 2009a, 2009b, 2009c). Preliminary research from our laboratory revealed that the crude extract from *Eleutherine americana* produced good antibacterial effect on *S. aureus* isolated from foods. Its MIC values of the food isolates ranged from 0.06-1.00 mg/ml, MIC_{90} was 0.5 mg/ml while MIC_{50} was at 0.25 mg/ml. Treatment with the crude extract at 4 MIC (1 mg/ml) reduced the viability of *S. aureus* by at least 5 log cycle and kept the inoculum at lag phase for 24 hours. At 2 MIC concentration, it could inhibit the organisms by 3 log reduction. The partially-purified fractions from the crude extract, Ea 6.3 and Ea 9, produced MIC value at 0.125-0.25 mg/ml with MBCs at 0.25 and 0.5 mg/ml. These fractions were further tested against MRSA isolated from foods. Fraction

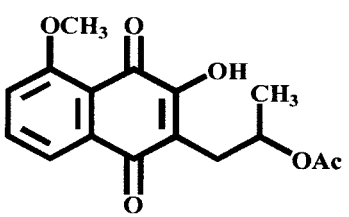
Ea 6.3 had MIC_{90} at 0.25 mg/ml while Ea 9.0 gave MIC_{50} at 0.125 mg/ml. The MBC values ranged from 0.25≥1.00 mg/ml for the two fractions on all the strains tested. Time-kill curve in the presence of Ea 6.3 at 4 MIC resulted in total killing of the cells at 20 hours for ATCC 23235 and ATCC 27664, enterotoxin-producing reference strains, and 24 hours for MRSA while fraction Ea 9.0 reduced the inoculum size by 7 log cycle (Voravuthikunchai *et al.*, 2008b). In addition, preliminary work from our research group has indicated that *Eleutherine americana* possess inhibitory activity against *S. pyogenes* isolated from throat swab as well as commonly foodborne *Campylobacter* spp. isolated from raw chickens (unpublished data).

Medicinal plants rich in naphthoquinones have been reported to possess antibacterial activity (Ambrogi *et al.*, 1970; Machado *et al.*, 2003). Naphthoquinone derivatives can act as bacterial growth inhibitors by participating in electron transport with the cell components (Holmes *et al.*, 1964). Naphthoquinones are polyphenols and known to form soluble complexes of high molecular weight with proteins, thereby, reacting with the cell enzymes in the cytoplasm and the cell wall. Also they can bind to bacterial adhesin, complex with cell wall and thus inactivate enzymes (Cowan, 1999). The improved antibacterial activity of the partially-purified fraction from *Eleutherine americana* may be attributed to the naphthoquinones. Bioactive compounds from bulbs of this plant such as naphthoquinones, eleutherin, and elecanacin have been isolated and identified (Hara *et al.*, 1997; Xu *et al.*, 2006). Two new naphthoquinones **(1-2)**, together with eleutherin **(3)**, isoeleutherin **(4)**, elecanacin **(5)**, 3,4-dihydro-8-hydroxy-1,3-dimethyl-1H-naphtho[2,3-c]pyran-5,10-dione **(6)**, eleutherol **(7)**, 8-hydroxy-3,4-dimethoxy-1-methylanthraquinone-2-carboxylic acid methyl ester **(8)**, 4,8-dihydroxy-3-methoxy-1-methylanthraquinone-2-carboxylic acid methyl ester **(9)** were further isolated from the bulb of *Eleutherine americana* by our research group (Mahabusarakam *et al.*, 2009). Compounds **(2)**, **(3)**, **(4)**, **(7)**, **(8)**, and **(9)** exhibited antibacterial activity against *S. aureus*.

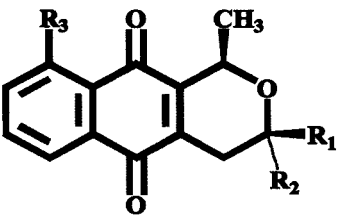
APPLICATIONS OF NATURAL PRODUCTS IN STAPHYLOCOCCAL INFECTIONS



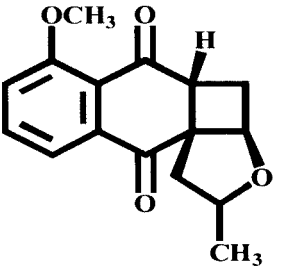
(1)



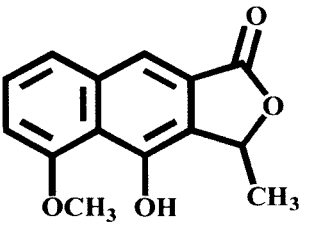
(2)



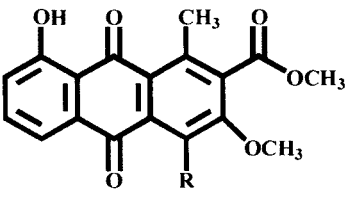
(3) $R_1 = CH_3$; $R_2 = H$; $R_3 = OCH_3$
(4) $R_1 = H$; $R_2 = CH_3$; $R_3 = OCH_3$
(6) $R_1 = CH_3$; $R_2 = H$; $R_3 = OH$



(5)



(7)



(8) R = OCH_3
(9) R = OH

Fig 6. Two new naphthoquinones (**1-2**), eleutherin (**3**), isoeleutherin (**4**), elecanacin (**5**), 3,4-dihydro-8-hydroxy-1,3-dimethyl-1H-naphtho[2,3-c]pyran-5,10-dione (**6**), eleutherol (**7**), 8-hydroxy-3,4-dimethoxy-1-methylanthraquinone-2-carboxylic acid methyl ester (**8**), and 4,8-dihydroxy-3-methoxy-1-methylanthraquinone-2-carboxylic acid methyl ester (**9**) isolated from the bulb of *Eleutherine americana*.

Healthcare-associated infections (HAIs) are a global issue affecting both patients and healthcare workers. They are the forth leading cause of death in the U.S., costing the healthcare industry more than $5 billion annually (NineSigma, 2008). Due to the significant cost of HAI related complications, awareness among healthcare workers and patients is increasingly growing. There are a number of initiatives being implemented to improve patient outcomes as well as to lower costs associated with HAIs including mandatory reporting of key HAI metrics, medicare and medicaid policies, and screening for community-acquired and antibiotic-resistant organisms.

Herbs have been reported with antimicrobial properties for over 4,500 years. According to WHO, herbal medicines serve the health needs of about 80% of the world's population, especially for millions of people in the vast rural areas of developing countries (World Health Organization, 2003). Many hundreds of plants worldwide are used in traditional medicine as treatments for bacterial infections. Some of these have been documented as effective agents for staphylococcal diseases as *in vitro* level (Table 3).

Interestingly, herb resistant bacterial strains have never been reported, and are in stark contrast to resistance often seen in pathogens treated with modern antibiotic (Lai & Roy, 2004).

Antibacterial Agents and Disinfectants

Current infection prevention practices include alcohol-based disinfectants, glutaraldehyde disinfectants, quaternary amine disinfectants, silver-based compounds and steam or gas sterilization. Important natural products against *S. aureus* infections will be discussed with specific focus on some of our findings.

Essential oils are volatile, natural, complex compounds characterized by a strong odour and are formed by aromatic plants as secondary metabolites. They are liquid, volatile, limpid and rarely coloured, soluble in lipid and organic solvents with generally lower density than that of water, and usually obtained by steam or hydro-distillation. The oils can be sensitized by all plant organs including buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood, and bark (Burt, 2004; Bakkali *et al.*, 2008). Up to the present day, these characteristics have not changed much except that more is now known about some of their antimicrobial mechanisms, particularly at antimicrobial level. Tea tree oil (TTO) is a complex mixture of terpene hydrocarbons and tertiary alcohols distilled from the leaves of the Australian native plant, *Melaleuca alternifolia* (Carson *et al.*, 2006). The pharmacology of TTO has been identified in part through bacteriologic and animal studies. Several published reports have addressed MIC and MBC of this oil against clinical isolates of *S. aureus* (Carson *et al.*, 2002; Hada *et al.*, 2003; Halcon & Milkus, 2004). The plant compounds are considered an effective decolonization agent for MRSA both *in vitro* (LaPlante, 2007) and *in vivo* (Caelli *et al.*, 2000).

Tea, a beverage has been safely consumed worldwide for centuries. Chinese green tea (*Camellia sinensis* L.) and its extract, in particular, have shown many health benefits to human and animal, including chemopreventive, anti-carcinogenic, anti-viral, and anti-microbial activities (Park & Pezzuto, 2002; Cabrera *et al.*, 2006). Important pathogens including *S. aureus*, *E. coli*, *Salmonella typhimurium*, *Listeria monocytogenes*, and *Campylobacter jejuni* have been reported to be inhibited by tea component which may be from different types of tea or tea extract (Lee *et al.*, 2003; Kim *et al.*, 2004; Taguri *et al.*, 2004; Song & Seong, 2007). Although, the effectiveness of tea catechins as anti-staphylococcal activity has been reported to be compromised by relative chemical instability and poor bioavailability, but tea catechins could be applied as a viable option as alternative medicine or as a synergistic combination therapy (Shiota *et al.*, 1999; Hamilton-Miller & Shah, 2000; Stapleton *et al.*, 2004a, 2004b; Sudano Roccaro *et al.*, 2004; Tiwari *et al.*, 2005; van Nederkassel *et al.*, 2005). The commercial product of green tea extract was particularly rich in epicatechin gallate (ECG) and

epigallocatechin gallate (EGCG), at 26% and 15%, respectively (Si *et al.*, 2006). These two principles were reported to be active against *S. aureus*, particularly EGCG with the lowest MIC_{90} values against MSSA (58 mg/l) and MRSA (37 mg/l) (Si *et al.*, 2006). Several studies have further indicated that epicatechin gallate and epigallocatechin gallate had the capacity to reduce oxacillin resistance in *S. aureus* (Shiota *et al.*, 1999; Hamilton-Miller & Shah, 2000; Stapleton & Taylor, 2002; Stapleton *et al.*, 2004a, 2004b).

Berries are traditionally an important-part of diet in many countries (Shukitt-Hale *et al.*, 2008). The most well-known and also commercially most important wild berries are lingonberry, bilberry, raspberry, cloudberry, buckthorn berry, and crowberry. Berry fruits are rich sources of bioactive compounds, such as phenolics and organic acids, which have antimicrobial activities against human pathogens (Puupponen-Pimia *et al.*, 2005a; 2005b). It was found that phenolic compounds such as ellagitannins present in berries selectively inhibited the growth of human pathogens, salmonella and staphylococcus. Especially, cranberry, cloudberry, raspberry, strawberry, and bilberry possess clear effects. Antimicrobial activity of phenolic extracts of Nordic berries was studied against selected human pathogenic microbes. The most sensitive bacteria with berry phenolics were *B. cereus*, *H. pylori,* and *S. aureus* (Nohynek *et al.*, 2006). Contrastly, flavonoids, phenolic acids, and eight extracts from common Finnish berry extracts inhibited the growth of Gram-negative but not Gram-positive bacteria (Puupponen-Pimia *et al.*, 2001). Myricetin inhibited the growth of all lactic acid bacteria derived from the human gastrointestinal tract but it did not affect the pathogenic strain.

We have previously demonstrated clearly the effectiveness of *Quercus infectoria* extract against *S. aureus*, an extremely important pathogen with an ability to develop various kinds of antibiotic resistance (Voravuthikunchai & Kitpipit, 2005; Chusri & Voravuthikunchai, 2008). In addition, the nutgalls have been reported as an approach for alternative treatment against a wide range of bacterial infections according to its extremely broad spectrum of antibacterial activity (Voravuthikunchai *et al.*, 2004a, 2004b, 2007a, 2007b, 2008a). The extraction method of this plant extract has been patented (Voravuthikunchai & Chusri, 2007). Parts of our detailed studies on semi-purified fractions have been published (Voravuthikunchai & Suwalak, 2008) as well as its active principles are being investigated. Studies on its antibacterial mechanisms are being carried out in our laboratories and some early findings have been documented (Chusri & Voravuthikunchai, 2009).

Quercus infectoria extract could inhibit the release of Verocytotoxin from *E. coli* O157: H7, a very harmful pathogen commonly contaminated in food, and can result in serious complications (Voravuthikunchai & Suwalak, 2008). It would be of great advantage to find some safe and effective compound which could inhibit both the growth of the organisms and the release of the toxins. Furthermore, the nutgalls possess a wide antimicrobial activity against other groups of microorganisms including fungi (Digraki *et al.*, 1999).

Recently, our colleagues at Natural Products Research Center, Prince of Songkla University have reported its activity against intestinal parasites (Sawangjaroen *et al.*, 2004).

According to its broad spectrum of antimicrobial activity against a wide range of microorganisms, the extracts from *Quercus infectoria* could be useful to apply as novel approaches to surface disinfection, instrument disinfection, and self-sanitizing materials. Examples of applications may include the following to eliminate the potential for microbial contamination or colonization: (i) novel means to disinfect surfaces in hospitals, long-term care facilities and clinics, (ii) novel approaches to the disinfection of surgical instruments, (iii) novel devices that allow for the disease treatment or surgical procedures.

The broad spectra of activities of the extracts from *Quercus infectoria* support their usage in the treatment of multiple drug resistant bacterial infections. The nutgall has been reviewed by the Food and Drug Administration and determined to be generally recognized as safe (GRAS). Therefore, we are very interested in its applications as natural preservative in infection prevention replacing alcohol-based disinfectants. We have experienced complaints from nurses about dryness of their hands after long term use of alcohol. Our scientific studies have demonstrated that the proposed approach has been shown to be functional and effective in reducing or eliminating the potential for infection. A big advantage of the study is that we may find some alternative natural preservative which is of low cost (10 US dollars per kilogram), with high yield approximately 70%, easy to prepare, and safe for consumers.

Rhodomyrtus tomentosa showed remarkably good antibacterial activity against Gram-positive bacteria with its MICs and MBCs ranged from 3.9-7.8 and 7.8-125 μg/ml, respectively. Its extremely good anti-Gram-positive activity suggests a potency for drug development. Compounds in acylphloroglucinal group are very promising as a good candidate for new principles for the development of a remedy for Gram-positive infections.

Food Preservatives

Inspite of modern improvements in food production techniques, food safety is an increasing important public health issue (World Health Organization, 2003). Due to the awareness on chemical preservatives, the food industry is now reflected by the consumers' opinions for safer additives and thus focusing on GRAS preservatives (Dillion & Board, 1994). At the same time, Western Society appears to be experiencing a trend of green 'consumerism' (Tuley de Silva, 1996) desiring fewer synthetic food additives and antimicrobials due to the potential health problems associated with their use and also the resistance of *S. aureus* to antibiotics in the case of MRSA. During the past decades, medicinal plant extracts and their essential oils have been extensively

reported to possess antimicrobial activities in the hope for some new safe food additives.

Food safety is a major concern to both consumers and food industry as there are an increasing number of reported cases of food-associated infections. Therefore, there is a need for naturally-derived compounds and other natural products with antimicrobial properties (Gould, 1996). Most commonly used food chemical preservatives include weak acids or their salts or esters such as lactic acid, citric acid, acetic acid, sodium benzoate, potassium sorbate, butylated hydroxyanisole, and butylated hydroxytoluene. However, the presence of chemical residues in foods and labeling of preservatives on food packages are major concerns to consumers. Chemical preservatives have increasingly been scrutinized, and recently there have been some concern with sodium benzoate degrading to the carcinogen benzene (Food & Drug Administration, 2006). Consumers' demand for natural products has increased. Most consumers prefer high quality, nutritious, and foods with prolonged shelf life with no chemical preservatives. Natural preservatives can then be used as additives instead of synthetic compounds while the microorganisms are eliminated and food is safe for consumption. The interest in plants with antimicrobial activities has increased due to the resistance of microorganisms to synthetic antibiotics, the potential health problem that could accompany the use of synthetic drugs, and the increasing costs of synthetic drugs for the maintenance of personal health.

There has been an increase in availability of a class of chemical compounds designated as 'natural preservatives'. Many are extracts from food with known stabilities such as rosemary extract, grape seed extract, tea extract. Although some show promise, many are limited by microbial effectiveness or other considerations including product taste, stability, and safety concerns. Many spices which are herbal products and their essential oils extracts have been reported. The essential oils and terpenoid alcohols of spices contribute to their smell, taste, and tactile sensation. Antibacterial properties and potential applications of certain essential oils in foods have been documented (Burt, 2004).

Khan *et al.* (2001) found that the methanol extract of *Cassia alata* could inhibit the bacterium at a concentration of 4 mg/ml while both water and ethanol extract of the same plant inhibited *S. aureus* at 10 mg/ml (Somchit *et al.*, 2003). Crude extract of Cinnamon stick (*Cinnamomum burmanii*) produced MIC and MBC greater than 2500 µg/ml against *S. aureus* (Shan *et al.*, 2007). Cinnamic acid, a phenolic component of several spices including cinnamon, generally regarded as safe for use in foods and is already used as a component in several food flavouring, for example, as antimicrobial in fish paste by the Japanese (Shimada *et al.*, 1991). Cinnamic acid has been found to dissolve in ethanol and was tested as a dip or spray at a range of concentrations on whole fruits and fruit slices, stored at ambient and

refrigeration temperatures (Roller, 1995). The antibacterial activities of essential oils of onions, garlic, and allicin from garlic against *S. aureus* were also reported (Kyung *et al.*, 2002; Benkeblia *et al.*, 2004).

The combined effect of extracts from Chinese chive, cinnamon, corni fructus, and their purified-fractions against *S. aureus* in food system has been shown (Mau *et al.*, 2001). Phytochemicals such as olive extracts and extracts of *Helichrysum italicum* have been reported to prevent the growth, protein secretion, and inhibition of SEB and SEC production (Novick *et al.*, 2001; Nostro *et al.*, 2001a; Nostro *et al.*, 2002). Methanol extract of pomegranate, a compound isolated from *Punica granatum*, was also effective at reducing or inhibiting SEA production (Braga *et al.*, 2005). The effect of lemon, orange, bergamot essential oils and their components on survival of *S. aureus in vitro* and in food system is documented. Citral and linalool vapours produced 6 log reductions in *S. aureus* populations on cabbage leaf after 8-10 h exposure (Fisher & Phillips, 2006).

Research work suggest applications on the use of *Quercus infectoria* and *Eleutherine americana* as food additive to prevent a number of foodborne infections. Both plant species are classified as GRAS. We have recently investigated *Eleutherine americana* and identified natural preservatives with proven anti-MRSA capabilities. Furthermore, its stability in food at elevated temperature has been ensured.

CURRENT AND FUTURE DEVELOPMENTS

Medicinal plants have significant promise in disease prevention and the promotion of human health. Phytotherapy markets are still not well-regulated (Hasler, 2002; Halsted, 2003; Bagchi *et al.*, 2004). Concerns from scientists, professionals, and customers continuously arise, due to increases in the use of these products and therapies. Governments, scientists, health professionals, nutritionists, toxicologists, and other related parties should strategically work together to plan appropriate regulations to provide the ultimate therapeutic benefits to mankind. Quality, safety, long-term adverse effects, and toxicity are primary concerns. For manufacturing processes of the products, quality controls such as the composition and contents of active constituents in natural plants, and maintenance are critically important. To establish product safety and efficacy, extensive safety studies including acute, subacute, subchronic, chronic, and long-term toxicity studies, reproductive toxicology, as well as supplementation studies in animals and clinical trials in humans are necessary.

The safety assessment of botanicals and botanical preparations in food and food supplements is complicated and should at least involve the characterization and quality of the material, its quality control; the intended use and consequent exposure; toxicological information, and risk evaluation (Schilter *et al.*, 2003; Kroes & Walker, 2004). Extracts and essential oils from medicinal plants have been found to exhibit antibacterial activity *in*

vitro, however, a greater concentration is needed to achieve the same effect in foods (Shelef, 1983; Smid & Gorris, 1999). This may be attributed to the greater availability of nutrients in foods compared to laboratory media, thus enable microbial cells to repair from stress more efficiently (Gill *et al.*, 2002). The intrinsic and extrinsic properties of foods have also been found to influence bacterial sensitivity (Shelef, 1983; Tassou *et al.*, 1995). Application of hurdle technology may allow the use of low levels of antibacterials in combination with other food preservation techniques such as refrigeration and modified temperatures.

Advances in biotechnology as well as other technologies in diagnosis, product quality control, and service style will greatly enhance these markets. In this era of genomics, DNA microarray technology may be used to examine the safety and efficacy of drugs, chemicals, food supplements and nutraceuticals (Roy *et al.*, 2004).

CONCLUSIONS

In vitro studies are both informative and revealing the effectiveness of a number of plant species in preventing, treating, and eradicating *S. aureus* infections which should ultimately be determined by clinical studies. Our results indicated that rhodomyrtone was highly effective against *S. aureus* with the MIC value at 0.5 μg/ml which is very closed to that of vancomycin, the effective agent at present. Other important plant species including *Quercus infectoria* and *Eleutherine americana* have potentials for use as alternative food additives, however, extensive safety studies should be carried out. Effective use of herbs and spices in food as additive will also require organoleptic approach where consumer taste would be given priority. The stability of these natural preservatives during food processing will also need to be studied. Antagonism or synergism between food components and antibacterials require more study prior to commercial applications.

ACKNOWLEDGEMENTS

This research work has been supported by Prince of Songkla University Budget, fiscal year 2001; Thai Government Budget, fiscal year 2001-2003; Prince of Songkla University Collaborative Research Fund, fiscal year 2003; Thailand-Tropical Diseases Research Programme, National Center for Genetic Engineering and Biotechnology, fiscal year 2004-2005; The Thailand Research Fund (8th Golden Jubilee, 9th Golden Jubilee); The Thailand Research Fund (Basic Research), fiscal year 2005-2008; 2008-2011. The first author wish to thank our research colleagues at collaborative laboratories at Research Institute for Microbial Diseases, Osaka University, Japan; School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Australia; Department of Pharmaceutical Biology, Groningen Research Institute of Pharmacy, University of Groningen, The Netherlands and Department of Microbiology and Immunology, University of Columbia, Canada.

REFERENCES

Abulrob, A.N., Suller, M.T., Gumbleton, M., Simons, C. and Russell, A.D. (2004). Identification and biological evaluation of grapefruit oil components as potential novel efflux pump modulators in methicillin-resistant *Staphylococcus aureus* bacterial strains. *Phytochemistry*, **65**: 3021-3027.

Adams, M.R. and Moss, M.O. (1995). Food Microbiology. The Royal Society of Chemistry, Cambrige, 398 pp.

Akiyama, H., Yamasaki, O., Tada, J. and Arata, J. (2000). Adherence characteristics and susceptibility to antimicrobial agents of *Staphylococcus aureus* strains isolated from skin infections and atopic dermatitis. *J. Dermatol. Sci.*, **23**: 155-160.

Alkofahi, A., Masaadeh, H. and Al-Khalil, S. (1996). Antimicrobial evaluation of some plant extracts of traditional medicine of Jordan. *Alex. J. Pharm. Sci.*, **10**: 123-126.

Alonzo, V. (2001). Effects of *Helichrysum italicum* extract on growth and enzymatic activity of *Staphylococcus aureus*. *Int. J. Antimicrob. Ag.*, **17**: 517-520.

Alves, T.M.A., Kloos, H. and Leomar, C. (2003). Eleutherinone, a novel fungitoxic naphthoquinone from *Eleutherine bulbosa*. *Mem. Inst. Oswaldo Cruz, Rio de janerio*, **98**: 709-712.

Ambrogi, V., Artini, I., Carneri, I., Castellino, S., Dradi, E., Logemann, W., Meinardi, G., Somma, M. and Tosolini, G. (1970). Studies on the antibacterial and antifungal properties of 1, 4-naphthoquinones. *Brit. J. Pharmacol.*, **40**: 871-880.

Annuk, H., Hirmo, S., Turi, E., Mikelsaar, M., Arak, E. and Wadstrom, T. (1999). Effect on cell surface hydrophobicity and susceptibility of *Helicobacter pylori* to medicinal plant extracts. *FEMS Microbiol. Lett.*, **172**: 41-45.

Anupurba, S., Sen, M.R., Nath, G., Sharma, B.M., Gulati, A.K. and Mohapatra, T.M. (2003). Prevalence of methicillin resistant *Staphylococcus aureus* in a tertiary referral hospital in eastern Uttar Pradesh. *Indian J. Med. Microbiol.*, **21**: 49-51.

Arakere, G., Nadig, S., Swedberg, G., Macaden, R., Amarnath, S.K. and Raghunath, D. (2005). Genotyping of methicillin-resistant *Staphylococcus aureus* strains from two hospitals in Bangalore, South India. *J. Clin. Microbiol.*, **43**: 3198-3202.

Arda, B., Yamazhan, T., Sipahi, O.R., Islekel, S., Buke, C. and Ulusoy, S. (2005). Meningitis due to methicillin-resistant *Staphylococcus aureus* (MRSA): review of 10 cases. *Int. J. Antimicrob. Agent*, **25**: 414-418.

Assadian, O., Daxboeck, F., Aspoeck, C., Blacky, A., Dunkl, R. and Koller, W. (2003). National surveillance of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* in Austrian hospitals: 1994-1998. *J. Hosp. Infect.*, **55**: 175-179.

Bagchi D., Preuss, H.G. and Kehrer, J.P. (2004). Nutraceutical and functional food industries: aspects on safety and regulatory requirements. *Toxicol. Lett.*, **150**: 1-2.

Bakkali, F., Averbeck, S., Averbeck, D. and Idaomar, M. (2008). Biological effects of essential oils-a review. *Food Chem. Toxicol.*, **46**: 446-475.

Balaban, N. and Rasooly, A. (2000). Staphylococcal enterotoxins. *Int. J. Food Microbiol.*, **61**: 1-10.

Bell, J.M. and Turnidge, J.D. (2002). High prevalence of oxacillin-resistant *Staphylococcus aureus* isolates from hospitalized patients in Asia-Pacific and South Africa: results from SENTRY antimicrobial surveillance program, 1998-1999. *Antimicrob. Agents Chemother.*, **46**: 879-881.

Benkeblia, N. (2004). Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*). *Lebensmittel Wissenschaft und-Technologie.*, **37**: 263-268.

Benkeblia, N., Onodera, S., Yoshihira, T., Kosaka, S. and Shiomi, N. (2004). Effect of temperature on soluble invertase activity, and glucose, fructose and sucrose status of onion bulbs (*Allium cepa*) in store. *Int. J. Food Sci. Nutr.*, **55**: 325-331.

Bennett, R.W. and Lancette, G.A. (1998). *Staphylococcus aureus In*: FDA, Bacteriological Analytical Manual, 8th ed, Revision A/1998. AOAC International, Gaithersburg, MD. Chapter 12. pp. 1-6.

Bergdoll, M.S. (1988). Monkey feeding test for staphylococcal enterotoxin. *Methods Enzymol.*, **165**: 324-333.

Bergdoll, M.S. (1989). *Staphylococcus aureus. In*: Doyle M.P., *ed.*, Foodborne Bacterial Pathogens. Marcel Dekker, New York, pp. 463-523.

Betley, M.J., Borst, D.W. and Regassa, L.B. (1992). Staphylococcal enterotoxins, toxic shock syndrome toxin and streptococcal pyrogenic exotoxins: a comparative study of their molecular biology. *Chem. Immunol.*, **55**: 1-35.

Biao-Yi, Z., Yu, Y. and Zeng-Liang, Y. (2008). Investigation of antimicrobial model of *Hemsleya pengxianensis* W.J. Chang and its main active component by metabolomics technique. *J. Ethnopharmacol.*, **116**: 89-95.

Blaiotta, G., Ercolini, D., Pennacchia, C., Fusco, V., Casaburi, A., Pepe, O. and Villani, F. (2004). PCR detection of staphylococcal enterotoxin genes in *Staphylococcus* spp. strains isolated from meat and dairy products. Evidence for new variants of seG and seI in *S. aureus* AB-8802. *J. Appl. Microbiol.*, **97**: 719-730.

Bolton, K.J., Dodd, C.E.R., Mead, G.C. and Waites, W.M. (1988). Chlorine resistance of strains of *S. aureus* isolated from poultry processing plants. *Lett. Appl. Microbiol.*, **6**: 31-34.

Braga, L.C., Shupp, J.W., Cummings, C., Jett, M., Takahashi, J.A., Carmo, L.S., Chartone-Souza, E. and Nascimento, A.M. (2005). Pomegranate extract inhibits *Staphylococcus aureus* growth and subsequent enterotoxin production. *J. Ethnopharmacol.*, **96**: 335-339.

Bremer, P.J., Fletcher, G.C. and Osborne, C. (2004). *Staphylococcus aureus*. New Zealand Institute for Crop and Food Research Limited. Christchurch, New Zealand. pp. 1-6.

Brook, I. 2002. Secondary bacterial infections complicating skin lesions. *J. Med. Microbiol.*, **51**: 808-812.

Bruinsma, G.M., van der Mei, H.C. and Busscher, H.J. (2001). Bacterial adhesion to surface hydrophilic and hydrophobic contact lenses. *Biomaterials*, **22**: 3217-3224.

Buenz, E.J., Bauer, B.A., Schnepple, D.J., Wahner-Roedler, D.L., Vandell, A.G. and Howe, C.L. (2007). A randomized Phase I study of *Atuna racemosa*: a potential new anti-MRSA natural product extract. *J. Ethnopharmacol*, **114**: 371-376.

Buerke, M., Sibelius, U., Grandel, U., Buerke, U., Grimminger, F., Seeger, W., Meyer, J. and Darius, H. (2002). *Staphylococcus aureus* alpha toxin mediates polymorphonuclear leukocyte-induced vasocontraction and endothelial dysfunction. *Shock*, **17**: 30-35.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods-a review. *Int. J. Food Microbiol.*, **94**: 223-253.

Butzle, J.P. and Oosterom, J. (1991). *Campylobacter*: pathogenicity and significance in foods. *Int. J. Food Microbiol.*, **12**: 1-8.

Cabrera, C., Artacho, R. and Gimenez, R. (2006). Beneficial effects of green tea-a review. *J. Am. Coll. Nutr.*, **25**: 79-99.

Caelli, M., Porteous, J., Carson, C.F., Heller, R. and Riley, T.V. (2000). Tea tree oil as an alternative topical decolonization agent for methicillin-resistant *Staphylococcus aureus*. *J. Hosp. Infect.*, **46**: 236-237.

Carson, C.F., Mee, B.J. and Riley, T.V. (2002). Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. *Antimicrob. Agents Chemother.*, **46**: 1914-1920.

Carson, C.F., Hammer, K.A. and Riley, T.V. (2006). *Melaleuca alternifolia* (tea tree) oil: a review of antimicrobial and other medicinal properties. *Clin. Microbiol. Rev.*, **19**: 50-62.

Caturla, N., Vera-Samper, E., Villalain, J., Mateo, C.R. and Micol, V. (2003). The relationship between the antioxidant and the antibacterial properties of galloylated catechins and the structure of phospholipid model membranes. *Free Radic. Biol. Med.*, **34**: 648-662.

Celebi, S., Hacimustafaoglu, M., Ozdemir, O. and Ozakin, C. (2007). Nosocomial Gram-positive bacterial infections in children: results of a 7-year study. *Pediatr. Int.*, **49**: 875-882.

Centers for Disease Control and Prevention. (1997). Reduced susceptibility of *Staphylococcus aureus* to vancomycin-Japan (1996). MMWR *Morb. Mortal. Wkly. Rep.*, **46**: 624-626.

Centers for Disease Control and Prevention. (2000). Food-related illness and death in the United States. *Emerg. Infect. Dis.*, **5**: 607-625.

Chambers, H.F. (1997). Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. *Clin. Microbiol. Rev.*, **10**: 781-791.

Chambers, H.F., Hartman, B.J. and Tomasz, A. (1985). Increased amounts of a novel penicillin-binding protein in a strain of methicillin-resistant *Staphylococcus aureus* exposed to nafcillin. *J. Clin. Invest.*, **76**: 325-331.

Chang, S., Sievert, D.M., Hageman, J.C., Boulton, M.L., Tenover, F.C., Downes, F.P., Shah, S., Rudrik, J.T., Pupp, G.R., Brown, W.J., Cardo, D. and Fridkin, S.K. (2003). Infection with vancomycin-resistant *Staphylococcus aureus* containing the *vanA* resistance gene. *N. Engl. J. Med.*, **348**: 1342-1347.

Cheong, I., Tan, S.C., Wong, Y.H., Zainudin, B.M. and Rahman, M.Z. (1994). Methicillin-resistant *Staphylococcus aureus* (MRSA) in a Malaysian hospital. *Med. J. Malaysia*, **49**: 24-28.

Chopra, R.N., Nayar, S.L. and Chopra, I.C. (1956). Glossary of Indian Medicinal Plants. Council of Scientific and Industrial Research. New Delhi, 330 pp.

Chusri, S. and Voravuthikunchai, S.P. (2008). *Quercus infectoria*: A candidate for the control of methicillin-resistant *Staphylococcus aureus* infections. *Phytother. Res.*, **22**: 560-562.

Chusri, S. and Voravuthikunchai, S.P. (2009). Enhancement of antibacterial activity of β-lactam antibiotics in methicillin-resistant *Staphylococcus aureus* by *Quercus infectoria* (Oliv.) extract and its components. *J. Appl. Microbiol.* **106**: 89-96.

Ciftci, I.H., Koken, R., Bukulmez, A., Ozdemir, M., Safak, B. and Cetinkaya, Z. (2007). Nasal carriage of *Staphylococcus aureus* in 4-6 age groups in healthy children in Afyonkarahisar, Turkey. *Acta. Paediatr.*, **96**: 1043-1046.

Clarke, S.R., Mohamed, R., Bian, L., Routh, A.F., Kokai-Kun, J.F., Mond, J.J., Tarkowski, A. and Foster, S.J. (2007). The *Staphylococcus aureus* surface protein IsdA mediates resistance to innate defenses of human skin. *Cell Host Microbe.*, **1**: 199-212.

Corzo-Martinez, M., Corzo, N. and Villamiel, M. (2007). Biological properties of onions and garlic. *Trends Food Sci. Tech.*, **18**: 609-625.

Cosgrove, S.E., Sakoulas, G., Perencevich, E.N., Schwaber, M.J., Karchmer, A.W. and Carmeli, Y. (2003). Comparison of mortality associated with methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteremia: a meta-analysis. *Clin. Infect. Dis.*, **36**: 53-59.

Cowan, M.M. (1999). Plant products as antimicrobial agents. *Clin. Microbiol. Rev.*, **12**: 564-582.

Cox, S.D., Mann, C.M. and Markham, J.L. (2001). Interactions between components of the essential oil of *Melaleuca alternifolia*. *J. Appl. Microbiol.*, **91**: 492-497.

Cushnie, T.P. and Lamb, A.J. (2005a). Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents*, **26**: 343-356.

Cushnie, T.P. and Lamb, A.J. (2005b). Detection of galangin-induced cytoplasmic membrane damage in *Staphylococcus aureus* by measuring potassium loss. *J. Ethnopharmacol.*, **101**: 243-248.

Cushnie, T.P., Hamilton, V.E., Chapman, D.G., Taylor, P.W. and Lamb, A.J. (2007). Aggregation of *Staphylococcus aureus* following treatment with the antibacterial flavonol galangin. *J. Appl. Microbiol.*, **103**: 1562-1567.

Dachryanus, S., Sargent, M.V., Skelton, B.W., Soediro, I., Sutisna, M., White, A.H. and Yulinah, E. 2002. Rhodomyrtone, an antibotic from *Rhodomyrtus tomentosa*. *Aust. J. Chem.* **55**: 229-232.

D'Aoust, J.Y. (1989). *Salmonella*. *In*: Doyle, M.P., *ed.*, Foodborne Pathogens. Marcel Dekker Inc., New York, pp. 327-445.

Dar, M.S., Ikram, M. and Fakouhi, T. (1976). Pharmacology of *Quercus infectoria*. *J. Pharm. Sci.*, **65**: 1791-1794.

Daud, A., Gallo, A. and Riera, A.S. (2005). Antimicrobial properties of *Phrygilanthus acutifolius*. *J. Ethnopharmacol.*, **99**: 193-197.

De Kimpe, S.J., Kengatharan, M., Thiemermann, C. and Vane, J.R. (1995). The cell wall components peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* act in synergy to cause shock and multiple organ failure. *Proc. Natl. Acad. Sci. USA.*, **92**: 10359-10363.

Denniston, S. and Riordan, F.A. (2006). *Staphylococcus aureus* bacteraemia in children and neonates: a 10 year retrospective review. *J. Infect.*, **53**: 387-393.

Digraki, M., Alma, M.H., Ilcim, A. and Sen, S. (1999). Antibacterial and antifungal effects of various commercial plant extracts. *Pharm. Biol.*, **37**: 216-220.

Dillion, V.M. and Board, R.G. (1994). Future prospect for natural antimicrobial food

preservation systems. *In*: Dillon, V.M. and Board, R.G., *eds*., Natural Antimicrobial Systems and Food Preservation, CAB, Washington, pp. 297-305.

Dinges, M.M., Orwin, P.M. and Schlievert, P.M. (2000). Exotoxins of *Staphylococcus aureus*. *Clin. Microbiol. Rev*., **13**: 16-34.

Dolah, S., Charoenjiratrakul, W. and Voravuthikunchai, S.P. (2007). Antibacterial activities of ethanolic extract of *Rhodomyrtus tomentosa* (Ait.) Hassk. against *Bacillus cereus* isolated from food. p. 92, Programme and Abstracts, 33rd Science and Technology of Thailand, 18-20 October, 2007 at Nakhon Si Thammarat, Thailand.

Doyle, M.P. and Padhye, V.V. (1989). *Escherichia coli*. *In*: Doyle, M.P., *ed*., Foodborne Pathogens. Marcel Dekker Inc., New York, pp. 235-281.

Drinka, P., Faulks, J.T., Gauerke, C., Goodman, B., Stemper, M. and Reed, K. (2001). Adverse events associated with methicillin-resistant *Staphylococcus aureus* in a nursing home. *Arch. Intern. Med.*, **161**: 2371-2377.

Dykes, G.A., Amarowicz, R. and Pegg, R.B. (2003). An antioxidant bearberry (*Arctostaphylos uva-ursi*) extract modulates surface hydrophobicity of a wide range of food-related bacteria: implications for functional food safety. *Food Control*, **14**: 515-518.

Evans, C.E. (1996). Trease and Evan's Pharmacognosy. WB Saunders Co. Ltd., London, 600 pp.

Evenson, M.L., Hinds, M.W., Bernstein, R.S. and Bergdoll, M.S. (1988). Estimation of human dose of staphylococcal enterotoxin A from a large outbreak of staphylococcal food poisoning involving chocolate milk. *Int. J. Food Microbiol*., **7**: 311-316.

Farooqi, A.H.A., Sharma, S., Khan, A., Kumar, R. and Kumar, S. (2001). Formulation useful as a natural herbal tooth powder. United States Patent 6264926.

Fazeli, M.R., Amin, G., Attari, M.M.A., Ashtiani, H., Jamalifar, H. and Samadi, N. (2007). Antimicrobial activities of Iranian sumac and avishan-e shirazi. (*Zataria multiflora*) against some food-borne bacteria. *Food Control*, **18**: 646-649.

Fidalgo, S., Vazquez, F., Mendoza, M.C., Perez, F. and Mendez, F.J. (1990). Bacteremia due to *Staphylococcus epidermidis*: microbiologic, epidemiologic, clinical, and prognostic features. *Rev. Infect. Dis*., **12**: 520-528.

Fisher, K. and Phillips, C.A. (2006). The effect of lemon, orange and bergamot essential oils and their components on the survival of *Campylobacter jejuni*, *Escherichia coli* O157, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus in vitro* and in food system. *J. Appl. Microbiol*., **101**: 1232-1240.

Fitzgerald, J.R., Monday, S.R., Foster, T.J., Bohach, G.A., Hartigan, P.J., Meaney, W.J. and Smyth, C.J. (2001). Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. *J. Bacteriol*., **183**: 63-70.

Food and Drug Administration. (2006). Data on Benzene in Soft Drinks and Other Beverages. United States Food and Drug Administration. http://www.cfsan.fda.gov/~dms/benzdata.html

Fowler, V.G., McIntyre, L.M., Yeaman, M.R., Peterson, G.E., Reller, L.B., Corey, G.R., Wray, D. and Bayer, A.S. (2000). *In vitro* resistance to thrombin-induced platelet microbicidal protein in isolates of *Staphylococcus aureus* from endocarditis patients correlates with an intravascular device source. *J. Infect. Dis*., **182**: 1251-1254.

Furneri, P.M., Garozzo, A., Musumarra, M.P., Scuderi, A.C., Russo, A. and Bonfiglio, G. (2003). Effects on adhesiveness and hydrophobicity of sub-inhibitory concentrations of netilmicin. *Int. J. Antimicrob. Agents*, **22**: 164-167.

Gaspar-Marques, C., Rijo, P., Simoes, M.F., Duarte, M.A. and Rodriguez, B. (2006). Abietanes from *Plectranthus grandidentatus* and *P. hereroensis* against methicillin- and vancomycin-resistant bacteria. *Phytomedicine*, **13**: 267-271.

Ghuysen, J.M. (1994). Molecular structures of penicillin-binding proteins and β-lactamases. *Trends Microbiol*., **2**: 372-380.

Gill, A.O., Delaquis, P., Russo, P. and Holley, R.A. (2002). Evaluation of antilisterial action of cilantro oil on vacuum packed ham. *Int. J. Food Microbiol*., **73**: 83-92.

Goffin, C. and Ghuysen, J.M. (1998). Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. *Microbiol. Mol. Biol. Rev*., **62**: 1079-1093.

Gould, G.W. (1996). Industry perspectives on the use of natural antimicrobials and inhibitors for food applications. *J. Food Prot*. **(Suppl.1)**: 82-86.

Granum, P.E. (1990). *Clostridium perfringens* toxins involved in food poisoning. *Int. J. Food Microbiol.*, **10**: 101-112.

Grivet, M., Morrier, J.J., Benay, G. and Barsotti, O. (2000). Effect of hydrophobicity on *in vitro* streptococcal adhesion to dental alloys. *J. Mater. Sci. Mater. Med.*, **11**: 637-642.

Habeeb, F., Shakir, E., Bradbury, F., Cameron, P., Taravati, M.R., Drummond, A.J., Gray, A.I. and Ferro, V.A. (2007). Screening methods used to determine the antimicrobial properties of *Aloe vera* inner gel. *Methods*, **42**: 315-320.

Hada, T., Inoue, Y., Shiraishi, A. and Hamashima, H. (2003). Leakage of K^+ ions from *Staphylococcus aureus* in response to tea tree oil. *J. Microbiol. Methods*, **53**: 309-312.

Halcon, L. and Milkus, K. (2004). *Staphylococcus aureus* and wounds: a review of tea tree oil as a promising antimicrobial. *Am. J. Infect. Control*, **32**: 402-408.

Halsted, C.H. (2003). Dietary supplements and functional foods: 2 sides of a coin? *Am. J. Clin. Nutr.*, **77**: 1001S-1007S.

Hamilton-Miller, J.M. and Shah, S. (1999). Disorganization of cell division of methicillin-resistant *Staphylococcus aureus* by a component of tea (*Camellia sinensis*): a study by electron microscopy. *FEMS Microbiol. Lett.*, **176**: 463-469.

Hamilton-Miller, J.M. and Shah, S. (2000). Activity of the tea component epicatechin gallate and analogues against methicillin-resistant *Staphylococcus aureus*. *J. Antimicrob. Chemother.*, **46**: 852-853.

Hanlon, G.W., Denyer, S.P., Hodges, N.A., Brant, J.A., Lansley, A.B. and Al-Rustamani, W.A. (2004). Biofilm formation and changes in bacterial cell surface hydrophobicity during growth in a CAPD model system. *J. Pharm. Pharmacol.*, **56**: 847-854.

Hara, H., Maruyama, N., Yamashita, S., Hayashi, Y., Lee, K., Bastow, K.F., Chairul, M.R., Marumoto, R. and Imakura, Y. (1997). Elecanacin, a novel naphthoquinone from the bulb of *Eleutherine americana*. *Chem. Pharm. Bull.*, **45**: 1714-1716.

Hasler, C.M. (2002). Functional foods: benefits, concerns and challenges-a position paper from the American council on science and health. *J. Nutr.*, **132**: 3772-3781.

Hauschild, A.H.W. (1989). '*Clostridium botulinum*. *In*: Doyle, M.P., *ed.*, Foodborne Bacterial Pathogens. Marcel Dekker, New York; pp. 111-189.

Hennebelle, T., Sahpaz, S., Joseph, H. and Bailleul, F. (2008). Ethnopharmacology of *Lippia alba*. *J. Ethnopharmacol.*, **116**: 211-222.

Heumann, D., Barras, C., Severin, A., Glauser, M.P. and Tomasz, A. (1994). Gram-positive cell walls stimulate synthesis of tumor necrosis factor alpha and interleukin-6 by human monocytes. *Infect. Immun.*, **62**: 2715-2721.

Heying, R., van de Gevel, J., Que, Y.A., Moreillon, P. and Beekhuizen, H. (2007). Fibronectin-binding proteins and clumping factor A in *Staphylococcus aureus* experimental endocarditis: FnBPA is sufficient to activate human endothelial cells. *Thromb. Haemost.*, **97**: 617-626.

Hiramatsu, K., Aritaka, N., Hanaki, H., Kawasaki, S., Hosoda, Y., Hori, S., Fukuchi, Y. and Kobayashi, I. (1997). Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet*, **350**: 670-1673.

Hiranrat, A., Mahabusarakam, W. (2008). New acylphloroglucinols from the leaves of *Rhodomyrtus tomentosa*. Tetrahedron **64**: 11193-11197.

Hisata, K., Kuwahara-Arai, K., Yamanoto, M., Ito, T., Nakatomi, Y., Cui, L., Baba, T., Terasawa, M., Sotozono, C., Kinoshita, S., Yamashiro, Y. and Hiramatsu, K. (2005). Dissemination of methicillin-resistant staphylococci among healthy Japanese children. *J. Clin. Microbiol.*, **43**: 3364-3372.

Ho, P.L., Wang, T.K., Ching, P., Mak, G.C., Lai, E., Yam, W.C. and Seto, W.H. (2007). Epidemiology and genetic diversity of methicillin-resistant *Staphylococcus aureus* strains in residential care homes for elderly persons in Hong Kong. *Infect. Control Hosp. Epidemiol.*, **28**: 671-678.

Hoeger, P.H., Lenz, W., Boutonnier, A. and Fournier, J.M. (1992). Staphylococcal skin colonization in children with atopic dermatitis: prevalence, persistence, and transmission of toxigenic and nontoxigenic strains. *J. Infect. Dis.*, **165**: 1064-1068.

Holmes, H.L., Currie, D.J., Maltman, J.R., Silver, R.F., Lough, C.E. and Lesky, G.J. (1964). Evidence for mode of chemical action of 1,4-naphthoquinones in bacteriostasis. *Chemotherapy*, **9**: 241-247.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T. (1994). Bergey's Manual of Determinative Bacteriology. 9th ed. Williams and Wilkins. Baltimore, 787 pp.

Horiuchi, K., Shiota, S., Hatano, T., Yoshida, T., Kuroda, T. and Tsuchiya, T. (2007). Antimicrobial activity of oleanolic acid from *Salvia officinalis* and related compounds on vancomycin-resistant enterococci (VRE). *Biol. Pharm. Bull.*, **30**: 1147-1149.

Hou, A.J., Wu, Y.J. and Liu, Y.Z. (1999). Flavones glycosides and ellagitannin from downy rosemyrtle (*Rhodomyrtus tomentosa). Zhongcaoyao*, **30**: 645-648.

Hsu, L.Y., Koh, T.H., Singh, K., Kang, M.L., Kurup., A. and Tan, B.H. (2005). Dissemination of multisusceptible methicillin-resistant *Staphylococcus aureus* in Singapore. *J. Clin. Microbiol.*, **43**: 2923-2925.

Hsueh, P.R., Chen, W.H., Teng, L.J. and Luh, K.T. (2005). Nosocomial infections due to methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci at a university hospital in Taiwan from 1991 to 2003: resistance trends, antibiotic usage and *in vitro* activities of newer antimicrobial agents. *Int. J. Antimicrob. Agents*, **26**: 43-49.

Hui, W.H. and Li, M.M. (1976). Two new triterpenoids from *Rhodomyrtus tomentosa. Phytochemistry*, **15**: 1741-1743.

Hui, W.H., Li, M.M. and Luk, K. (1975). Triterpenoids and steroids from *Rhodomyrtus tomentosa. Phytochemistry*, **14**: 833-834.

Hwang, J.K., Kong, T.W., Baek, N.I. and Pyun, Y.R. (2000). alpha-Glycosidase inhibitory activity of hexagalloylglucose from the galls of *Quercus infectoria. Planta Med.*, **66**: 273-274.

Hwang, J.K., Shim, J.S. and Chung, J.Y. (2004). Anticariogenic activity of some tropical medicinal plants against *Streptococcus mutans. Fitoterapia*, **75**: 596-598.

Ifesan, B.O.T. and Voravuthikunchai, S.P. (2009a). Inhibitory effect of *Eleutherine americana* Merr. extract on *Staphylococcus aureus. J. Food Sci.*, **74**: M31-M36.

Ifesan, B.O.T.and Voravuthikunchai, S.P. (2009b). Effect of *Eleutherine americana* Merr. extract on enzymatic activity and enterotoxin production of *Staphylococcus aureus* in broth and cooked pork. *Foodborne Pathog. Dis.*, **6**: 699-704.

Ifesan, B.O.T., Mahabusarakam, W., Hamtasin, C. and Voravuthikunchai, S.P. (2009a). Assessment of antistaphylococcal activity of partially-purified fractions and pure compounds from *Eleutherine americana. J. Food Prot.*, **72**: 354-359.

Ifesan, B.O.T., Siripongvutikorn, S. and Voravuthikunchai, S.P. (2009b). Application of *Eleutherine americana* crude extract as additive in homemade salad dressing. *J. Food Prot.*, **72**: 650-665.

Ifesan, B.O.T., Siripongvutikorn, S., Hutadilok-Towatana, N. and Voravuthikunchai, S.P. (2009c). Evaluation of the ability of *Eleutherine americana* crude extract as food additive in cooked pork. *J. Food Sci.* **74**: M352-M357.

Igbeneghu, O.A., Iwalewa, E.O. and Lamikanra, A. (2007). A study of the *in vivo* activity of the leaf extract of *Alchornea cordifolia* against multiply antibiotic resistant *S. aureus* isolate in mice. *Phytother. Res.*, **21**: 67-71.

Ikigai, H., Nakae, T., Hara, Y. and Shimamura, T. (1993). Bactericidal catechins damage the lipid bilayer. *Biochim. Biophys. Acta*, **1147**: 132-136.

Ikram, M. and Nowshad, F. (1977). Constituents of *Quercus infectoria. Planta Med.*, **31**: 286-287.

Ishida, K., de Mello, J.C., Cortez, D.A., Filho, B.P., Ueda-Nakamura, T. and Nakamura, C.V. (2006). Influence of tannins from *Stryphnodendron adstringens* on growth and virulence factors of *Candida albicans. J. Antimicrob. Chemother.*, **58**: 942-949.

Ito, T. and Hiramatsu, K. (2003). MRSA (methicillin-resistant *Staphylococcus aureus*). *Nippon Rinsho.*, **61(Suppl 3)**: 164-170.

Iwatsuki, K., Yamasaki, O., Morizane, S. and Oono, T. (2006). Staphylococcal cutaneous infections: invasion, evasion and aggression. *J. Dermatol. Sci.*, **42**: 203-214.

Jablonski, L.M. and Bohach, G.A. (2001). *Staphylococcus aureus. In*: Beuchat, M.P. and Montville, L.R. (*eds.*), Food Microbiology: Fundamentals and Frontiers, ASM Press, Washinghton, D.C; pp. 411-434.

Jay, J.M. (2000). Modern Food Microbiology. 6th ed. Gaithersberg Aspen, Maryland.

Jeong, S.I., Han, W.S., Yun, Y.H. and Kim, K.J. (2006). Continentalic acid from *Aralia continentalis* shows activity against methicillin-resistant *Staphylococcus aureus. Phytother. Res.*, **20**: 511-514.

Jevons, M.P., Coe, A.W. and Parker, M.T. (1963). Methicillin resistance in staphylococci. *Lancet*, **1**: 904-907.

Jones, T.F., Kellum, M.E., Porter, S.S., Bell, M. and Schaffner, W. (2002). An outbreak of community-acquired foodborne illness caused by methicillin-resistant *Staphylococcus aureus*. *Emerg. Infect. Dis.*, **8**: 82-84.

Jonsson, I.M., Mazmanian, S.K., Schneewind, O., Verdrengh, M., Bremell, T. and Tarkowski, A. (2002). On the role of *Staphylococcus aureus* sortase and sortase-catalyzed surface protein anchoring in murine septic arthritis. *J. Infect. Dis.*, **185**: 1417-1424.

Joshi, A.S., Li, X.C., Nimrod, A.C., elSohly, H.N., Walker, L.A. and Clark, A.M. (2001). Dihydrochalcones from *Piper longicaudatum*. *Planta Med.*, **67**: 186-188.

Kapperud, G. (1991). *Yersinia enterocolitica* in food hygiene. *Int. J. Food Microbiol.*, **12**: 53-65.

Kaur, G., Hamid, H., Ali, A., Alam, M.S. and Athar, M. (2004). Antiinflammatory evaluation of alcoholic extract of galls of *Quercus infectoria*. *J. Ethnopharmacol.*, **90**: 285-292.

Kerdudou, S., Laschke, M.W., Sinha, B., Preissner, K.T., Menger, M.D. and Herrmann, M. (2006). Fibronectin binding proteins contribute to the adherence of *Staphylococcus aureus* to intact endothelium *in vivo*. *Thromb. Haemost.*, **96**: 183-189.

Kerttula, A.M., Lyytikainen, O., Karden-Lilja, M., Ibrahem, S., Salmenlinna, S., Virolainen, A. and Vuopio-Varkila, J. (2007). Nationwide trends in molecular epidemiology of methicillin-resistant *Staphylococcus aureus*, Finland, 1997-2004. *BMC Infect. Dis.*, **7**: 94.

Kesah, C., Ben Redjeb, S., Odugbemi, T.O., Boye, C.S., Dosso, M., Ndinya Achola, J.O., Koulla-Shiro, S., Benbachir, M., Rahal, K. at and Borg, M. (2003). Prevalence of methicillin-resistant *Staphylococcus aureus* in eight African hospitals and Malta. *Clin. Microbiol. Infect.*, **9**: 153-156.

Khan, M.R., Kihara, M. and Omoloso, A.D. (2001). Antimicrobial activity of *Cassia alata*. *Fitoterapia*, **72**: 561-564.

Khera, S., Woldemichael, G.M., Singh, M.P., Suarez, E. and Timmermann, B.N. (2003). A novel antibacterial iridoid and triterpene from *Caiophora coronata*. *J. Nat. Prod.*, **66**: 1628-1631.

Kim, E.S., Song, J.S., Lee, H.J., Choe, P.G., Park, K.H., Cho, J.H., Park, W.B., Kim, S.H., Bang, J.H., Kim, D.M., Park, K.U., Shin, S., Lee, M.S., Choi, H.J., Kim, N.J., Kim, E.C., Oh, M.D., Kim, H.B. and Choe, K.W. (2007). A survey of community-associated methicillin-resistant *Staphylococcus aureus* in Korea. *J. Antimicrob. Chemother.*, **60**: 1108-1114.

Kim, K.J., Yu, H.H., Jeong, S.I., Cha, J.D., Kim, S.M. and You, Y.O. (2004). Inhibitory effects of *Caesalpinia sappan* on growth and invasion of methicillin-resistant *Staphylococcus aureus*. *J. Ethnopharmacol.*, **91**: 81-87.

Kim, K.J., Yu, H.H., Cha, J.D., Seo, S.J., Choi, N.Y. and You, Y.O. (2005). Antibacterial activity of *Curcuma longa* L. against methicillin-resistant *Staphylococcus aureus*. *Phytother. Res.*, **19**: 599-604.

Kim, K.Y., Davidson, P.M. and Chung, H.J. (2001). Antibacterial activity in extracts of *Camellia japonica* L. petals and its application to a model food system. *J. Food Prot.*, **64**: 1255-1260.

Kim, S., Ruengwilysup, C. and Fung, D.Y. (2004). Antibacterial effect of water-soluble tea extracts on foodborne pathogens in laboratory medium and in a food model. *J. Food Prot.*, **67**: 2608-2612.

Kirov, S.M. (1993). The public health significance of *Aeromonas* ssp. in foods. *Int. J. Food Microbiol.* **20**: 179-198.

Kitai, S., Shimizu, A., Kawano, J., Sato, E., Nakano, C., Uji, T. and Kitagawa, H. (2005). Characterization of methicillin-resistant *Staphylococcus aureus* isolated from retail raw chicken meat in Japan. *J. Vet. Med. Sci.*, **67**: 107-110.

Klein, E., Smith, D.L. and Laxminarayan, R. (2007). Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999-2005. *Emerg. Infect. Dis.*, **13**: 1840-1846.

Kloss, W.E. and Bannerman, T.L. (1995). Staphylococcus and Micrococcus. *In*: Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C. and Yolken, R.H. *eds.*, Manual of Clinical Microbial., 6th *ed.* ASM Press, Washington, D.C. pp. 282-298.

Kramer, J.M. and Gilbert, R.J. (1989). *Bacillus cereus* and other *Bacillus* species. *In*: Doyle, M.P., *ed*., Foodborne Pathogens. Marcel Dekker, New York, pp. 21-70.

Kroes, R. and Walker, R. (2004). Safety issues of botanicals and botanical preparations in functional foods. *Toxicology*, **198**: 213-220.

Kustos, T., Kustos, I., Kilar, F., Rappai, G. and Kocsis, B. (2003). Effect of antibiotics on cell surface hydrophobicity of bacteria causing orthopedic wound infections. *Chemotherapy*, **49**: 237-342.

Kusuda, M., Inada, K., Ogawa, T.O., Yoshida, T., Shiota, S., Tsuchiya, T. and Hatano, T. (2006). Polyphenolic constituent structures of *Zanthoxylum piperitum* fruit and the antibacterial effects of its polymeric procyanidin on methicillin-resistant *Staphylococcus aureus Biosci. Biotechnol. Biochem.*, **70**: 1423-1431.

Kusumaningrum, H.D., Riboldi, G., Hazeleger, W.C. and Beumer, R.R. (2003). Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *Int. J. Food Microbiol.*, **85**: 227-236.

Kwon, D.Y., Kang, O.H., Choi, J.G., Lee, Y.S., Oh, Y.C., Chae, H.S., Lee, G.H., Park, P.S., Kim, Y.C., Sohn, D.H., Park, H. and Lee, J.H. (2007). Antibacterial effect of *Dryopteris crassirhizoma* against methicillin-resistant *Staphylococcus aureus. Fitoterapia*, **78**: 430-433.

Kyung, K.H., Kim, M.H., Park, M.S. and Kim, Y.E. (2002). Allinase-independent inhibition of *Staphylococcus aureus* B33 by heated garlic. *J. Food Sci.*, **67**: 780-785.

Lai, P.K. and Roy, J. (2004). Antimicrobial and chemopreventive properties of herbs and spices. *Curr. Med. Chem.*, **11**: 1451-1460.

Lambert, R.J., Skandamis, P.N., Coote, P.J. and Nychas, G.J. (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J. Appl. Microbiol.*, **91**: 453-462.

LaPlante, K.L. (2007). *In vitro* activity of lysostaphin, mupirocin, and tea tree oil against clinical methicillin-resistant *Staphylococcus aureus. Diagn. Microbiol. Infect. Dis.*, **57**: 413-418.

Laporta, O., Funes, L., Garzon, M.T., Villalain, J. and Micol, V. (2007). Role of membranes on the antibacterial and anti-inflammatory activities of the bioactive compounds from *Hypoxis rooperi* corm extract. *Arch. Biochem. Biophys.*, **467**: 119-131.

Latiff, A.M. (1992). *Rhodomyrtus tomentosa* (Aiton) Hassk. *In*: Plant Resources of South-East Asia. No. 2: Edible fruits and nuts, Coronel, R.E. and Verheij, E.W.M. *eds*. Prosea Foundation, Bogor, Indonesia, pp. 267-277.

Le Loir, Y., Baron, F. and Gautier, M. (2003). *Staphylococcus aureus* and food poisoning. *Genet. Mol. Res.*, **2**: 63-76.

Lee, J.H. (2003). Methicillin (Oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. *Appl. Environ. Microbiol.*, **69**: 6489-6494.

Lee, J.H., Shim, J.S., Lee, J.S., Kim, J.K., Yang, I.S., Chung, M.S. and Kim, K.H. (2006). Inhibition of pathogenic bacterial adhesion by acidic polysaccharide from green tea (*Camellia sinensis*). *J. Agric. Food Chem.*, **54**: 8717-8723.

Lee, Y.L., Cesario, T., Wang, Y., Shanbrom, E. and Thrupp, L. (2003). Antibacterial activity of vegetables and juices. *Nutrition*, **19**: 994-996.

Lerebour, G., Cupferman, S. and Bellon-Fontaine, M.N. (2004). Adhesion of *Staphylococcus aureus* and *Staphylococcus epidermidis* to the Episkin reconstructed epidermis model and to an inert 304 stainless steel substrate. *J. Appl. Microbiol.*, **97**: 7-16.

Leski, T.A. and Tomasz, A. (2005). Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of *Staphylococcus aureus*: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A. *J. Bacteriol.* **187**: 1815-1824.

Letertre, C., Perelle, S., Dilasser, F. and Fach, P. (2003). Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus. J. Appl. Microbiol.*, **95**: 38-43.

Liang, X. and Ji, Y. (2007). Comparative analysis of staphylococcal adhesion and internalization by epithelial cells. *Methods Mol. Biol.*, **391**: 145-151.

Libert, M., Elkholti, M., Massaut, J., Karmali, R., Mascart, G. and Cherifi, S. (2008). Risk factors for meticillin resistance and outcome of *Staphylococcus aureus* bloodstream infection in a Belgian university hospital. *J. Hosp. Infect.*, **68**: 17-24.

Limsuwan, S. and Voravuthikunchai, S.P. (2008). *Boesenbergia pandurata* (Roxb.) Schltr., *Eleutherine americana* Merr. and *Rhodomyrtus tomentosa* (Aiton) Hassk. as anti-biofilm producing and anti-quorum sensing in *Streptococcus pyogenes*. *Fem. Immunol. Med. Microbiol.*, **53**: 429-436.

Limsuwan, S. and Voravuthikunchai, S.P. (2009). Medicinal plants with significant activity against important pathogenic bacteria. *Pharm. Biol.* **47**: 683-689.

Limsuwan, S., Trip, E.N., Hiranra, A., Mahabusarakam, W., Voravuthikunchai, S.P. and Kayser, O. (2009). Rhodomyrtone: A candidate natural drug from *Rhodomyrtus tomentosa* for antibacterial property. *Phytomedicine* **16**: 645-651.

Liu, Y.Z., Hou, A.J., Ji, C.R. and Wu, Y.J. (1997). A new C-glycosidic hydrolyzable tannin from *Rhodomyrtus tomentosa*. *Chinese Chem. Lett.*, **8**: 39-40.

Liu, Y.Z., Hou, A.J., Ji, C.R. and Wu, Y.J. (1998). Isolation and structure of hydrolysable tannins from *Rhodomyrtus tomentosa*. *Tianran Chanwu Yanjiu Yu Kaifa.*, **10**: 14-19.

Lowry, F.D. (1998). *Staphylococcus aureus*. *N. Engl. J. Med.*, **339**: 520-535.

Machado, T.B., Pinto, A.V., Pinto, M.C., Leal, I.C., Silva, M.G., Amaral, A.C., Kuster, R.M. and Netto-dosSantos, K.R. (2003). *In vitro* activity of Brazilian medicinal plants, naturally occurring naphthoquinones and their analogues, against methicillin-resistant *Staphylococcus aureus*. *Int. J. Antimicrob. Agent*, **21**: 279-284.

Magwa, M.L., Gundidza, M., Gweru, N. and Humphrey, G. (2006). Chemical composition and biological activities of essential oil from the leaves of *Sesuvium portulacastrum*. *J. Ethnopharmacol.*, **103**: 85-89.

Mahabusarakam, W., Hamtasin, C., Chakthong, S., Voravuthikunchai, S.P. and Ifesan B.O.T. 2009. Napthoquinones, anthroquinones and naphthalene derivatives from the bulb of *Eleutherine americana*. *Planta Med.* doi. 10.1055/s-0029-1186143.

Maillard, J.Y. (2002). Bacterial target sites for biocide action. *Symp. Ser. Soc. Appl. Microbiol.*, **92**: 16S-27S.

Malm, A., Biernasiuk, A., Los, R., Kosikowska, U., Juda, M., Korona-Glowniak, I. and Gorniewski, G. (2005). Slime production and cell surface hydrophobicity of nasopharyngeal and skin staphylococci isolated from healthy people. *Pol. J. Microbiol.*, **54**: 117-121.

Mandell, G., Douglas, J. and Bennett, R. (1995). Principles and Practices of Infectious Diseases. 4th *ed.* Churchill Livingstone, Ltd., Edinburgh, United Kingdom.

Marwah, R.G., Fatope, M.O., Deadman, M.L., Ochei, J.E. and Al-Saidi, S.H. (2007). Antimicrobial activity and the major components of the essential oil of *Plectranthus cylindraceus*. *J. Appl. Microbiol.*, **103**: 1220-1226.

Mata, R., Martinez, E., Bye, R., Morales, G., Singh, M.P., Janso, J.E., Maiese, W.M. and Timmermann, B. (2001). Biological and mechanistic activities of xanthorrizol and 4-(1',5'-dimethylhex-4'-enyl)-2-methylphenol isolated from *Iostephane heterophylla*. *J. Nat. Prod.*, **64**: 911-914.

Mau, J., Chen, C. and Hsieh, P. (2001). Antimicrobial effect of extracts from Chinese chive, cinnamon, and corni fructus. *J. Agric. Food Chem.*, **49**: 183-188.

McRae, J.M., Yang, Q., Crawford, R.J. and Palombo, E.A. (2008). Antibacterial compounds from *Planchonia careya* leaf extracts. *J. Ethnopharmacol.*, **116**: 554-560.

Melzer, M., Eykyn, S.J., Gransden, W.R. and Chinn, S. (2003). Is methicillin-resistant *Staphylococcus aureus* more virulent than methicillin-susceptible *S. aureus*? A comparative cohort study of British patients with nosocomial infection and bacteremia. *Clin. Infect. Dis.*, **37**: 1453-1460.

Menzies, B.E. and Kourteva, I. (2000). *Staphylococcus aureus* alpha-toxin induces apoptosis in endothelial cells. *FEMS Immunol. Med. Microbiol.*, **29**: 39-45.

Micol, V., Mateo, C.R., Shapiro, S., Aranda, F.J. and Villalain, J. (2001). Effects of (+)-totarol, a diterpenoid antibacterial agent, on phospholipid model membranes. *Biochem. Biophys. Acta*, **1511**: 281-290.

Miller, A.J., Call, J.E. and Eben, S.B. (1997). Growth, injury and survival of *Yersinia enterolitica*, *Listeria monocytogenes* and *Staphylococcus aureus* in brine chiller conditions. *J. Food Prot.*, **60**: 1334-1340.

Munson, S.H., Tremaine, M.T., Betley, M.J. and Welch, R.A. (1998). Identification and characterization of staphylococcal enterotoxin types G and I from *Staphylococcus aureus*. *Infect. Immun.*, **66**: 3337-3348.

Ndi, C.P., Semple, S.J., Griesser, H.J. and Barton, M.D. (2007a). Antimicrobial activity of some Australian plant species from the genus Eremophila. *J. Basic Microbiol.*, **47**: 158-164.

Ndi, C.P., Semple, S.J., Griesser, H.J., Pyke, S.M. and Barton, M.D. (2007b). Antimicrobial compounds from *Eremophila serrulata*. *Phytochemistry*, **68**: 2684-2690.

Neilsen, L.B. and Wege, D. (2006). The enantioselective synthesis of elecanacin through an intramolecular naphthoquinone-vinyl ether photochemical cycloaddition. *Org. Biomol. Chem.*, **4**: 868-876.

Nimmo, G.R., Pearson, J.C., Collignon, P.J., Christiansen, Coombs, G.W., Bell, J.M. and McLaws, M.L. (2007). Prevalence of MRSA among *Staphylococcus aureus* isolated from hospital inpatients, (2005): report from the Australian Group for Antimicrobial Resistance. *Commun. Dis. Intell.*, **31**: 288-296.

NineSigma, (2008). Natural Antimicrobial Ingredients for Low pH Beverages. http://www.ninesigma.com/mx/50703-1 (16 September 2009, last day accessed).

Nishizawa, M., Yamagishi, T., Nonaka, G.I. and Nishioka, I. (1983). Tannins and related compounds. Part 9. Isolation and characterization of polygalloylglucoses from Turkish galls (*Quercus infectoria*). *J. Chem. Soc.*, **1**: 961-965.

Noble, W.C., Virani, Z. and Cree, R.G. (1992). Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. *FEMS Microbiol. Lett.*, **72**: 195-198.

Nohynek, L.J., Alakomi, H.L., Kahkonen, M.P., Heinonen, M., Helander, I.M., Oksman-Caldentey, K.M. and Puupponen-Pimia, R.H. (2006). Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens. *Nutr. Cancer*, **54**: 18-32.

Norajit, K., Laohakunjit, N. and Kerdchoechuen, O. (2007). Antibacterial effect of five Zingiberaceae essential oils. *Molecules*, **12**: 2047-2060.

Normanno, G., Firinu, A., Virgilio, S., Mula, G., Dambrosio, A., Poggiu, A., Decastelli, L., Mioni, R., Scuota, S., Bolzoni, G., Di Giannatale, E., Salinetti, A.P., La Salandra, G., Bartoli, M., Zuccon, F., Pirino, T., Sias, S., Parisi, A., Quaglia, N.C. and Celano, G.V. (2005). Coagulase-positive *Staphylococci* and *Staphylococcus aureus* in food products marketed in Italy. *Int. J. Food Microbiol.*, **98**: 73-79.

Normanno, G., La Salandra, G., Dambrosio, A., Quaglia, N.C., Corrente, M., Parisi, A., Santagada, G., Firinu, A., Crisetti, E. and Celano, G.V. (2007). Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. *Int. J. Food Microbiol.*, **115**: 290-296.

Nostro, A., Bisignano, G., Cannatelli, M.A., Crisafi, G., Germano, M.P. and Alonzo, V. (2001a). Effects of *Helichrysum italicum* extract on growth and enzymatic activity of *Staphylococcus aureus*. *Int. J. Antimicrob. Ag.*, **17**: 517-520.

Nostro, A., Cannatelli, M.A., Crisafi, G. and Alonzo, V. (2001b). The effect of *Nepeta cataria* extract on adherence and enzyme production of *Staphylococcus aureus*. *Int. J. Antimicrob. Agents*, **18**: 583-585.

Nostro, A., Cannatelli, M.A., Musolino, A.D., Procopio, F. and Alonzo, V. 2002. *Helichrysum italicum* extract interferes with the production of enterotoxins by *Staphylococcus aureus*. *Lett. Appl. Microbiol.*, **35**: 181-184.

Nostro, A., Cannatelli, M.A., Crisafi, G., Musolino, A.D., Procopio, F. and Alonzo, V. (2004). Modifications of hydrophobicity, *in vitro* adherence and cellular aggregation of *Streptococcus mutans* by *Helichrysum italicum* extract. *Lett. Appl. Microbiol.*, **38**: 423-427.

Nottermans, S. and Heuvelman, C.J. (1983). Combined effect of water activity, pH and sub-optimal temperature on growth and enterotoxin production. *J. Food Sci.*, **48**: 1832-1835.

Novick, R.P., Schlievert, P. and Ruzin, A. (2001). Pathogenicity and resistance islands of staphylococci. *Microbes. Infect.*, **3**: 585-594.

Okusa, P.N., Penge, O., Devleeschouwer, M. and Duez, P. (2007). Direct and indirect antimicrobial effects and antioxidant activity of *Cordia gilletii* De Wild (Boraginaceae). *J. Ethnopharmacol.*, **112**: 476-481.

Oliveira, D.R., Leitao, G.G., Santos, S.S., Bizzo, H.R., Lopes, D., Alviano, C.S., Alviano, D.S. and Leitao, S.G. (2006). Ethnopharmacological study of two *Lippia* species from Oriximina, Brazil. *J. Ethnopharmacol.*, **108**: 103-108.

Oonmetta-aree, H., Suzuki, T., Gasaluck, P. and Eumkeb, G. (2006). Antimicrobial properties and action of galangal (*Alpinia galanga* Linn.) on *Staphylococcus aureus*. *LWT-Food Sci. Technol.*, **39**: 1214-1220.

Oumeish, I., Oumeish, O.Y. and Bataineh, O. (2000). Acute bacterial skin infections in children. *Clin. Dermatol.*, **18**: 667-678.

Pan, W.H., Li, P.L. and Liu, Z. (2006). The correlation between surface hydrophobicity and adherence of Bifidobacterium strains from centenarians' faeces. *Anaerobe*, **12**: 148-152.

Park, B.S., Kim, J.G., Kim, M.R., Lee, S.E., Takeoka, G.R., Oh, K.B. and Kim, J.H. (2005). *Curcuma longa* L. constituents inhibit sortase A and *Staphylococcus aureus* cell adhesion to fibronectin. *J. Agric. Food Chem.*, **53**: 9005-9009.

Park, E.J. and Pezzuto, J.M. (2002). Botanicals in cancer chemoprevention. *Cancer Metastasis Rev.*, **21**: 231-255.

Park, W.B., Kim, S.H., Kang, C.I., Cho, J.H., Bang, J.W., Park, K.W., Lee, Y.S., Kim, N.J., Oh, M.D., Kim, H.B. and Choe, K.W. (2007). *In vitro* ability of *Staphylococcus aureus* isolates from bacteraemic patients with and without metastatic complications to invade vascular endothelial cells. *J. Med. Microbiol.*, **56**: 1290-1295.

Patil, R., Baveja, S., Nataraj, G. and Khopkar, U. (2006). Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in community-acquired primary pyoderma. *Indian J. Dermatol. Venereol. Leprol.*, **72**: 126-128.

Peacock, S.J., Silva, L. and Lowry, F.D. (2001). What determines nasal carriage of *Staphylococcus aureus*? *Trends Microbiol.*, **9**: 605-610.

Peacock, S.J., Foster, T.J., Cameron, B.J. and Berendt, A.R. (1999). Bacterial fibronectin-binding proteins and endothelial cell surface fibronectin mediate adherence of *Staphylococcus aureus* to resting human endothelial cells. *Microbiology*, **145**: 3477-3486.

Pereira, E.M., Machado Tde, B., Leal, I.C., Jesus, D.M., Damaso, C.R., Pinto, A.V., Giambiagi-deMarval, M., Kuster, R.M. and Santos, K.R. (2006). *Tabebuia avellanedae* naphthoquinones: activity against methicillin-resistant staphylococcal strains, cytotoxic activity and *in vivo* dermal irritability analysis. *Ann. Clin. Microbiol. Antimicrob.*, **5**: 5 doi :10.1186/1476-0711-5-5.

Pesavento, G., Ducci, B., Comodo, N. and Nostro, L. (2007). Antimicrobial resistance of *Staphylococcus aureus* isolated from raw meat: A reseach for methicillin resistant *Staphylococcus aureus* (MRSA). *Food Control.*, **18**: 196-200.

Pesewu, G.A., Cutler, R.R. and Humber, D.P. (2008). Antibacterial activity of plants used in traditional medicines of Ghana with particular reference to MRSA. *J. Ethnopharmacol.*, **116**: 102-111.

Prabu, G.R., Gnanamani, A. and Sadulla, S. (2006). Guaijaverin-a plant flavonoid as potential antiplaque agent against *Streptococcus mutans*. *J. Appl. Microbiol.*, **101**: 487-495.

Puupponen-Pimia, R., Nohynek, L., Meier, C., Kahkonen, M., Heinonen, M., Hopia, A. and Oksman-Caldentey, K.M. (2001). Antimicrobial properties of phenolic compounds from berries. *J. Appl. Microbiol.*, **90**: 494-507.

Puupponen-Pimia, R., Nohynek, L., Alakomi, H.L. and Oksman-Caldentey, K.M. (2005a). Bioactive berry compounds-novel tools against human pathogens. *Appl. Microbiol. Biotechnol.*, **67**: 8-18.

Puupponen-Pimia, R., Nohynek, L., Hartmann-Schmidlin, S., Kahkonen, M., Heinonen, M., Maatta-Riihinen, K. and Oksman-Caldentey, K.M. (2005b). Berry phenolics selectively inhibit the growth of intestinal pathogens. *J. Appl. Microbiol.*, **98**: 991-1000.

Reddy, M.K., Gupta, S.K., Jacob, M.R., Khan, S.I. and Ferreira, D. (2007). Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from *Punica granatum* L. *Planta Med.*, **73**: 461-467.

Regerat, F., Pourrat, H. and Pourrat, A. (1989). Hydrolysis by fermentation of tannins from gall nuts. *J. Am. Leather Chem. Assoc.*, **84**: 323-328.

Reichling, J., Weseler, A. and Saller, R. (2001). A current review of the antimicrobial activity of *Hypericum perforatum* L. *Pharmacopsychiatry*, **34(Suppl 1)**: S116-118.

Ren, K., Bannan, J.D., Pancholi, V., Cheung, A.L., Robbins, J.C., Fischetti, V.A. and Zabriskie, J.B. (1994). Characterization and biological properties of a new staphylococcal exotoxin. *J. Exp. Med.*, **180**: 1675-1683.

Rennie, R.P., Jones, R.N. and Mutnick, A.H. (2003). Occurrence and antimicrobial susceptibility patterns of pathogens isolated from skin and soft tissue infections: report from the SENTRY Antimicrobial Surveillance Program (United States and Canada, 2000). *Diagn. Microbiol. Infect. Dis.*, **45**: 287-293.

Rhody, C. (2000). Bacterial infections of the skin. *Prim. Care.*, **27**: 459-473.

Rice, L.B. (2006). Antimicrobial resistance in gram-positive bacteria. *Am. J. Infect. Control*, **34**: S11-19; discussion S64-73.

Rivas, J.M., Speziale, P., Patti, J.M. and Hook, M. (2004). MSCRAMM-targeted vaccines and immunotherapy for staphylococcal infection. *Curr. Opin. Drug Discov. Devel.*, **7**: 223-227.

Roberts, F.J., Geere, I.W. and Coldman, A. (1991). A three-year study of positive blood cultures, with emphasis on prognosis. *Rev. Infect. Dis.*, **13**: 34-46.

Roller, S. (1995). The Quest for Natural Antimicrobials as Novel Means of Food Preservation: Status Report on an European Research Project. *Int. Biodeter. Biodegrad.*, pp. 333-345.

Rott, O. and Fleischer, B. (1994). A superantigen as virulence factor in an acute bacterial infection. *J. Infect. Dis.*, **169**: 1142-1146.

Roy, S., Rink, C., Khanna, S., Phillips, C., Bagchi, D., Bagchi, M. and Sen, C.K. (2004). Body weight and abdominal fat gene expression profile in response to a novel hydroxycitric acid-based dietary supplement. *Gene Exp.* **11**: 251-262.

Roychoudhury, S., Dotzlaf, J.E., Ghag, S. and Yeh, W.K. (1994). Purification, properties, and kinetics of enzymatic acylation with beta-lactams of soluble penicillin-binding protein 2a. A major factor in methicillin-resistant *Staphylococcus aureus*. *J. Biol. Chem.*, **269**: 12067-12073.

Rukachaisirikul, T., Innok, P., Aroonrerk, N., Boonamnuaylap, W., Limrangsun, S., Boonyon, C., Woonjina, U. and Suksamrarn, A. (2007). Antibacterial pterocarpans from *Erythrina subumbrans*. *J. Ethnopharmacol.*, **110**: 171-175.

Rukachaisirikul, V., Phainuphong, P., Sukpondma, Y., Phongpaichit, S. and Taylor, W.C. (2005). Antibacterial caged-tetraprenylated xanthones from the stem bark of *Garcinia scortechinii*. *Planta Med.*, **71**: 165-170.

Russell, A.D. and Chopra, I. (1996). Understanding antibacterial action and resistance. Eills Herwood. England, pp. 96-149.

Saising, J., Hiranrat, A., Mahabusarakum, W., Ongsakul, M. and Voravuthikunchai, S.P. (2008). Rhodomyrtone from *Rhodomyrtus tomentosa* (Aiton) Hassk. as an antibiotic for staphylococcal infections. *J. Health Sci.*, **54**: 589-595.

Samuelsson, G. (1999). Drugs of Natural Origin. 4th ed. Swedish Pharmaceutical Press. Stockholm., pp. 551.

Savas, L., Onlen, Y., Duran, N. and Savas, N. (2007). Causes of nosocomial pneumonia and evaluation of risk factors in a university hospital in Turkey. *Saudi Med. J.*, **28**: 114-120.

Sawangjaroen, N., Sawangjaroen, K. and Poonpanang, P. (2004). Effects of *Piper longum* fruit, *Piper sarmentosum* root and *Quercus infectoria* nut gall on caecal amoebiasis in mice. *J. Ethnopharmacol.*, **91**: 357-360.

Schilter, B., Andersson, C., Anton, R., Constable, A., Kleiner, J., O'Brien, J., Renwick, A.G., Korver, O., Smit, F. and Walker, R. (2003). Guidance for the safety assessment of botanicals and botanical preparations for use in food and food supplements. *Food Chem. Toxicol.*, **41**: 1625-1649.

Schmid, D., Gschiel, E., Mann, M., Huhulescu, S., Ruppitsch, W., Bohm, G., Pichler, J., Lederer, I., Hoger, G., Heuberger, S. and Allerberger, F. (2007). Outbreak of acute gastroenteritis in an Austrian boarding school, Sept., 2006. *Euro Surveill.*, **12**: 224.

Schuchat, A., Swaminathan, B. and Broome, C.V. (1991). Epidermiology of human listeriosis. *Clin. Microbiol. Rev.*, **4**: 169-183.

Shan, B., Cai, Y., Brooks, J.D. and Corke, H. (2007). Antibacterial Properties and Major Bioactive Components of Cinnamon Stick (*Cinnamomum burmannii*): Activity against Foodborne Pathogenic Bacteria. *J. Agric. Food Chem.*, **55**: 5484-5490.

Shelef, L.A. (1983). Antimicrobial effect of spices. *J. Food Safety*, **6**: 29-44.

Shen, C.C., Syu, W.J., Li, S.Y., Lin, C.H., Lee, G.H. and Sun, C.M. (2002). Antimicrobial activities of naphthazarins from *Arnebia euchroma*. *J. Nat. Prod.*, **65**: 1857-1862.

Shimada, K., Kimura, E., Yasui, Y., Tanaka, H., Matsushita, S., Nagakmura, M. and Kawashisa, M. (1991). Styrene formation by the decomposition of *Pichia carsonii* of trans-cinnamic acid added to a ground fish product. *Appl. Environ. Microbiol.*, **58**: 1577-1582.

Shimizu, A., Fujita, M., Igarashi, H., Takagi, M., Nagase, N., Sasaki, A. and Kawano, J. (2000). Characterization of *Staphylococcus aureus* coagulase type VII isolates from staphylococcal food poisoning outbreaks (1980-1995) in Tokyo, Japan, by pulsed-field gel electrophoresis. *J. Clin. Microbiol.*, **38**: 3746-3749.

Shiota, S., Shimizu, M., Sugiyama, J., Morita, Y., Mizushima, T. and Tsuchiya, T. (2004). Mechanisms of action of corilagin and tellimagrandin I that remarkably potentiate the activity of beta-lactams against methicillin-resistant *Staphylococcus aureus*. *Microbiol. Immunol.*, **48**: 67-73.

Shiota, S., Shimizu, M., Mizushima, T., Ito, H., Hatano, T., Yoshida, T. and Tsuchiya, T. (1999). Marked reduction in the minimum inhibitory concentration (MIC) of beta-lactams in methicillin-resistant *Staphylococcus aureus* produced by epicatechin gallate, an ingredient of green tea (*Camellia sinensis*). *Biol. Pharm. Bull.*, **22**: 1388-1390.

Shiu, W.K. and Gibbons, S. (2006). Anti-staphylococcal acylphloroglucinols from *Hypericum beanii*. *Phytochemistry*, **67**: 2568-2572.

Shmuely, H., Pitlik, S., Drucker, M., Samra, Z., Konisberger, H. and Leibovici, L. (2000). Prediction of mortality in patients with bacteremia: the importance of pre-existing renal insufficiency. *Ren. Fail.*, **22**: 99-108.

Shorr, A.F. (2007). Epidemiology of staphylococcal resistance. *Clin. Infect. Dis.*, **45**: S171-S176.

Shukitt-Hale, B., Lau, F.C. and Joseph, J.A. (2008). Berry fruit supplementation and the aging brain. *J. Agric. Food Chem.*, **56**: 636-641.

Si, W., Gong, J., Tsao, R., Kalab, M., Yang, R. and Yin, Y. (2006). Bioassay-guided purification and identification of antimicrobial components in Chinese green tea extract. *J. Chromatogr. A.*, **1125**: 204-210.

Singh, R., Jain, A., Panwar, S., Gupta, D. and Khare, S.K. (2005). Antimicrobial activity of some natural dyes. *Dyes and Pigments*, **66**: 99-102.

Smid, E.J. and Gorris, L.G.M. (1999). Natural antimicrobials for food preservation. *In*: Rahman, M.S., *ed.*, Handbook of Food Preservation. Dekker, New York, pp. 285-308.

Smith, E., Williamson, E., Zloh, M. and Gibbons, S. (2005). Isopimaric acid from *Pinus nigra* shows activity against multidrug-resistant and EMRSA strains of *Staphylococcus aureus*. *Phytother. Res.*, **19**: 538-542.

Soderquist, B., Sundqvist, K.G. and Vikerfors, T. (1999). Adhesion molecules (E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)) in sera from patients with *Staphylococcus aureus* bacteraemia with or without endocarditis. *Clin. Exp. Immunol.*, **118**: 408-411.

Somchit, M.N., Reezal, I., Nur, I.E. and Mutalib, A.R. (2003). *In vitro* antimicrobial activity of ethanol and water extracts of *Cassia alata*. *J. Ethnopharmacol.*, **84**: 1-4.

Song, J.M. and Seong, B.L. (2007). Tea catechins as a potential alternative anti-infectious agent. *Expert Rev. Anti. Infect. Ther.*, **5**: 497-506.

Spika, J.S., Peterson, P.K., Wilkinson, B.J., Hammerschmidt, D.E., Verbrugh, H.A., Verhoef, J. and Quie, P.G. (1982). Role of peptidoglycan from *Staphylococcus aureus* in leukopenia, thrombocytopenia, and complement activation associated with bacteremia. *J. Infect. Dis.*, **146**: 227-234.

Stapleton, P.D. and Taylor, P.W. (2002). Methicillin resistance in *Staphylococcus aureus*: mechanisms and modulation. *Sci. Prog.*, **85**: 57-72.

Stapleton, P.D., Gettert, J. and Taylor, P.W. (2006). Epicatechin gallate, a component of green tea, reduces halotolerance in *Staphylococcus aureus*. *Int. J. Food Microbiol.*, **111**: 276-279.

Stapleton, P.D., Shah, S., Anderson, J.C., Hara, Y., Hamilton-Miller, J.M.T. and Taylor, P.W. (2004a). Modulation of beta-lactam resistance in *Staphylococcus aureus* by catechins and gallates. *Int. J. Antimicrob. Agents*, **23**: 462-467.

Stapleton, P.D., Shah, S., Hamilton-Miller, J.M., Hara, Y., Nagaoka, Y., Kumagai, A., Uesato, S. and Taylor, P.W. (2004b). Anti-*Staphylococcus aureus* activity and oxacillin resistance modulating capacity of 3-*O*-acyl-catechins. *Int. J. Antimicrob. Agents*, **24**: 374-380.

Stojanovic, G., Radulovic, N., Hashimoto, T. and Palic, R. (2005). *In vitro* antimicrobial activity of extracts of four *Achillea* species: the composition of *Achillea clavennae* L. (Asteraceae) extract. *J. Ethnopharmacol.*, **101**: 185-190.

Su, Y.C. and Wong, A.C. (1995). Identification and purification of a new staphylococcal enterotoxin H. *Appl. Environ. Microbiol.*, **61**: 1438-1443.

Sudano Roccaro, A., Blanco, A.R., Giuliano, F., Rusciano, D. and Enea, V. (2004). Epigallocatechin-gallate enhances the activity of tetracycline in staphylococci by inhibiting its efflux from bacterial cells. *Antimicrob. Agents Chemother.*, **48**: 1968-1973.

Sukpondma, Y., Rukachaisirikul, V. and Phongpaichit, S. (2005). Xanthone and sesquiterpene derivatives from the fruits of *Garcinia scortechinii*. *J. Nat. Prod.*, **68**: 1010-1017.

Suwalak, S. and Voravuthikunchai, S.P. (2009). Morphological and ultrastructural changes in the cell structure of enterohaemorrhagic *Escherichia coli* O157: H7 following treatment with *Quercus infectoria* nut galls. *J. Electron Microsc.*, **58**: 315-320.

Syu, W.J., Shen, C.C., Lu, J.J., Lee, G.H. and Sun, C.M. (2004). Antimicrobial and cytotoxic activities of neolignans from *Magnolia officinalis*. *Chem. Biodivers.*, **1**: 530-537.

Tack, K.J., Hedrick, J.A., Rothstein, E., Nemeth, M.A., Keyserling, C. and Pichichero, M.E. (1997). A study of 5-day cefdinir treatment for streptococcal pharyngitis in children. Cefdinir Pediatric Pharyngitis Study Group. *Arch. Pediatr. Adolesc. Med.*, **151**: 45-49.

Taguri, T., Tanaka, T. and Kouno, I. (2004). Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. *Biol. Pharm. Bull.*, **27**: 1965-1969.

Tassou, C.C., Drosinos, E.H. and Nychas, G.J. (1995). Effects of essential oil from mint (*Mentha piperita*) on *Salmonella enteritidis* and *Listeria monocytogenes* in model food systems at 4 °C and 10 °C. *J. Appl. Bacteriol.*, **78**: 593-600.

Tatsimo, S.J., Tane, P., Srinivas, P.V., Sondengam, B.L., Melissa, J., Okunji, C.O., Schuster, B.M., Iwu, M.M. and Khan, I.A. (2005). Novel antimicrobial diterpenoids from *Turraeanthus africanus*. *Planta Med.*, **71**: 1145-1151.

Tenover, F.C. and Gaynes, R.P. (2000). The epidermiology of *Staphylococcus* infections. *In*: Fischetti, V.A., Novick, R.P., Ferretti, J.J., Portnoy, D.A. and Rood, J.I. *eds*., Gram-Positive Pathogens. American Society for Microbiology, Washington D.C., pp. 414-421.

Tenover, F.C., Lancaster, M.V., Hill, B.C., Steward, C.D., Stocker, S.A., Hancock, G.A., O'Hara, C.M., McAllister, S.K., Clark, N.C. and Hiramatsu, K. (1998). Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. *J. Clin. Microbiol.*, **36**: 1020-1027.

Thiemermann, C. (2002). Interactions between lipoteichoic acid and peptidoglycan from *Staphylococcus aureus*: a structural and functional analysis. *Microbes. Infect.*, **4**: 927-935.

Thongpiyapoom, S., Narong, M.N., Suwalak, N., Jamulitrat, S., Intaraksa, P., Boonrat, J., Kasatpibal, N. and Unahalekhaka, A.V. (2004). Device-associated infections and patterns of antimicrobial resistance in a medical-surgical intensive care unit in a university hospital in Thailand. *J. Med. Assoc. Thai.*, **87**: 819-824.

Thring, T.S.A., Springfield, E.P. and Weitz, F.M. (2007). Antimicrobial activities of four plant species from the Southern Overberg region of South Africa. *Afr. J. Biotechnol.* **6**: 1779-1784.

Tiemersma, E.W., Bronzwaer, S.L., Lyytikainen, O., Degener, J.E., Schrijnemakers, P., Bruinsma, N., Monen, J., Witte, W. and Grundman, H. (2004). Methicillin-resistant *Staphylococcus aureus* in Europe, 1999-2002. *Emerg. Infect. Dis.*, **10**: 1627-1634.

Tiwari, T.P., Bharti, S.K., Kaur, H.D., Dikshit, R.P. and Hoondal, G.S. (2005). Synergistic antimicrobial activity of tea & antibiotics. *Indian J. Med. Res.* **122**: 80-84.

Trombetta, D., Castelli, F., Sarpietro, M.G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G. and Bisignano, G. (2005). Mechanisms of antibacterial action of three monoterpenes. *Antimicrob. Agents Chemother.*, **49**: 2474-2478.

Tuley de Silva, K. (1996). A Manual on the Essential Oil Industry. United Nations Industrial Development Organization. Vienna.

Turnidge, J.D., Nimmo, G.R., Pearson, J., Gottlieb, T. and Collignon, P.J. (2007). Epidemiology and outcomes for *Staphylococcus aureus* bacteraemia in Australian hospitals, (2005-06): report from the Australian Group on Antimicrobial Resistance. *Commun. Dis. Intell.*, **31**: 398-403.

Uckay, I., Harbarth, S. and Pittet, D. (2007). Management of Gram-positive bacteraemia. *Curr. Opin. Infect. Dis.*, **20**: 561-567.

van Belkum, A. (2006). Staphylococcal colonization and infection: homeostasis versus disbalance of human (innate) immunity and bacterial virulence. *Curr. Opin. Infect. Dis.*, **19**: 339-344.

van Heerden, F.R., Viljoen, A.M. and van Wyk, B.E. (2000). The major flavonoid of *Dodonaea angustifolia*. *Fitoterapia*, **71**: 602-604.

van Nederkassel, A.M., Daszykowski, M., Massart, D.L. and Heyden, Y.V. (2005). Prediction of total green tea antioxidant capacity from chromatograms by multivariate modeling. *J. Chromatogr. A.*, **1096**: 177-186.

Vann, J.M. and Proctor, R.A. (1987). Ingestion of *Staphylococcus aureus* by bovine endothelial cells results in time- and inoculum-dependent damage to endothelial cell monolayers. *Infect. Immun.*, **55**: 2155-2163.

Vann, J.M. and Proctor, R.A. (1988). Cytotoxic effects of ingested *Staphylococcus aureus* on bovine endothelial cells: role of *S. aureus* alpha-hemolysin. *Microb. Pathog.*, **4**: 443-453.

Vercellotti, G.M., Lussenhop, D., Peterson, P.K., Furcht, L.T., McCarthy, J.B., Jacob, H.S. and Moldow, C.F. (1984). Bacterial adherence to fibronectin and endothelial cells: a possible mechanism for bacterial tissue tropism. *J. Lab. Clin. Med.*, **103**: 34-43.

Verdrengh, M. and Tarkowski, A. (2000). Role of macrophages in *Staphylococcus aureus*-induced arthritis and sepsis. *Arthritis Rheum.*, **43**: 2276-2282.

Visutthi, M., Srimanote, P., Klaysing, B., Pootong, A., Khantisitthiporn, O. and Voravuthikunchai, S.P. (2008). Influence of the ethanolic extract of *Rhodomyrtus tomentosa* (Aiton) Hassk. leaves on protein expression in methicillin-resistant *Staphylococcus aureus*. Programme and Abstracts, 1st Scientific Meeting in Allied Health Sciences: Trends of Research in Biomedical Sciences, 20 February, 2008 at Bangkok, Thailand.

von Eiff, C., Proctor, R.A. and Peters, G. (2000). Small colony variants of staphylococci: a link to persistent infections. *Berl. Munch. Tierarztl. Wochenschr.*, **113**: 321-325.

Voravuthikunchai, S.P. and Kitpipit, L. (2005). Activity of medicinal plant extracts against hospital isolates of methicillin-resistant *Staphylococcus aureus*. *Clin. Microbiol. Infect.*, **11**: 510-512.

Voravuthikunchai, S.P. and Limsuwan, S. (2006). Medicinal plant extracts as anti-*Escherichia coli* O157: H7 agents and their effects on bacterial cell aggregation. *J. Food Prot.*, **69**: 2336-2341.

Voravuthikunchai, S.P. and Chusri, S. (2007). Extraction method of bioactive compounds from *Quercus infectoria*. Thai Patent. 0701 003210 (22/06/2007).

Voravuthikunchai, S.P. and Mitchell, H. (2008). Inhibitory and killing activities of medicinal plants against multiple antibiotic-resistant strains of *Helicobacter pylori*. *J. Health Sci.*, **54**: 81-88.

Voravuthikunchai, S.P. and Siripongvutikorn, S. (2008). Food additive with antibacterial activity from *Eleutherine americana* Merr. and its application in food preservation. Thai Patent. Application No. 0801004660 (11/09/2008).

Voravuthikunchai, S.P. and Suwalak, S. (2008). Antibacterial activities of semi-purified fractions of *Quercus infectoria* (nut galls) against enterohaemorrhagic *Escherichia coli* O157: H7. *J. Food Prot.*, **71**: 87-91.

Voravuthikunchai, S.P. and Suwalak, S. (2009). Changes in cell surface properties of shiga toxigenic *Escherichia coli* by *Quercus infectoria* G. Olivier. *J. Food. Prot.* **72**: 265-270.

Voravuthikunchai, S.P., Popaya, V. and Supawita, T. (2004a). Antibacterial activity of crude extracts of medicinal plants used in Thailand against pathogenic bacteria. *Ethnopharmacologia*, **33**: 60-70.

Voravuthikunchai, S.P., Lortheeranuwat, A., Jeeju, W., Sririrak, T., Phongpaichit, S. and Supawita, T. (2004b). Effective medicinal plants against enterohaemorrhagic *Escherichia coli* O157: H7. *J. Ethnopharmacol.*, **94**: 49-54.

Voravuthikunchai, S.P., Chusri, S. and Kleiner, P. (2006a). Inhibitory activity and killing activity of extracts from the gall of *Quercus infectoria* against methicillin-resistant *Staphylococcus aureus. Clin. Microbiol. Infect.*, **12(Suppl. 4)**: R1885-R2270.

Voravuthikunchai, S.P., Limsuwan, S. and Mitchell, H. (2006b). Effects of *Punica granatum* pericarps and *Quercus infectoria* nutgalls on cell surface hydrophobicity and cell survival of *Helicobacter pylori. J. Health Sci.*, **52**: 154-159.

Voravuthikunchai, S.P., Suwalak, S. and Supawita, T. (2006c). Antibacterial activity of fractions of *Quercus infectoria* (nut galls) against enterohaemorrhagic *Escherichia coli. Clin. Microbiol. Infect.* **12(Suppl. 4)**: P679-P940.

Voravuthikunchai, S.P., Limsuwan, S. and Chusri, S. (2007a). 'New perspectives on herbal medicines for bacterial infections. *In:* J.N. Govil, V.K. Singh and Naila T. Siddqui *eds.*, Recent Progress in Medicinal Plants Vol. 18: Natural Products II. Studium Press LLC, USA, pp. 41-101.

Voravuthikunchai, S.P, Limsuwan, S. and Subhadhirasakul, S. (2007b). Screening for medicinal plants with broad spectrum of antibacterial activity. *Int. J. Antimicrob. Agents,* **29(Suppl. 2)**: S599.

Voravuthikunchai, S.P., Chusri, S. and Suwalak, S. (2008a). *Quercus infectoria* Oliv.: Activity as a broad-spectrum antibacterial drug. *Pharm. Biol.*, **46**: 367-372.

Voravuthikunchai, S.P., Ifesan, B.O.T., Mahabusarakam, W. and Hamtasin, C. (2008b). Antistaphylococcal activity of semi-purified fractions from *Eleutherine americana. Clin. Microbiol. Infect.*, **14(Suppl. 7)**: P580.

Voravuthikunchai and, S.P., Mahabusarakam, W., Hiranrat, A. and Limsuwan S. (2008c). Extraction method for bioactive compounds from *Rhodomyrtus tomentosa* (Ait.) Hassk. Thai Patent. Application No. 0801003113 (15/07/2008).

Wachsmuth, I.K. and Morris, G.K. (1989). 'Shigella'. *In*: Doyle, M.P. *eds.* Foodborne Pathogens. Marcel Dekker, New York and Basel; pp. 447-462.

Wachsmuth, I.K., Blake, P.A. and Olsvik, O. (1994). *Vibrio cholerae* and Cholera. *ASM Press*, Washington, D.C., 465 pp.

Wada, A. and Watanabe, H. (1998). Penicillin-binding protein 1 of *Staphylococcus aureus* is essential for growth. *J. Bacteriol.* **180**: 2759-2765.

Walencka, E., Wieckowska-Szakiel, M., Rozalska, S., Sadowska, B. and Rozalska, B. (2007). A surface-active agent from *Saccharomyces cerevisiae* influences staphylococcal adhesion and biofilm development. *Z. Naturforsch. [C]*, **62**: 433-438.

Wang, F.D., Chen, Y.Y., Chen, T.L. and Liu, C.Y. (2008). Risk factors and mortality in patients with nosocomial *Staphylococcus aureus* bacteremia. *Am. J. Infect. Control*, **36**: 118-122.

Wann, E.R., Gurusiddappa, S. and Hook, M. (2000). The fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen. *J. Biol. Chem.*, **275**: 13863-13871.

Wiart, C. and Kumar, A. (2001). Practical Handbook of Pharmacognosy. Person Education Malaysia Sdn Bhd. Malaysia, pp. 241.

Wieneke, A.A., Roberts, D. and Gilbert, R.J. (1993). Staphylococcal food poisoning in the United Kingdom, 1969-90. *Epidemiol. Infect.*, **110**: 519-531.

Wilkinson, B. (1997). 'The staphylococci in human disease'. *In*: Crossley, K.B. and Archer, *eds.*, Biology. Churchill Livingstone; pp. 1-38.

Willcox, P.A., Rayner, B.L. and Whitelaw, D.A. (1998). Community-acquired *Staphylococcus aureus* bacteraemia in patients who do not abuse intravenous drugs. *Qjm.*, **91**: 41-47.

Williams, G.J., Denyer, S.P., Hosein, I.K., Hill, D.W. and Maillard, J.Y. (2007). The development of a new three-step protocol to determine the efficacy of disinfectant wipes on surfaces contaminated with *Staphylococcus aureus*. *J. Hosp. Infect.*, **67**: 329-335.

Winotai, A., Wright, T. and Goolsby, J.A. (2005). Herbivores in Thailand on *Rhodomyrtus tomentosa* (Myrtaceae), an invasive weed in Florida. *Florida Entomologist.*, **88**: 104-105.

Wojnicz, D. and Jankowski, S. (2007). Effects of subinhibitory concentrations of amikacin and ciprofloxacin on the hydrophobicity and adherence to epithelial cells of uropathogenic *Escherichia coli* strains. *Int. J. Antimicrob. Agents*, **29**: 700-704.

Woldemichael, G.M., Wachter, G., M.P. Singh, Maiese, W.M. and Timmermann, B.N. (2003b). Antibacterial diterpenes from *Calceolaria pinifolia*. *J. Nat. Prod.* **66**: 242-246.

Woldemichael, G.M., Singh, M.P., Maiese, W.M. and Timmermann, B.N. (2003a). Constituents of antibacterial extract of *Caesalpinia paraguariensis* Burk.

World Health Organization, (2003). Traditional medicine. *The World Heath Organization Fact Sheet*, **134**: 1-3.

Wu, T., Yeaman, M.R. and Bayer, A.S. (1994). *In vitro* resistance to platelet microbicidal protein correlates with endocarditis source among bacteremic staphylococcal and streptococcal isolates. *Antimicrob. Agents Chemother.*, **38**: 729-732.

Xu, J., Qui, F., Duan, W., Qu, G., Wang, N. and Yao, X. (2006). New Bioactive constituents from *Eleutherine americana*. *Front. Chem. China*, **3**: 320-323.

Yi, Z.B., Yan, Y., Liang, Y.Z. and Bao, Z. (2007). Evaluation of the antimicrobial mode of berberine by LC/ESI-MS combined with principal component analysis. *J. Pharm. Biomed. Anal.*, **44**: 301-304.

Yu, Y., Yi, Z.B. and Liang, Y.Z. (2007a). Validate antibacterial mode and find main bioactive components of traditional Chinese medicine *Aquilegia oxysepala*. *Bioorg. Med. Chem. Lett.*, **17**: 1855-1859.

Yu, Y., Yi, Z.B. and Liang, Y.Z. (2007b). Main antimicrobial components of *Tinospora capillipes*, and their mode of action against *Staphylococcus aureus*. *FEBS Lett.*, **581**: 4179-4183.

Zhang, Z., ElSohly, H.N., Jacob, M.R., Pasco, D.S., Walker, L.A. and Clark, A.M. (2002). New sesquiterpenoids from the root of *Guatteria multivenia*. *J. Nat. Prod.*, **65**: 856-859.

Zhao, W.H., Hu, Z.Q., Okubo, S., Hara, Y. and Shimamura, T. (2001). Mechanism of synergy between epigallocatechin gallate and beta-lactams against methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.*, **45**: 1737-1742.

3

Pharmacological Activities, Phytochemical Investigations and *In vitro* Studies of *Gymnema sylvestre* R.Br. – A Historical Review

A. Bakrudeen Ali Ahmed[1,4], N. Komalavalli[2], M. Muthukumar[1], J.H.F. Benjamin[1], A.S. Rao[3], Se-kwon Kim[4] and M.V. Rao*[1]

ABSTRACT

Gymnema sylvestre R.Br., a liane belonging to the family Asclepiadaceae, has been employed to control diabetes, obesity, asthma etc., by traditional medicinal practitioners of India for nearly two millennia. The active principle from the leaf extracts is assumed to be gymnemic acid and its derivatives which are of triterpenoids in chemical nature have the ability to renew the islet cell mass for the possible cure of diabetes. The present review deals with botanical description, phenology, geographical distribution, chemical constituents and medicinal properties, pharmacological, phytochemical and in vitro plant tissue culture studies from the past to recent developments. Other aspects such as current status, progress and future challenges are also discussed.

Key words : *Gymnema sylvestre* R.Br, antidiabetic, botanical description, geographical distribution, phenology, medicinal uses,

1. Department of Plant Science, Bharathidasan University, Tiruchirappalli - 620 024, Tamilnadu, India.
2. Department of Botany, Govt. Arts college for Women, Pudukkottai - 622 001, Tamilnadu, India.
3. Department of Biotechnology, Bharathidasan University, Tiruchirappalli - 620 024, Tamilnadu, India.
4. Department of Chemistry, Pukyong National University, Busan - 608 737, South Korea.

* *Corresponding author* : E-mail : mvrao_456@yahoo.co.in

phytochemistry, pharmacological activity, *in vitro* studies, secondary metabolites production

INTRODUCTION

Gymnema sylvestre R.Br. is a well known and highly valuable medicinal plant in folk, Ayurvedic, Siddha and Homeopathy systems of medicine for the treatment of diabetes (Dixit & Pandey, 1984; Mitra *et al.*, 1996). More than 148 plant species of 50 families have shown hypoglycaemic activity (Handa *et al.*, 1989). All the species were helpful only in balancing the blood glucose level, while *G. sylvestre* brings blood glucose homeostasis through increased serum insulin levels provided by repair or regeneration of the endocrine pancreas (Shanmugasundaram *et al.*, 1983, 1990). This character gives a distinct advantage over other plant and synthetic drugs used for diabetes treatment. Besides antidiabetic activity, *G. sylvestre* has various medicinal uses discussed in the later part of this review. Several products, under brand names such as Body Slatto Tea®, Gymnema®, Gymnema Diet®, Sugar Off®, Glucoset™, Cinndrome X ™, and Pilisoft ™ are commercially available in markets of Japan, Germany and USA as health foods and cosmetics.

Several scientists during the period 1907 – 2009 have attempted and succeeded in the pharmacology, phytochemical studies (isolation and structure elucidation) and *in vitro* plant tissue culture studies (Figs. 1 & 2). The composition of triterpenoids of this plant has been thoroughly investigated. However, there are many references about the traditional uses of this plant, often they are contradictory. A systematic review related to phamacognosy, phytochemistry, pharmacology and clinical applications of *G. sylvestre* was made (Shailendra Gurav *et al.*, 2007). However, it deals only with minimum literature and not discussed about the contradictory views, structure of all the gymnemic acid derivatives, biotechnological aspects and future prospects.

Alternative *in vitro* (plant tissue culture) methods would be beneficial in accelerating large scale multiplication of secondary metabolites and conservation of the plant. Gymnemic acid is an important source of new drug for diabetes but to attain that numerous challenges are encountered including the procurement of plant material, the selection and implementation of appropriate high-throughout screening bioassays and the scale-up of active compounds. Hence an attempt is made to review *G. sylvestre* historically which helps the plant to utilize completely for the near future.

BOTANICAL DESCRIPTION AND PHENOLOGY

Gymnema sylvestre (syn. *Asclepias geminata* Roxb, *Periploca sylvestris* Retz) is a woody climber running over the tops of high trees. The vernacular

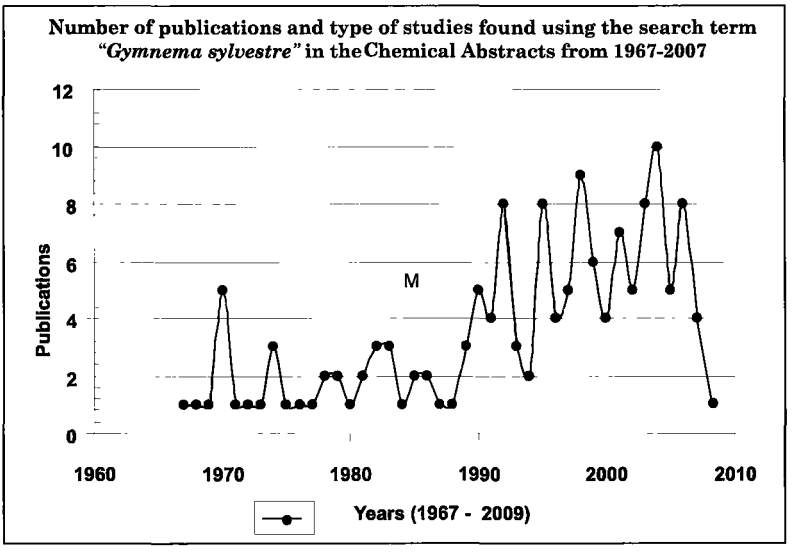


Fig 1. Number of publications in *Gymnema sylvestre* during the year 1967 – 2009

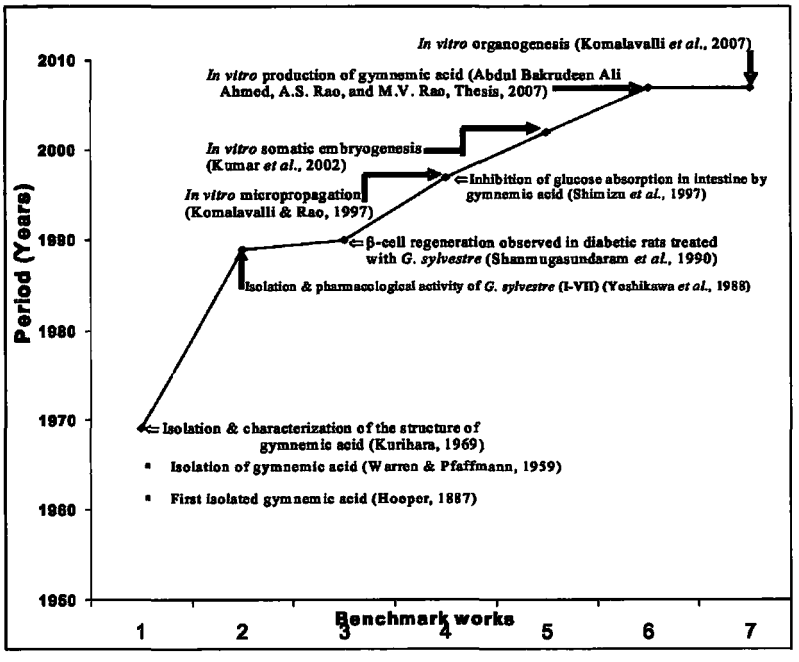


Fig 2. Benchmark studies of *Gymnema sylvestre* (1967 - 2009)

names of *G. sylvestre* are in Tamil-Sirukurinjan, English-Periploca of the woods, Hindi-Gurmar and Sanskrit-Ajabolli. The young stems and branches are pubescent and often densely so, terete. Leaves 3.2-5 by 1.3-3.2 cm, elliptic to obovate, base obtuse, apex abruptly acute, penninerved; petioles 6-13 mm long, pubescent, flowers dull yellow, small, in pedunculate or nearly sessile cymes; peduncles densely pubescent, shorter than the petioles and arising between them, sometimes producing successive umbels or whorls of flowers; pedicels 3-13 mm long, pubescent; bracts min, ovate-oblong, hairy ciliate; calyx lobes obovate, 1 mm, scabrous without, obtuse; corolla 4 mm across, pollinial bags 0.2 mm; fruits of 2 (or 1), dark green smooth follicular mericarps; seeds ovate, margined, ending in a silky coma, cotyledons elliptic, radicle cylindric (Gamble, 1956; Caius *et al.*, 1975; Mathew & Rani, 1983). Flowering: August-March; Fruiting: from October onwards; The *Gymnema* species are diploid with a chromosome number of 2n = 22 (Nadkarni *et al.*, 1967; Sredeevi & Namboodiri, 1977).

GEOGRAPHICAL DISTRIBUTION

G. sylvestre native to the tropical forests of central and southern India had wider distribution and it grows in the plains from the coast, in scrub jungles and in thickets at an altitude ranging from 300–700 m (Gamble, 1956; Mathew & Rani, 1983). The genus *Gymnema* comprises 40 species distributed from Western Africa to Australia (Caius *et al.*, 1975). *G. acuminatum* (Roxb.) Wall, *G. aurantiacum*, *G. balsamicum, G. elegans* W&A, *G. hirsutum* W&A, *G. lactiferum*, *G. latifolium*, *G. montanum* Hook.f., *G. sylvestre* R.Br., *G. tingens* W&A, *G. indorum, G. yunnanse* and *G. spartum* are some of the important species of genus *Gymnema* (Gamble, 1956; Caius *et al.*, 1975; Mathew & Rani, 1983). They are mainly distributed in the Deccan peninsula parts of northern, western India, Tropical Africa, Australia, Vietnam, Malaysia and Sri Lanka (Nakamura *et al.*, 1997; Ye *et al.*, 2000).

CHEMICAL CONSTITUENTS AND MEDICINAL PROPERTIES

Gymnema sylvestre leaf extract (ethanol and water) contain triterpene saponins, belonging to oleanane and damarane classes. Besides, flavones, anthraquinenes, hentri-acontane, alpha and beta cholorphylls, phytin, resins, inositol, d-quercitol, alkaloid, tartaric acid, formic acid, butyric acid, lupeol, β-amyrins, stigmasterol were isolated and characterised (Kapoor, 1990). The dry leaves were analysed for various chemical constituents and it consists of ash (11.45%), petroleum ether soluble residue (6.21%), ether soluble extract (1.72%), alcohol soluble residue (12.16%), albumin (0.45%), organic acids (5.50%), paraben (7.26%), calcium oxalate (7.30%), lignin (4.80%) and cellulose (22.65%). Analysis of the leaf ash consists of K_2O (14.73%), Na_2O (8.56%), CaO (20.72%), MgO (2.75%), Fe_2O_3 (5.44%), Al_2O_3 (0.92%), Mn (1.31%), CO_2 (11.66%), SO_3 (6.04%), P_2O_5 (6.73%), SiO (insol.) (11.90%), SiO_2 (sol.) (5.79%) and Cl (3.35%) (Caius *et al.*, 1975).

The various medicinal properties include antidiabetic, antisweetness, stimulant, stomachic, diuretic, laxative, acrid, biliousness, astringent, alexiteric, refrigerant, expectorant, emetic, cardiotonic and antihelmintic (Gharpurey, 1926; Sastri, 1956; Chopra, 1958; Yackzan, 1969; Liu *et al.*, 1992).

PHARMACOLOGICAL STUDIES

Gymnema species have numerous pharmacological activities reported by several authors and many experiments were carried out in *G. sylvestre*. In most of the laboratory, investigations on this plant have employed on *G. sylvestre* leaves, which are slightly bitter and are non toxic to humans in gram quantities. The pharmacopoeia standards of the leaf of this plant have been recently published by Indian Council of Medical Research (ICMR), New Delhi, India (Anonymous, 2003).

Antisweet activity

This plant is famous for its fascinating ability to antagonize the sweet taste of sugar thus known as Gurmar in 'Hindi' (Gupta & Seth, 1962; Gupta, 1963; Gupta & Variyar, 1964; Mitra *et al.*, 1975; Terasawa *et al.*, 1994). It was observed that *G. sylvestre* crude leaf extract reduced the sweetness of eight sweeteners namely acesulfame K, aspartame, sodium cyclamate, fructose, glucose, sucrose, stevioside and xylitol to 77% in human (Frank *et al.*, 1992). The major active compound gymnemic acid from *G. sylvestre* (Stocklin, 1969; Dateo & Long, 1973) binds bovine tongue and inhibits the sweet taste observed in fungiform papillae (Izutani *et al.*, 2002), human and chimpanzee (Oakley, 1985; Hellekant *et al.*, 1985; Hooper, 1887; Shore, 1892; Shimizu *et al.*, 1997; Rafiullah *et al.*, 2006). Due to anti-sweet activity of gymnemic acid, *G. sylvestre* appeared on the US market several years ago, and it was hyped as a "sugar blocker" (Murray *et al.*, 1999).

Antidiabetic activity

G. sylvestre leaf extract reduces blood sugar level in diabetic rabbits (Gharpurey, 1926; Chopra, 1958; Sanjappa & Sathayanda, 1979; Kirtikar & Basu, 1975; Shanmugasundaram *et al.*, 1983; Hirata *et al.*, 1992; Yoshikawa *et al.*, 1997; Agarwal *et al.*, 2000; Rafiullah *et al.*, 2006), rats (Srivastava *et al.*, 1985) and human (Shanmugasundaram *et al.*, 1990). It decreases glucose by increasing insulin releases (Shanmugasundaram *et al.*, 1981). It was found to contain an active principle which would cure the diabetes (Shanmugasundaram *et al.*, 1990; Gupta, 1961; Jain & Sharma, 1967; Dixit & Pandey, 1984). There has been a lot of research on its involvement in carbohydrate metabolism from the viewpoint of phamacognosy, nutrition and food science. Various effects have been reported, such as suppression of glucose absorption in the small intestine of rats, reduction of plasma glucose absorption in the small intestine of rats, reduction of plasma glucose

increment in the oral sucrose tolerance test, significantly lowered blood glucose and insulin values in dogs as well as suppression of insulin increase in glucose tolerance tests in men and the alleviation of diabetic symptoms in patients with non- insulin dependent diabetes mellitus (Shanmugasundaram *et al.*, 1990).

The beneficial effect was observed in oral treatment to cure Non-Insulin Dependent Diabetes Mellitus (NIDDM) to use the 400 mg/kg of leaf extract, where there is a significant reduction of blood glucose, glycosylated haemoglobin and plasma protein and increase in serum insulin levels (Shanmugasundaram *et al.*, 1990). Single dose oral administration of leaf powder (1.0 g/kg) to normal and Streptozotocin (STZ) induced rats led to significant reduction of blood glucose of OGTT (Oral Glucose Tolerance Test) and no significant effect in immuno reactive insulin (IRI) (Dixit & Pandey, 1984). Administration of leaf extract of *G. sylvestre* (120 mg/kg/day P.O) for 7 days in STZ induced rats reduced amylase activity in serum, increased β-cell function, regenerated β-cells in pancreatic islets and showed higher levels of serum C-peptide (Shanmugasundaram *et al.*, 1990).

The *G. sylvestre* alcoholic extract also stimulates insulin secretion from rat islets of Langerhans and several pancreatic β-cells lines (Persaud *et al.*, 1999). It was found to increase the activities of the enzyme affording the utilization of glucose by insulin dependent pathway and it controlled phosphorylase level, gluconeogenic enzymes, sorbital dehydrogenase (Shanmugasundaram *et al.*, 1983). It also significantly lowered the hepatoglycogen content of the glucose fed rats (Chattopadhyay, 1998; Kar *et al.*, 1999) and it controls the blood sugar in beryllium nitrate treated rats (Prakash *et al.*, 1986). Leaf extract of 13.4 g/kg of gymnemic acid was potentially effective in the amelioration of corticosteroid – induced diabetes mellitus (Gholap & Kar, 2003) and also potentially regulates dexamethasone induced hyperglycaemia in mice (Gholap & Kar, 2005). In Alloxan induced diabetic rabbits it decreases the activity of gluconeogenic enzymes and reversal of pathological changes in the liver initiated during the hyperglycaemic phase (Shanmugasundaram *et al.*, 1983).

Oral administration of different concentrations of *G. sylvestre* leaf extract (50, 100, 200 and 400 mg/kg) with normal and STZ induced rats showed significant dose dependent hypoglycaemic activity (Prakash *et al.*, 1986; Chattopadhyay, 1999) and it has hypoglycaemic activity in nature (Shanmugasundaram *et al.*, 1981; Khare *et al.*, 1983; Shanmugasundram et *al.*, 1990; Tripathi & Chaturvedi, 1995; Khan *et al.*, 2005). It lowers plasma glucose and insulin levels (Kurihara, 1969; Hirata *et al.*, 1992; Porchezhian & Dobriyal, 2003). It inhibits glyceraldehyde-3-phosphate dehydrogenase activity (Izutani *et al.*, 2005). Gymnemic acid IV derived from *G. sylvestre* inhibits the glucose absorption (glycosidase inhibition), increase glucose uptake in striated muscles, lowered blood glucose and increases the insulin secretion

in β-cells (Sugihara *et al.*, 2000), and gymnemic acid IV has multidirectional antihyperglycaemic activity (Kimura, 2006).

The various herbal formulation such as Dianex (Mutalik *et al.*, 2005), D-400 (Maji & Singh, 1995), hyponid (Babu & Prince, 2004) contain G. *sylvestre* extract lower blood glucose and cures diabetes mellitus. However, the absence of antidiabetic effect of *G. sylvestre* in non-diabetic and alloxan-diabetic rats was reported in the commercialized herbal preparations in the Brazil (Ricardo galletto *et al.*, 2004).

Obesity

G. sylvestre leaf extract was found to have reduction of cholesterol and triglyceride levels in diabetic rats (Terasawa *et al.*, 1994). The effect of *G. sylvestre* on body weight, glucose absorption and lipid metabolism was examined by using a breed of fatty rats with genetic obese-hyperglycaemia. Simultaneous feeding with *Gymnema* aqueous extract decreased the body weight in fatty and lean rats (4.2% and 6.1% respectively) compared to animals consuming only the test diet (high carbohydrate-low fat) over a 21 weeks period. Plasma glucose was lowered by 18% in the *Gymnema* treated animals compared to control. The plasma glucose increase following an oral glucose tolerance test was almost normalised, but not hypercholesterolemia. Two fractions of *Gymnema* extract (containing 160-360 mg/g of gymnemagenin) decreased body weight gain and food intake dose-dependently when given orally (0.05-1 g/kg) to rats for 22 days. Administration of *Gymnema* fraction (1.0 g/kg) containing 363 mg/g of gymnemagenin increased faecal excretion of cholesterol, total neutral steroids, total bile acids and cholic acid-derived bile acid (Nakamura *et al.*, 1997). Furthermore, the lipid lowering properties (Wang *et al.* 1998; Shigematsu *et al.*, 2001) hypolipidaemic and antiatherosclerotic effect in rats (Bishayee & Chatterjee, 1994) have also been reported. It is recently reported that *G. sylvestre* leaf contains the gymnemate promoted weight loss by its ability to reduce hyperlipidemia (Luo *et al.*, 2006). In contradictory, dried powdered leaf prepared commercially in Brazil lacks hypolipidemic effect (Ricardo galletto *et al.*, 2004). This may due to the adulteration present in the preparations.

In human, leaf extract (2 g thrice a day) given oral led to significant reduction of OGTT (Oral Glucose Tolerance Test) (Khare *et al.*, 1983). In addition, it reduces the serum lipid level to near normal levels (Shanmugasundaram *et al.*, 1990). The mechanism behind this is that gymnemic acid inhibits the glucose uptake (Imoto *et al.*, 1991; Shimizu *et al.*, 1996; Shimizu *et al.*, 1997) and maltose absorption (Imoto *et al.*, 1991) in intestine and also inhibits glucose-stimulated gastric inhibitory peptide secretion (Shimizu *et al.*, 1996), thereby suppressing the body weight (Agarwal *et al.*, 2000). OB-200G polyherbal formulation extracts works

against obesity of mice (Kaur & Kulkarni, 2001) and body weight managements (Preuss *et al.*, 2004).

Miscellaneous uses

G. sylvestre has strong reducing effect on urinary disorder (Sanjappa & Sathayanda, 1979; Kirtikar & Basu, 1975). The *G. sylvestre* cures the major diseases such as diuretic and cough (Kirtikar & Basu, 1935) and mycosis of the toes (Reddy *et al.*, 1989). The active constituent gymnemic acid is also useful for the prevention of the formation of dental plaque and caries (Kirtikar & Basu, 1935) and also possesses antiviral effect (Sinsheimer *et al.*, 1968). The roots of *G. sylvestre* are macerated and drunk as a remedy for snake bite (Shanmugasundaram *et al.*, 1983; Nagaraju & Rao, 1990) and the root extract is also found to have antioxidant activity against free radicals and LDL levels (Ohmori *et al.*, 2005; Aldona Dembinska-Kiec *et al.*, 2008), antimicrobial activity (Satdive *et al.*, 2003), anti-inflammatory activity (Diwan *et al.*, 1995) and larvicidal effect against *Culex qinquifaciatus* mosquito larvae (Gopiesh Khanna & Kannabiran, 2007). Gastric sensation was observed in humans fed with *G. sylvestre* leaf tea extract (Schroeder and Flannery-Schroeder, 2005). Diabecon herbal formulation protects the lens against sugar induced cataract (Moghaddam *et al.*, 2005). The pharmacology studies of *G. sylvestre* are presented in Table 1.

Table 1. Pharmacological studies in *Gymnema sylvestre*

Plant part	Pharmacological acitivity	Reference(s)
Leaf	Anti-sweet tested in human	Shore, 1892
Leaf	Diabetes mellitus, stomachic and diurea	Sastri, 1956
Root	Anti-sweet activity	Stocklin *et al.*, 1967
Leaf	Decreases gluconeogenic enzyme and pathological changes	Shanmugasundaram *et al.*, 1983
Whole plant	Reduced glucose fasting and OGTT in human	Khare *et al.*, 1983
Leaf	Snake bite	Nagaraju & Rao, 1990
Leaf	Lowered the insulin requirement in IDDM; stimulating insulin production from pancreas and Pancreatic islets production from pancreas	Shanmugasundaram *et al.*, 1990
Leaf	Inhibitions glucose absorption in rat intestine conduritol A gymnemic acid	Hirata *et al.*, 1992
Leaf extract	Inhibits sweetness of eight sweeteners	Frank *et al.*, 1992
Leaf extract	Rats – body weight, plasma glucose, serum glucose, serum triglyceride, total cholesterol and insulin.	Terasawa *et al.*, 1994
Leaf extract	Hypolipidaemic and antiatherosclerotic effects	Bishayee & Chatterjee, 1994
Leaf	Anti-inflammatory	Diwan *et al.*, 1995

Table 1. *Contd.*

Plant part	Pharmacological acitivity	Reference(s)
Leaf extract	Stimulate insulin production in rat pancreatic islet cells and the insulin secreting hamster β-cell line (HIT cells *in vitro*)	Diwan *et al.*, 1995
Whole plant	Inflammation studies	Persaud *et al.*, 1996
Whole plant	Triterpenoid glycosides – inhibits glucose utilization in muscles	Shimizu *et al.*, 1996
Leaf extract	Alcohol extract – lower hepatic	Nakamura *et al.*, 1997
Leaf extract	Gymnemic acid – health food	Shimizu *et al.*, 1997
Leaf	Inhibit glucose absorption in rats	Chattopadhay, 1998
Leaf extract	Anti-microbial activity	Satdive *et al.*, 2003
Leaf extract	Diabetes and obesity – clinical practice	Porchezian & Dobriyal, 2003
Leaf extract	Diabetes and in food additives against obesity	Press *et al.*, 2004
Leaf extract	Anti hyperglycaemic and anti oxidant studies	Babu & Prince, 2004
Leaf extract	Diabecon – contract studies	Moghaddam *et al.*, 2005
Aqueous extract	Antioxidant activity – LDL and free radicals	Ohmori *et al.*, 2005
Leaf extract	Dianex herbal formulation – use in diabetes	Mutalik *et al.*, 2005
Leaf extract	Gustatory sensation studies	Schroeder & Flannery-Schroeder, 2005
Leaf extract	Regulated – induced hyperglycemia	Gholap & Kar, 2005

PHYTOCHEMICAL STUDIES

Phytochemical progress has been aided enormously by the development of rapid and accurate methods of screening plants for particular chemicals and the emphasis in this area is inevitably on chromatographic technique. The amount and type of compounds separated into the different fractions will, of course, vary from plant to plant. Also, such a procedure may have to be modified when labile substances are under investigation. Isolation and characterization of pharmacologically active compounds from medicinal plants continue today. More recently, drug discovery techniques have been applied to the standardization of herbal medicines, to elucidate analytical marker compounds (Balunas & Kinghorn, 2005). Since drug discovery from medicinal plants is time-consuming, faster and better methodologies for plant collection, bioassay screening, compound isolation and subsequently compound must be employed (Do & Bernard, 2004; Koehn & Carter, 2005).

In *Gymnema* species a number of phytochemical constituents have been reported by several authors. While considerable practical difficulty was experienced initially in purifying these labile compounds, about 20

distinct oleanane-type glycosides have now been individually characterized as sweetness inhibitory constituents. *G. sylvestre* cures many diseases and its constituents include two resins, gymnemic acids, saponins, stigmasterol, quercitol and the amino acid derivative of betaine, choline and trimethylamine, but its main active compound is gymnemic acid, saponins and oleanane type of triterpenoid (Kapoor, 1990). Although attempts regarding isolation and structure elucidation of the gymnemic acids have continued for many years, this area of endeavour caused a great deal of difficulty for phytochemical researchers, and somewhat controversial. The first attempt to isolate the active compound(s) from the leaves of *G. sylvestre* concluded that gymnemic acid is a glycoside (Hooper, 1887, 1889). Subsequently several efforts to purify gymnemic acid were unsuccessful and it able to obtain a relatively pure sample of gymnemic acid (Warren & Pfaffmann, 1969).

G. sylvestre contains oleanane type triterpene (Gymnemagenin) and gymnemic acid which itself is not pure entity, but composed of 4 components, A_1 - A_4, with gymnemic acid A_1 as the predominant one (Stocklin *et al.*, 1967). Gymnemagenin and gymnestogenin were isolated and crystallized (Stocklin, 1968; Kurihara, 1969). Earlier, reported the isolation of a new compound gymnemic acid A_1, which could be converted to gymnemic acid A_2 and it has anti-sweet activity against sucrose (Kurihara, 1969), but gymnemic acid A-D reported by some in different forms (Sinsheimer & Rao, 1970). Others studied the gymnemic acid production (Liu *et al.*, 1992; Stocklin *et al.*, 1967).

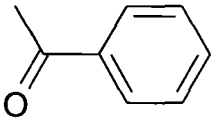
Yoshikawa and co-workers (1989a, b) isolated gymnemic acids from the hot water extract of *G. sylvestre* dry leaves, which they named gymnemic acids I, II, III, IV, V, VI and VII respectively and the anti-sweet activity was evaluated (Sugihara *et al.*, 2000; Stocklin, 1968; Sinsheimer *et al.*, 1970). In contrast, the next series of anti-sweet compounds, gymnemasaponins I, II, III, IV, and V were isolated from *G. sylvestre* (Yoshikawa *et al.*, 1991). Further work carried out in *G. sylvestre* led to isolate and characterization of other gymnemic acids VIII, IX, X, XI, XII, XIII, XIV, XV, XVI, XVII and XVIII (Liu *et al.*, 1992; Yoshikawa *et al.*, 1992; Yoshikawa *et al.*, 1993; Yoshikawa *et al.*, 1997).

The confirmed structure of gymnemagenin by X-ray crystallographic method and identified its antisweet activity (Liu *et al.*, 1992). Gymnemic acid and deacylgymnemic acid were isolated and identified by the use of high performance liquid chromatography (HPLC) and atmospheric pressure ionization mass spectrometry (Imoto *et al.*, 1991; Suzuki *et al.*, 1993).

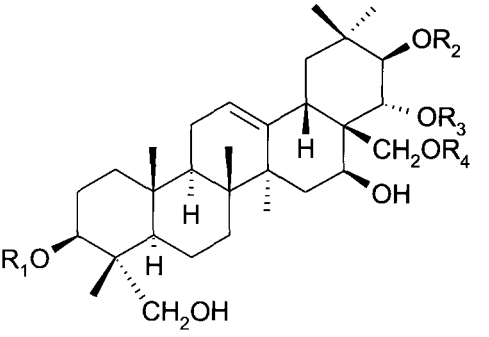
In *G. sylvestre* some of the antisweet saponin compounds were reported (Yoshikawa *et al.*, 1992; Ye *et al.*, 2001). Gymnemaside I, II, III, IV, V, VI and VII (Yoshikawa *et al.*, 1992) gymnemoside a, b, c, d and e (Yoshikawa *et al.*, 1997), gymnesins A, B, C and D (Sahu *et al.*, 1996) and some of triterpenes (Ye *et al.*, 2001) were also isolated from the leaves of *G. sylvestre*. Recently, two new flavonol glycosides were isolated from the leaves of *G. sylvestre* (Liu *et al.*, 2004) and 2-(trimethylsiyl) ethyl glycoside and flavonoid triglycoside from *G. sylvestre* (Mukhopadhyay & Field, 2006). High performance thin layer chromatography (HPTLC) method led to the determination of gymnestrogenin and its reference sample from *G. sylvestre* (Puratchimani & Jha, 2004). Gymnemic acid and gymnemogenins are isolated from *G. sylvestre* through HPTLC (Raju *et al.*, 2006). The various aspects of phytochemical investigations and structural details of isolated phytochemical compounds from *G. sylvestre* were presented in Table 2 and Figs 3-6.

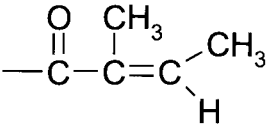
Table 2. Secondary metabolites isolated from *Gymnema sylvestre*

Plant part	Compounds	Reference(s)
Leaf	Gymnemic acid A_1	Hooper, 1887; Yackzan, 1966; Stocklin *et al.*, 1967; Sinsheimer *et al.*, 1968; Warren & Pfaffmann, 1969; Dateo & Long, 1973
Leaf	Gymnemic acid A_1, A_2, A_3 and A_4	Kurihara, 1969
Leaf	Gymnemogagenin and Gymnestrogenin	Stocklin, 1969; Rao & Insheimer, 1971
Leaf	Gymnemasaponin III, IV and V	Yoshikawa *et al.*, 1991
Leaf	Gymnemic acid I, II	Imoto *et al.*, 1991
Leaf	Gymnemic acid VII and VIII	Liu *et al.*, 1992
Leaf	Gymnemic acid I - IX, X, XI, XII, XIII and XIV	Yoshikawa *et al.*, 1992
Leaf	Gymnemic acid XV, XVI, XVII and XVIII	Yoshikawa *et al.*, 1993
Leaf	Gymnemoside A and B	Murakami, 1996
Leaf	Triterpenoids - saponins	Sahu *et al.*, 1996
Leaf	Gymnemoside A, B, C, D, E and F	Yoshikawa *et al.*,1997a
Leaf	Six Oleanane – Saponins and Two triterpene saponins	Ye *et al.*, 2001
Leaf	Triterpene glycosides (Oleanolic acid, Longispinogenin, Chichipegenin, Sitakisogenin, 30-hydroxylupeol)	Ye *et al.*, 2001
Leaf	Two new flavonol glycosides	Liu *et al.*, 2004
Leaf	Gymnemogagenin	Puratchimani & Jha, 2004

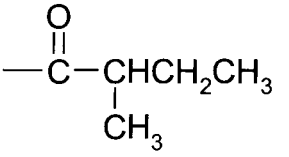


Benzyl group

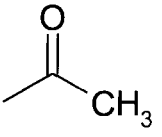




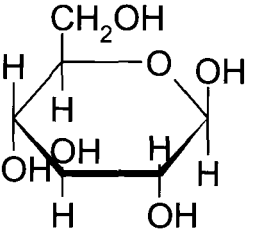
Tigloyl (Tig)



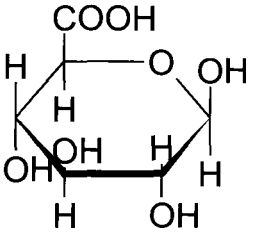
2-methyl butyloyl (Mb)



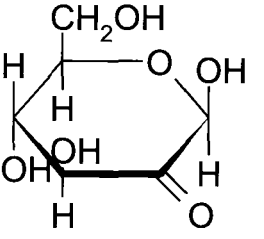
Acetyl Group



β – Glc



β – Glc A



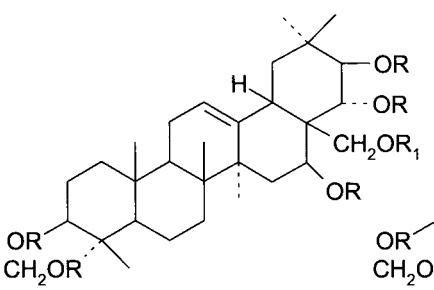
β – OG

Compounds	R1	R2	R3	R4	AP*	Reference(s)
Gymnemic acid I	β-gluA	tga	H	Ac	1	Yoshikawa *et al.*, 1989a
Gymnemic acid II	β-gluA	mba	H	Ac	1	Yoshikawa *et al.*, 1989a
Gymnemic acid III	β-gluA	mba	H	H	0.5	Yoshikawa *et al.*, 1989a
Gymnemic acid IV	β-gluA	tga	H	H	0.25	Yoshikawa *et al.*, 1989a
Gymnemic acid V	β-gluA	tga	tga	H	0.5	Yoshikawa *et al.*, 1989b
Gymnemic acid VI	β-gluA^3- β-glc	tga	H	H	0.5	Yoshikawa *et al.*, 1989b
Gymnemic acid VII	H	H	H	H	ND	Yoshikawa *et al.*, 1989b
Gymnemic acid VIII	β-gluA^3-β-OG	mba	H	H	ND	Liu *et al.*, 1992
Gymnemic acid IX	β-gluA^3-β-OG	tga	H	H	ND	Liu *et al.*, 1992
Gymnemic acid X	β-gluA	H	H	Ac	0.5	Yoshikawa *et al.*, 1992b
Gymnemic acid XI	β-gluA	tga	H	tga	1	Yoshikawa *et al.*, 1992b
Gymnemic acid XII	β-gluA^3- β-glc	tga	H	Ac	1	Yoshikawa *et al.*, 1992b
Gymnemic acid XIII	β-gluA	H	H	mba	0.5	Yoshikawa *et al.*, 1992b
Gymnemic acid XIV	β-gluA	H	H	tga	0.5	Yoshikawa *et al.*, 1993a
Gymnemic acid XV	H	mba	tga	H	1	Yoshikawa *et al.*, 1993a
Gymnemic acid XVI	tga	H	H	H	1	Yoshikawa *et al.*, 1993a
Gymnemic acid XVII	H	Bz	H	H	1	Yoshikawa *et al.*, 1993a
Gymnemic acid XVIII	H	H	H	Bz	1	Yoshikawa *et al.*, 1993a

* AP – Antisweet Potency x Gymnemic acid I; ND – Not detected

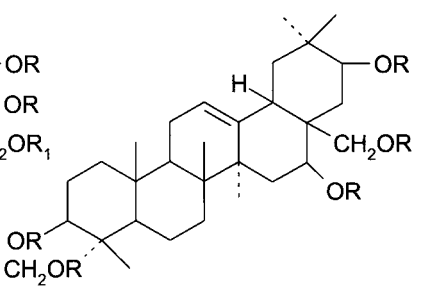
Fig 3. Structures of Gymnemic acid I – XVIII

Gymnemagenin I, III, IV, V



I : R = R_1 = H; III : R = R_1 = Ac;
IV : R = R_1 = Bz; V : R = H; R_1 = C(Ph)$_3$

Gymnemagenin II, IV



I : R = H; VI : R = Ac

Fig 4. *Contd.*

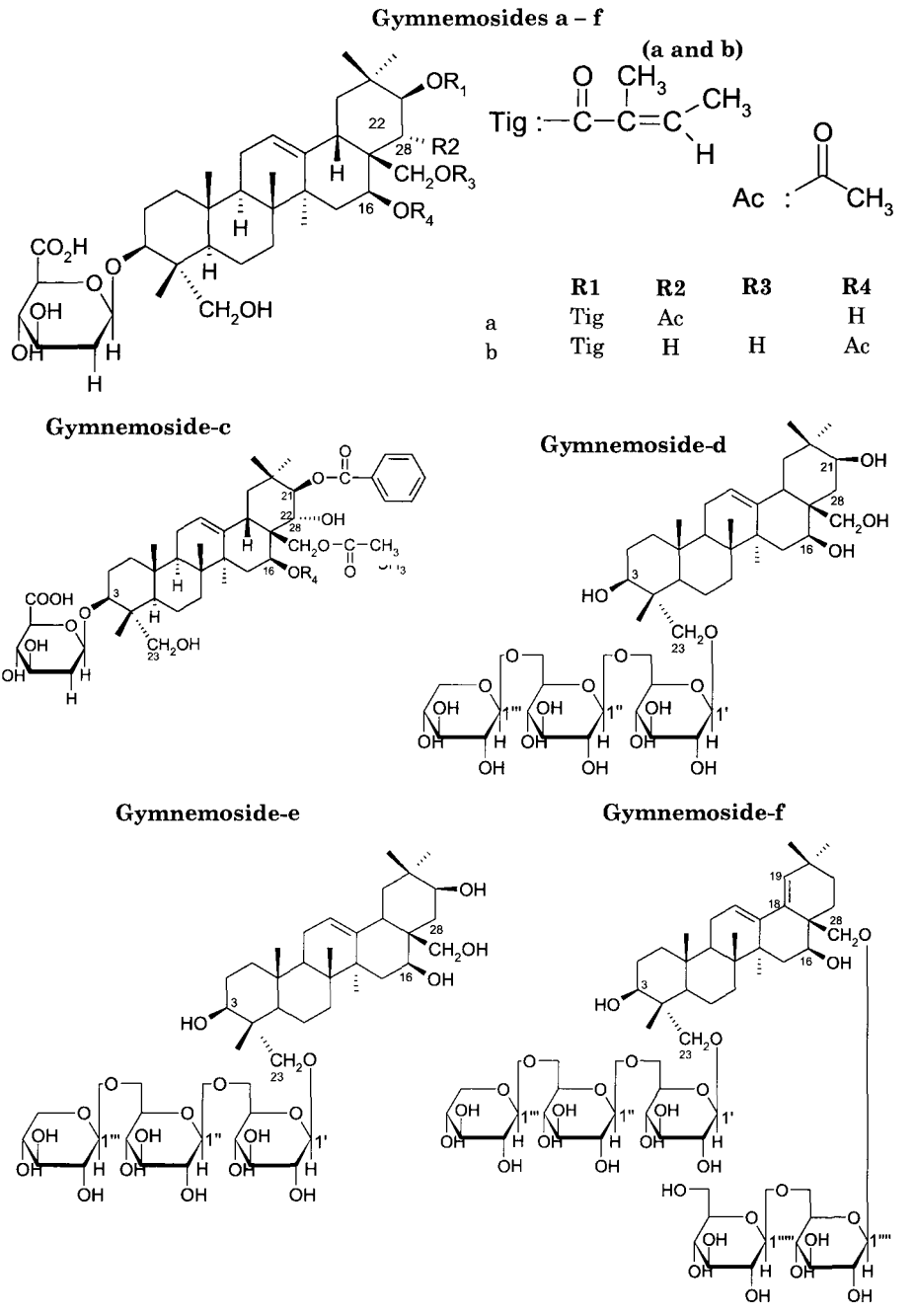


Fig 4. Structures of Gymnemagenin I, III – V, Gymnestrogenin II, IV, Gymnemosides a-f

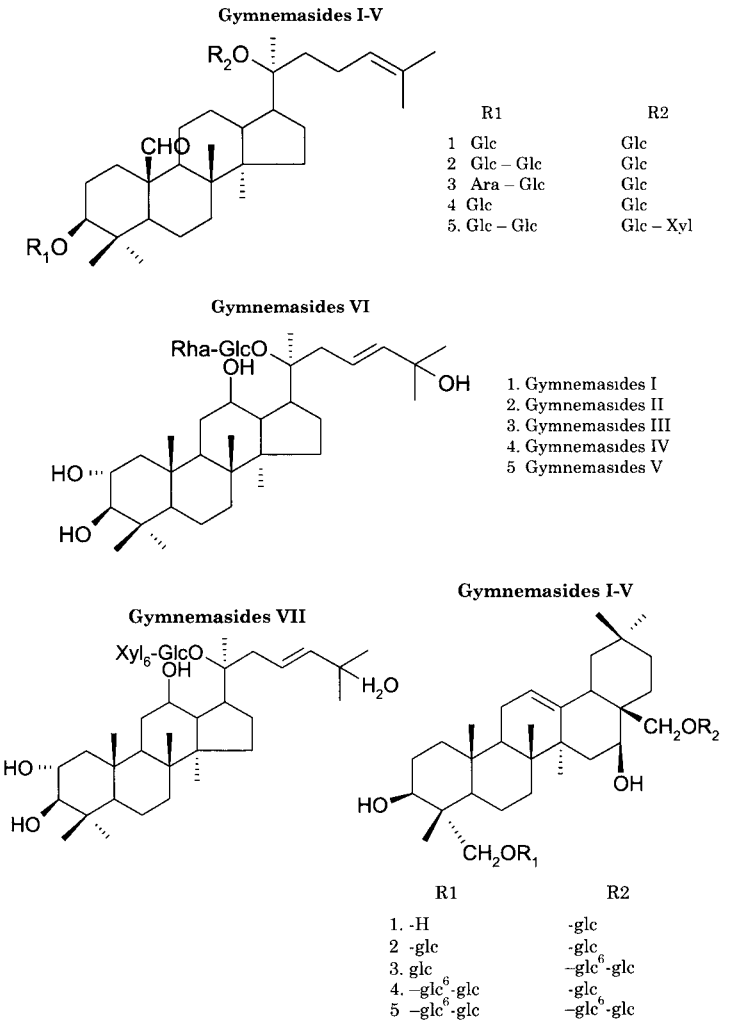


Fig 5. Structures of Gymnemasides I – VII and Gymnemasaponins I – V

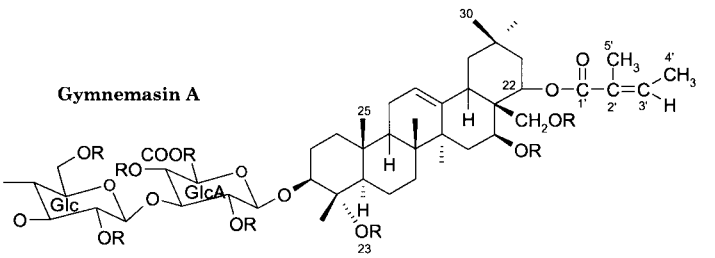
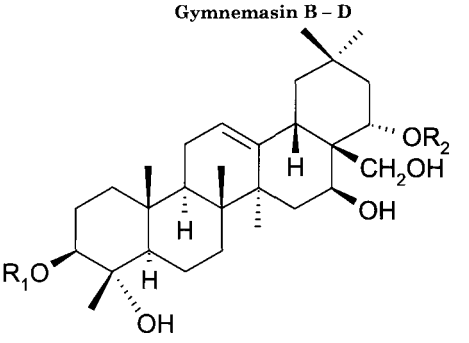
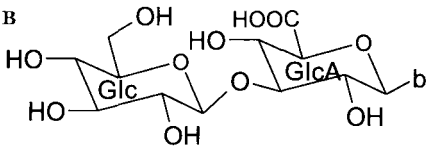
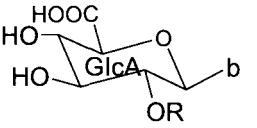
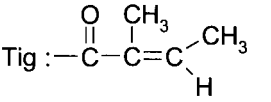
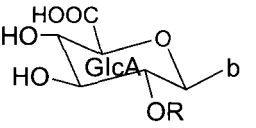


Fig 6. *Contd.*

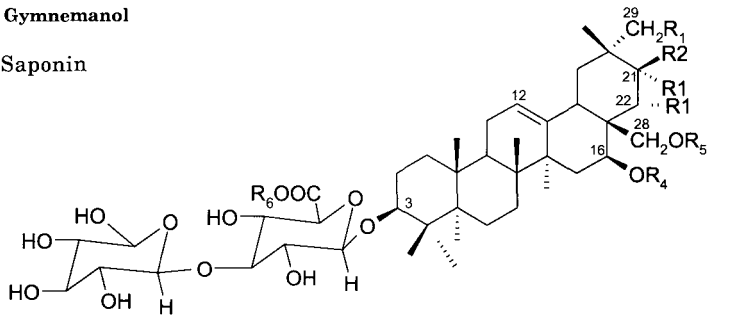
Gymnemasin B – D



	R1	R2
Gymnemasin B		H
Gymnemasin C		
Gymnemasin D		H
	H	H

Gymnemanol

Saponin



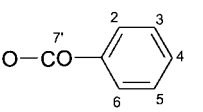
1. R1 = R3 = R4 = R5 = R6 = H; R2 = 
2. R1 = R2 = R3 = R4 = R5 = H; R6 = K^+
3. R1 = OH; R2 = R3 = R4 = R5 = H; R6 = K^+
4. R1 = R2 = H; R3 = OH; R4 = Ac, R5 = rhamnopy ranosyl, R6 = Na^+

Fig 6. *Contd.*

1. 21β-O-benzylsitakisogenin 5-O- β-D-glucopyranosyl (1-3)- β-D-glucuronopyranoside
2. Potassium salt of 29-hydroxylongispinogenin-3-O- β-D-glycopyranosyl (1-3) β-D-glucuronopyranoside
3. 3β, 16β, 28, 29-tetrahydroxyolean-12-ene
4. Sodium salt of alternoside II.

Fig 6. Gymnemasin A-D, Gymnemanol and Saponin

PLANT TISSUE CULTURE

Plant biotechnology offers an opportunity to exploit the cell, tissue, organ or entire plant by growing them *in vitro* and to genetically manipulate them to get desired compounds. Many facts of biotechnological approaches can be envisaged like plant callus culture, cell culture, shoot culture, root culture and scale up of cultures. Since the world population is increasing rapidly, there is extreme pressure on the available cultivable land to produce chemicals from plants and the available land should be used effectively. Hence, it is appropriate to develop modern technologies leading to plant improvement for better utilization of the land to meet the requirement. The search for new plant derived chemicals should thus be a priority in current and future efforts towards sustainable conservation and rational utilization of biodiversity (Phillipson, 1990). In the search for alternatives to the production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue culture, is found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Rao & Ravishankar, 2002).

Gymnema species natural stands are fast disappearing and are threatened with extinction due to its indiscriminate collection, over exploitation and natural resources for commercial purposes and to meet the requirements of pharmaceutical industry (Choudhury, 1998). Commercial exploitation for production and conventional propagation are hampered due to its poor seed viability, low rate of germination and poor rooting ability of vegetative cuttings. Alternative *in vitro* propagation methods would be beneficial in accelerating large scale multiplication and conservation of the plant.

There are only a few reports available on *in vitro* propagation of *Gymnema* species using different explants (Anu *et al.*, 1994; Reddy *et al.*, 1998; Komalavalli & Rao, 1997; Komalavalli & Rao, 2000; Kumar *et al.*, 2002). Micropropagation was observed in Murashige and Skoog (MS) medium supplemented with 6-benzyl aminopurine (BA 5.0 mg/l) combination of 1-naphthaleneacetic acid (NAA 0.2 mg/l) strength induced 7 shoots/explant and best root induction in ½ MS without growth regulators (Reddy *et al.*, 1998). However, the highest number of multiple shoots (57.2 shoots/explant)

observed in MS medium supplemented with 6-benzyl aminopurine (BA 1.0 mg/l), 6-furfurylaminopurine (KN 0.5 mg/l), 1-naphthaleneacetic acid (NAA 0.1 mg/l), malt extract (100 mg/l) and citric acid (100 mg/l), and the best root induction was in MS medium supplemented with indole -3-butyric acid (IBA 3.0 mg/l) (Komalavalli & Rao, 2000). Micropropagation studies carried out in *Gymnema* species, indicated that MS medium supplemented with 6-furfurylaminopurine (KN 0.4 ppm) and indole -3-acetic acid (IAA 4.0 ppm) induced 33.3 shoots/explant (Kumar *et al.*, 2002). It was reported that KN induced the highest shoot number in *Gymnema sylvestre* (Anu *et al.*, 1994). In *G. elegans* the highest number of shoots were induced from seedlings and mature auxiliary node explants on MS medium supplemented with BA (13.0 µm), KN (2.0 µm), NAA (3.0 µm), ascorbic acid (100 mg/l), coconut milk (10%), GA_3 (1.0 µm) and rooting in MS medium with IBA (5.0, 15.0 µm) (Komalavalli & Rao, 1997). Somatic embryogenesis and plant regeneration were achieved from hypocotyl, cotyledon and leaf explants excised from seedling of *G. sylvestre* in MS medium supplemented with 2,4-dicholorophenoxyacetic acid (2,4-D 0.5-5.0 µm), BA (0.5-2.0 µm) and 2% sucrose. The embryo germination and plantlet formation were achieved in fresh EM 8 medium (Kumar *et al.*, 2002). *In vitro* organogenesis of *G. sylvestre* from matured decoated explants (Komalavalli *et al.*, 2007). *In vitro* callus showed that the active compounds, gymnemic acid and gymnemagenin, were present in sufficiently large amount in the cultured undifferentiated cells (Gopi & Vatsala, 2006; Kanetkar *et al.*, 2006); external phytohormone, shaking speeds, pH of the medium, played important roles in growth and gymnemic acid production in suspension culture (Devi *et al.*, 2006); sucrose, inoculum density, auxins and aeration played important roles in cell growth in bioreactor cultures (Lee *et al.*, 2006). *In vitro* production of gymnemic acid through callus culture under abiotic stress conditions was also reported (Abdul Bakrudeen Ali Ahmed *et al.*, 2009). The various aspects of plant tissue culture of *G. sylvestre* is presented in Table 3.

Table 3. *In vitro* studies on *Gymnema sylvestre*

Explants	Culture medium	Growth response	Reference(s)
Mature nodal	MS + BA (5.0 mg/l) + NAA (0.2 mg/l)	Micropropagation	Reddy *et al.*, 1998
Axillary node	MS + BA (1.0 mg/l) + KN (0.5 mg/l) + NAA (0.1 mg/l)	Micropropagation	Komalavalli & Rao, 2000
Hypocotyl, cotyledon and leaf	EM 8 medium + 2,4-D (2.0 µm) + BA (1.0 µm)	Somatic embryogenesis	Kumar *et al.*, 2002
Leaf and nodal	MS + 2,4-D (0.5 mg/l) + NAA (2.5 mg/l) + BA (0.5 mg/l)	Callus and cell suspension culture	Gopi *et al.*, 2006
Leaf	MS + BA (1.0 mg/l) + IAA	Cell suspension culture	Devi *et al.*, 2006

Table 3. *Contd.*

Explants	Culture medium	Growth response	Reference(s)
Leaf	(0.5 mg/l) MS + BA (2.22 mg/l) + 2,4-D (0.44 mg/l)	Callus culture	Kanetkar *et al.*, 2006
Node	MS + BA (1.0 mg/l) + 2,4-D (1.0 mg/l)	Callus culture	Lee *et al.*, 2006
Seed	MS + BA (0.22 mg/l) + 2,4-D (0.44 mg/l)	Callus culture	
Leaf	MS + 2,4-D (1.0 mg/l) + KN (0.1 mg/l) + Sucrose, inoculum density, auxins	Batch culture – callus cell growth	
Mature decoated seeds	MS + NAA (8 µm) + citric acid (10 mg/l) + casein hydrolysate (100 mg/l) + BA (22.2 µm) + Ad (35.7 µm) + GA_3 (2.9 µm)	Organogenesis	Komalavalli *et al.*, 2007
Leaf	MS+2,4-D (1.5 mg/l)+KN (0.5 mg/l) + 3 mMol NH_4NO_3	Gymnemic acid production under abiotic stress	Abdul Bakrudeen Ali Ahmed *et al.*, 2009

CONCLUSIONS

G. sylvestre is a multipurpose potential medicinal plant having high market potential all over the world. Hence it is utmost important to monitor the progress of pharmacological, phytochemical and plant tissue culture literature to asses the efficacy before being recommended for various therapies, commercial propagation and *in vitro* production of gymnemic acid. Thus this paper gives an overview of *G. sylvestre* from antiquity to till date. It has addressed recent advances in key areas of *G. sylvestre,* namely plant tissue culture techniques and plant secondary metabolites production. Even with all the challenges facing *G. sylvestre* from medicinal plants, large scale of new secondary metabolites isolated from *G. sylvestre* can be predicted to remain an essential component in the search for new secondary metabolites and its pharmacological activities.

There is sufficient evidence of pharmacological and phytochemical studies to draw a definite conclusion about the efficacy of the gymnemic acid for the treatment of diabetes and obesity but, there is still inadequate literature related to plant tissue culture and other activities. Moreover, refinements in protocols and field performance data are necessary to get good quality regenerants and for large scale propagation. The reported studies on the *in vitro* production of gymnemic acid are not satisfactory to obtain sufficient quantities of gymnemic acid in high yields. Therefore further *in vitro* studies such as role of bioelicitors, precursors and biotransformation are needed to enhance the gymnemic acid production at laboratory conditions and also for commercial production at large scale. With the interest that has been generated both the general public and multinationals across the globe into *Gymnema sylvestre*, there is now more

than ever a golden opportunity to continue making worthwhile contribution to healthcare.

REFERENCES

Abdul Bakrudeen Ali Ahmed, Adhikarla Suryanarayana, Rao and Mandali Venkateswara, Rao (2009). *In vitro* production of gymnemic acid from *Gymnema sylvestre* (Retz.) R. Br. Ex Roemer and Schultes through callus culture under abiotic stress conditions. *In: Methods in molecular biology, protocols for in vitro cultures and secondary metabolite analysis of aromatic and medicinal plants, (Eds.* S. Mohan Jain and Praveen K. Saxena) Human press Inc., Totowa, NW, **547**: 370-390.

Agarwal, S.K., Singh, S.S., Verma, S., Lakshmi, V., Sharma, A. and Kumar, S. (2000). Chemistry and medicinal uses of *Gymnema sylvestre* (GUR-MAR) leaves – A review. *Indian Drugs*, **37**: 354-360.

Aldona, Dembinska-kiee, Otto Mykkanen, Beata, Kiee-Wilk and Hannu, Mykkanen (2008). Antioxidant phytochemicals against type 2 diabetes. *British Journal of Nutrition*, **99**: 109–117.

Anonymous, (2003). Quality standards of Indian medicinal plants, Indian Council of Medical Research, New Delhi.

Anu, K.I., Nazeem, P.A., Joseph, L. and Vijaykumar, N.K. (1994). Response of Gurmar (*Gymnema sylvestre* R.Br) for *in vitro* propagation. *South Indian Horticulture,* **42**: 365-368.

Babu, P.S. and Prince, P.S.M. (2004). Antihyperglycaemic and antioxidant effect of hyponidd, an ayurvedic herbomineral formulation in stereptozotocin-induce diabetic rats. *Journal of Pharmaceutical and Pharmacology,* **56**: 1435-1442.

Balunas, M.J. and Kinghorn, A.D. (2005). Drug discovery from medicinal plants. *Life Sciences,* **78**: 431-441.

Bishayee, A. and Chatterjee, M. (1994). Protective effects of *Gymnema sylvestre* in streptozotocin induced diabetic rats. *Phytotherapy Research,* **8**: 118-120.

Caius, J.F., Mhaskar, K.S. and Blatter, E. (1975). (*Ed.*), Bishen Singh and Mahaendra Pal Singh Publ. Dehardun, **3**: 1624.

Chattopadhyay, R.R. (1998). Possible mechanism of antihyperglycemic effect of *Gymnema sylvestre* leaf extract, Part I. *General Pharmacology,* **31**: 495-496.

Chattopadhyay, R.R. (1999). A comparative evaluation of some blood sugar lowering agents of plant origin. *Journal of Ethnopharmacology,* **67**: 367- 372.

Chopra, R.M. (1958). "Indigenous Drugs of India", 2nd (*Eds.*), Art Press, Calcutta, India, pp. 336.

Choudhury, B.P. (1998). Assessment and conservation of medicinal plant of Bhubaneswar and its neighbourhood. *In:* Indigenous Medicinal Plants. Today and Tomorrow's Printers & Publishers, New Delhi, India, pp. 211-219.

Dateo, J.G.P. and Long, J.L. (1973). Gymnemic acid, the antisaccharine principle of *Gymnema sylvestre* studies on the isolation and heterogeneity of gymnemic acid A1. *Journal of Agricultural Food Chemistry,* **21**: 899-903.

Devi, C.S., Murugesh, S. and Srinivasan, V.M. (2006). Gymnemic acid production in suspension cell cultures of *Gymnema sylvestre. Journal of Applied Science,* **6**: 2263-2268.

Diwan, P.V., Margaret, I. and Ramkrishna, S. (1995). Influence of *Gymnema sylvestre* on inflammation. *Inflammo Pharmacology,* **3**: 271-277.

Dixit, R.S. and Pandey, H.C. (1984). Plant used as folk-medicine in Jhansi and Lalitpur sections of Bundelkhand, Uttar Pradesh. *International Journal of Crude Drug Research,* **22**: 47-51.

Do, Q.T. and Bernard, P. (2004). Pharmacognosy and reverse pharmacognosy: a new concept for accelerating natural drug discovery. *Drugs*, **7**: 1107-1027.

Frank, R.A., Mize, S.J.S., Kennedy, L.M., Santos, H.C. and Green, S.J. (1992). The effect of *Gymnema sylvestre* extracts on the sweetness on eight sweeteners. *Chemical Senses,* **17**: 461-479.

Gamble, J.S. (1956). Asclepiadaceae. *In*: Flora of the Presidency Madras, Printed under the authority of the Government of India, Calcutta, Vol. II, pp. 589-590.

Gharpurey, K.G. (1926). *Gymnema sylvestre* in the treatment of diabetes. *Indian Med. Gaz.*, **61**: 155.

Gholap, S. and Kar, A. (2003). Efficacy of some plant extracts in regulating corticosteroid-induced hyperglycaemia in mice. *Pharmaceutical Biology,* **41**: 315.

Gholap, S. and Kar, A. (2005). Gymnemic acids from *Gymnema sylvestre* potentially regulates dexamethasone induced hyperglycemia in mice. *Pharmaceutical Biology,* **43**: 192.

Gopi, C. and Vatsala, T.M. (2006). *In vitro* studies on effects of plant growth regulators on callus and suspension culture biomass yield from *Gymnema sylvestre* R.Br. *African Journal of Biotechnology,* **5**: 1215-1219.

Gopiesh Khanna, V. and Kannabiran, K. (2007). Larvicidal effect of *Hemidesmus indicus, Gymnema sylvestre and Eclipta prostrate* against *Culex qinquifaciatus* mosquito larvae. *African Journal of Biotechnology,* **6**: 307-311.

Gupta, S.S. (1961). Inhibitory effect of *Gymnema sylvestre* (Gurmar) on adrenaline induced hyperglycemia in rats. *Indian Journal Med. Sci.,* **15**: 883-886.

Gupta, S.S. (1963). Effect of *Gymnema sylvestre* and *Pterocarpus marsupium* on glucose tolerance in albino rats. *Indian Journal Med Res.,* **51**: 716-721.

Gupta, S.S. and Seth, C.B. (1962). Experimental studies on pituitary diabetes, part II. Comparison of blood sugar level in normal and anterior pituitary extract induced hyperglycaemic rats treated with a few Ayurvedic remedies. *Indian Journal Med. Res.,* **50**: 708-714.

Gupta, S.S. and Variyar, M.C. (1964). Experimental studies on pituitary diabetes. Part IV. Effect of *Gymnema sylvestre* and *Coccinia indica* against the hyperglycaemic response of somatotropin and corticotrophin hormones. *Indian Journal Med. Res.,* **52**: 200-207.

Handa, S.S., Chawla, A.S. and Maninder (1989). Hypoglycaemic plants – A review. *Fitoterapia* **60**: 195-224.

Hellekant, G., Segerstad, C.H., Roberts, T.W., Vander, W.H., Brouwer, J.N., Glaser, D., Haynes, R. and Eichberg, J.W. (1985). Effects of gymnemic acid on the chorda tympani proper nerve responses to sweet, sour, salty and better taste stimuli in the chimpanzee. *Acta Physiol Scand.,* **124**: 399-408.

Hirata, S., Abe, T. and Imoto, T. (1992). Effect of crude gymnemic acid on oral glucose tolerance test in human being. *Journal of Yonago Medical Association,* **43**: 392-396.

Hooper, D. (1887). An examination of the leaves of *Gymnema sylvestre. Idem. J. Soc. Chem. Ind.,* **6**: 380.

Hooper, D. (1889). Gymnemic acid. *Chemical News,* **59**: 158-160.

Imoto, T., Miyasaka, A., Ishima, R. and Akasaka, K. (1991). A novel peptide isolated from the leaves of *Gymnema sylvestre* I. Characterization and its suppressive effect on the neural responses to sweet taste stimuli in the rat. *Comp. Biochem. Physiol.,* **100**: 309-314.

Imoto, T., Yamamoto, F., Miyasaka, A. and Hatano, H. (1991). High-performance liquid chromatography-atmospheric pressure ionization mass spectrometry of gymnemic acids. *Journal of Chromatography,* **557**: 383-389.

Izutani, Y., Hirai, H., Takei, T., Imoto, T., Ishijima, S. and Ohnishi, M. (2002). Studies on the proteins from fungiform papillae of bovine tongue: Glyceraldehyde-3-phosphate dehydrogenase is bound to gymnemic acid coupling gel. *Journal Biol. Macromol.,* **2**: 60-63.

Izutani, Y., Kanaori, K., Imoto, T. and Oda, M. (2005). Interaction of gymnemic acid with cyclodextrins analyzed by isothermal titration calorimetry, NMR and dynamic light scattering. *FEBS Journal.,* **272**: 6154-6160.

Jain, S.R. and Sharma, S.N. (1967). Hypoglycaemic drugs of Indian indigenous origin. *Planta Medica,* **15**: 439-442.

Kanetkar, P.V. and Singhal, R.S., Laddha, K.S. and Kamat, M.Y. (2006). Extraction and quantification of gymnemic acids through gymnemagenin from callus cultures of *Gymnema sylvestre. Phytochemical Analysis,* **17**: 409-413.

Kapoor, L.D. (1990). CRC Handbook of Ayurvedic Medicinal Plants; CRC Press: Boca Raton, FL, pp. 200-201.

Kar, A., Choudhary, B.K. and Bandyopadhyay, N.G. (1999). Preliminary studies of the inorganic constituents of some indigenous hypoglycaemic herbs on oral glucose tolerance test. *Journal of Ethnopharmacology,* **64**: 179-184.

Kaur, G. and Kulkarni, K. (2001). Investigation on possible serotonergic involvement in effects of OB-200G (polyherbal preparation) on food intake in female mice. *European Journal of Nutrition,* **40**: 127-133.

Khan, B., Arayne, M.S., Naz, S. and Mukhtar, N. (2005). Hypoglycemic activity of aqueous extract of some indigenous plants. *Journal of Pharmaceutical Science,* **18**: 62-64.

Khare, A.K., Tondon, R.N. and Tewari, J.P. (1983). Hypoglycaemic activity of an indigenous drug (*Gymnema sylvestre*, 'Gurmar') in normal and diabetic persons. *Indian Journal of Physiology and Pharmacology,* **27**: 257-258.

Kimura, I. (2006). Medicinal benefits of using natural compounds and their derivatives having multiple pharmacological action. *Yakugaku Zasshi,* **6**: 133-143.

Kirtikar, K.R. and Basu, B.D. (1935). Indian Medicinal Plants, 2nd edn., Lalit Mohan Basu Publishers, pp. 1624-1626.

Kirtikar, K.R. and Basu, B.D. (1975). Indian medicinal plants, 2nd edn., M.S. Bishen Singh Mahendrapal, New Delhi, Vol-III, pp. 1620.

Koehn, F.E. and Carter, G.T. (2005). The evolving role of natural products in drug discovery. *Nat. Rev. Drug. Discovery.,* **4**: 206-220.

Komalavalli, N. and Rao, M.V. (1997). *In vitro* micropropagation of *Gymnema elegans* W&A - A rare medicinal plant. *Indian Journal of Experimental Biology,* **35**: 1088-1092.

Komalavalli, N. and Rao, M.V. (2000). *In vitro micropropagation of Gymnema sylvestre - a multipurpose medicinal plant. Plant Cell Tissue and Organ Culture,* **61**: 97-105.

Komalavalli, N., Ahmed, B.A., Rao, A.S. and Rao, M.V. (2007). *In vitro* organogenesis of *Gymnema sylvestre* (Retz.) *R.Br.ex Roemer &Schultes-a multipurpose medicinal plant. In Plant Biotechnology: New Frontiers (Proceeding of National Symposium) 18-20 November, 2005.*

Kumar, H.G., Murthy, H.N. and Paek, K.Y. (2002). Somatic embryogenesis in *Gymnema sylvestre. Plant Cell Tissue and Organ Culture,* **71**: 85-88.

Kurihara, Y. (1969). Antisweet activity of gymnemic acid A1 and its derivatives. *Life Science,* **8**: 537-543.

Lee, E.J., Mobin, M., Hahn, E.J. and Peak, K.Y. (2006). Effects of sucrose, inoculum density, auxins, and aeration volume on cell growth of *Gymnema sylvestre. Journal Plant Biology,* **49**: 427-431.

Liu, H.M., Kiuchi, F. and Tsuda, Y. (1992). Isolation and structure elucidation of gymnemic acids, anti-sweet principles of *Gymnema sylvestre. Chemical Pharmaceutical Bulletin,* **40**: 1366-1371.

Liu, X., Ye, W., Yu, B., Zhao, S., Wu, H. and Che, C. (2004). Two new flavonol glycosides from *Gymnema sylvestre* and *Euphorbia ebracteolata. Carbohydrate Research,* **339**: 891-895.

Luo, H., Kashiwagi, A., Shibahara, T. and Yamada, K. (2006). Decreased bodyweight without rebound and regulated lipoprotein metabolism by gymnemate in genetic multifactor syndrome animal. *Mol. Cell Biochem.,* **113**: 6-12.

Maji, D. and Singh, A.K. (1995). Clinical trial of D-400, a herbomineral preparation in diabetes mellitus. *Journal of Diabetic Association India,* **35**: 1-4.

Mathew, K.M. and Rani, N. (1983). Asclepiadaceae. *In*: the flora of the Tamil Nadu Carnatic Part Two. Rapinat herbarium, Diocesan press, Madras, India, pp. 945-947.

Mitra, P.P., Chakraborty, T. and Ganguly, N. (1975). Hypoglycaemic effect on indigenous drug (*Gymnema sylvestre*) in normal and diabetic persons. *Indian Journal of Physiology and Pharmacology,* **27**: 257-258.

Mitra, S.K., Gopu Madhavan, S., Muralidhar, T.S. and Seshadri, S.J. (1996). Effect of D-400, a herbomineral formulation a liver glycogen content and microscopic structure of

pancreas and liver in streptozotocin induced diabetes in rats. *Indian Journal of Experimental Biology*, **34**: 964-967.

Moghaddam, M.S., Anil kumar, P., Reddy, G.B. and Ghole, V.S. (2005). Effect of Diabecon on sugar-induced lens opacity in organ culture: Mechanism of action. *Journal of Ethnopharmacology*, **97**: 397-403.

Mukhopadhyay, B. and Field, R.A. (2006). Convergent synthesis of a trisaccharide as its 2 - (trimethylsilyl) ethyl glycoside related to the flavonoid triglycoside from *Gymnema sylvestre*. *Carbohydrate Research*, **341**: 1697-1701.

Murray, M.T. and Pizzorno, J.E. (1999). Diabetes mellitus. *In*: Pizzorno, Jr J.E. and Murray M.T. (*Eds.*), Textbook of Natural Medicine. New York: Churchill Livingstone, pp. 1193-1218.

Mutalik, S., Chetana, M., Sulochana, B., Devi, P.U. and Udupa, N. (2005). Effect of Dianex, a herbal formulation on experimentally induced diabetes mellitus. *Phytotherapy Research*, **19**: 409-415.

Nadkarni, K.M., Nadkarni, A.K. and Chopra, R.N. (1967). Indian Material Medica. Popular Prakashan Pvt Ltd, Bombay, pp. 474.

Nagaraju, N., and Rao, K.N. (1990). A survey of plant crude drugs of Rayalaseema, Andhra Pradesh, India. *Jouranl of Ethnopharmacology*, **29**: 137-158.

Nakamura, Y., Tsumura, Y., Tonogai, Y. and Shibata, T. (1997). Contents of gymnemic acid in health foods using *Gymnema sylvestre*. *Journal of Food Hygienic Society Japan*, **38**: 178-184.

Oakley, B. (1985). Taste responses of human chorda tympani nerve. *Chemical Senses*, **10**: 469-482.

Ohmori, R., Iwamoto, T., Tago, M., Takeo, T., Unno, T., Itakura, H. and Kondo, K. (2005). Antioxidant of various teas against free radicals and LDL oxidation. *Lipids*, **40**: 849-853.

Persaud, S.J., Al-Majed, H., Raman, A. and Jones, P.M. (1999). *Gymnema sylvestre* stimulates insulin releases *in vitro* by increased membrane permeability. *Journal of Endocrinology*, **163**: 207-212.

Phillipson, J.D. (1990). Plants as source of valuable products. *In*: Charlwood, B.V. and Rhodes, J.C. (*Eds.*), Secondary Products from Plant Tissue Culture. Oxford: Clarendon Press, pp. 1-21.

Porchezhian, E. and Dobriyal, R.M. (2003). An overview on the advances of *Gymnema sylvestre*: Chemistry, Pharmacology and Patents. *Pharmazie*, **58**: 5-12.

Prakash, A.O., Mather, S. and Mathur, R. (1986). Effect of feeding *Gymnema sylvestre* leaves on blood glucose in beryllium nitrate treated rats. *Journal of Ethnopharmacology*, **18**: 143-146.

Preuss, H.G., Bagchi, D., Bagchi, M., Rao, C.V.S., Dey, D.K. and Satyanarayana, S. (2004). Effects of a natural extract of (-) - hydroxycitric acid (HCA-SX) and a combination of HCA-SX plus niacin-bound chromium and *Gymnema sylvestre* extract on weight loss. *Diabetes Obesity Metabolism*, **6**: 171-180.

Puratchimani, V. and Jha, S. (2004). Standardisation of *Gymnema sylvestre* R.Br., with reference of gymnemagenin by high-performance thin-layer chromatography. *Phytochemical Analysis*, **15**: 164-166.

Rafiullah, M.R.M., Siddiqui, A.W., Mir, S.R., Ali, M., Pillai, K.K. and Singh, S. (2006). Antidiabetic activity of some Indian medicinal plants. *Pharmaceutical Biology*, **44**: 95-99.

Raju, V.S.R., Kannababu, S. and Subbaraju, G.V. (2006). Standardization of *Gymnema sylvestre* R.Br., by high-performance thin-layer chromatography: an improved method. *Phytochemical Analysis*, **17**: 192-196.

Rao, S.R. and Ravishankar, G.A. (2002). Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnology Advances*, **20**: 101-153.

Reddy, M.B., Reddy, K.R. and Reddy, M.N.A. (1989). A survey of plant crude drugs of Anantapur district, Andhra Pradesh, India. *International Journal of Crude Drug Research*, **3**: 145-155.

Reddy, P.S., Gopal, G.R. and Sita, G.L. (1998). *In vitro* multiplication of *Gymnema sylvestre*, an important medicinal plant. *Current Science*, **75**: 843-845.

Ricardo Galletto, Vera Lucia Dias Siqueira, Edilene Bega Ferreira, Arildo Jose Braz Oliveira and Roberto Barbosa Bazotte (2004). *Brazilian Archives of Biology and Technology,* **47**: 545-551.

Sahu, N.P., Shashi, B.M., Sudip, K.S. and Gurudas, P. (1996). Triterpenoid saponins from *Gymnema sylvestre. Phytochemistry,* **41**: 1181-1185.

Sanjappa, M. and Sathayanda, N. (1979). IOPB chromosome number reports LXIV. *Taxon,* **28**: 391-408.

Sastri, B.N. (1956). *Wealth of India. Raw materials*, Delhi CSIR, Vol. IV, pp. 275.

Satdive, R.K., Abhilash, P. and Fulzele, D.P. (2003). Antimicrobial activity of *Gymnema sylvestre* leaf extract. *Fitoterapia,* **74**: 699-701.

Schroeder, J.A. and Flannery-Schroeder, E. (2005). Use of the herb *Gymnema sylvestre* to illustrate the principles of gustatory sensation: An undergraduate neuroscience laboratory exercise. *Journal of Undergraduate Neuroscience Education,* **3**: A59-A62.

Shailendra Gurav, Vijay Gulkari, Nandkishore Duragkar and Arun Patil (2007). Systematic review: Pharmacognosy, phytochemistry, pharmacology and clinical applications of *G. sylvestre* R.Br. *Pharmacognosy Reviews*, **1**: 338-343.

Shanmugasundaram, E.R.B., Leela, K.G., Radha, K.S. and Rajendran, V.M. (1990). Possible regeneration of the islets of Langerhans in streptozotocin - diabetic rats given *Gymnema sylvestre* leaf extracts. *Journal of Ethnopharmacology,* **30**: 265-279.

Shanmugasundaram, K.R. and Panneerselvam, C. (1981). The insulinotropic activity of *Gymnema sylvestre*, an Indian herb used in controlling diabetes mellitus. *Pharmacology Research Communication,* **13**: 475-485.

Shanmugasundaram, K.R., Panneerselvam, C., Samudram, P. and Shanmugasundaram, E.R.B. (1983). Enzyme changes and glucose utilization in diabetic rabbits the effect of *Gymnema sylvestre. Journal of Ethnopharmacology,* **7**: 205-234.

Shanmugasundram, E.R.B., Rajeswari, G., Baskaran, K., Rajeshkumar, B.R., Radha, K.S., and Ahamath, K. (1990). Use of *Gymnema sylvestre* leaf extract in the control of blood glucose in insulin–dependent diabetes mellitus. *Journal of Ethnopharmacology,* **30**: 281-294.

Shigematsu, N., Asano, R., Shimosaka, M. and Okazaki, M. (2001). *Biological Pharmaceutical Bulletin,* **24**: 643.

Shimizu, K., Abe, T., Nakajyo, S., Urakawa, N., Atsuchi, M. and Yamashita, C. (1996). Inhibitory effects of glucose utilization by gymnema acids in the guinea-pig ileal longitudinal muscle. *Journal Smooth Muscle Research,* **32**: 219-228.

Shimizu, K., Iino, A., Nakajima, J., Tanaka, K., Nakajyo, S., Urakawa, N., Atsuchi, M., Wada, T. and Yamashita, C. (1997). Suppression of glucose absorbtion by some fraction from *Gymnema sylvestre* leaves. *Journal of Vet. Med. Sci.,* **59**: 245-251.

Shore, L.E. (1892). A contribution to our knowledge of taste sensations. *Journal of Physiology,* **13**: 191-217.

Sinsheimer, J.E., Rao, G. and Mcllhenny, H.M. (1970). Constituents from *Gymnema sylvestre* leaves, isolation and preliminary characterization of the gymnemic acids. *Journal of Pharmaceutical Science,* **59**: 622-628.

Sinsheimer, J.E., Rao, G.S., McIlhenny, H.M., Smith, R.V., Maassab, H.F. and Cochran, K.W. (1968). Antiviral activities of gymnemic acids. *Experientia,* **24**: 302-303.

Sredeevi, P. and Namboodiri, A.N. (1977). IOPB chromosome numbers reports, LVI, *Taxon,* **26**: 257-274.

Srivastava, Y., Bhatt, H.V. and Prem, A.S. (1985). Hypoglycemic and life-prolonging properties of *Gymnema sylvestre* leaf extract in diabetic rats. *Isral Journal of Medical Science,* **21**: 540-542.

Stocklin, W. (1968). Gymnestrogenin, ein neues pentahydroytriterpene aus den Blattern von *Gymnema sylvestre. Helv. Chim. Acta.,* **51**: 1235-1242.

Stocklin, W. (1969). Chemistry and physiological properties of gymnemic acid, the antisaccharine principle of the leaves of *Gymnema sylvestre. Journal of Agricultural Food Chemistry,* **17**: 704-708.

Stocklin, W., Weiss, E. and Reichstein, T. (1967). Gymnemasiure, das antisaccharine Prinzip von *Gymnema sylvestre.* Isolierungen und Identifizierungen. *Helv. Chim. Acta,* **50**: 474-490.

Sugihara, Y., Nojima, H., Matsuda, H., Murakami, T., Yoshikawa, M. and Kimura, I. (2000). Antihyperglycemic effects of gymnemic acid IV, a compound derived from *Gymnema sylvestre* leaves in streptozotocin-diabetic mice. *Journal Asian Natural Product Research,* **2**: 321-327.

Suzuki, K., Ishihara, S., Uchida, M. and Komoda, Y. (1993). Quantitative analysis of deacylgymnemic acid by high-performance liquid chromatography. *Yakugaku Zasshi,* **113**: 316-320.

Terasawa, H., Miyoshi, M. and Imoto, T. (1994). Effects of long-term administration of *Gymnema sylvestre* watery-extract on variations of body weight, plasma glucose, serum triglyceride, total cholesterol and insulin in Wistar fatty rats. *Yonago Acta Med.,* **37**: 117-127.

Tripathi, Y.B. and Chaturvedi, P. (1995). Assessment of endocrine response of *Inula racemosa* in relation to glucose homeostasis in rats. *Indian Journal of Experimental Biology,* **33**: 686-689.

Wang, L.F., Luo, H., Miyoshi, M., Imoto, T., Hiji, Y. and Sasaki, T. (1998). Inhibitory effect of gymnemic acid on intestinal absorption of oleic acid in rats. *Canadian Journal of Physiology and Pharmacology,* **76**: 1017-1023.

Warren, R.M. and Pfaffmann, C.J. (1969). Suppression of sweet sensitivity by potassium gymnemate. *Applied Physiology,* **14**: 40-42.

Yackzan, K.S. (1969). Biological effects of *Gymnema sylvestre* fractions II. Electrophysiology-effect of gymnemic acid on taste receptor response. *Alabama Journal of Medical Science,* **6**: 455-463.

Ye, W.C., Liu, X., Zhang, Q., Che, C. and Zhao, S. (2001). Anti sweet saponins from *Gymnema sylvestre. Journal of Natural Product,* **64**: 232-235.

Ye, W.C., Liu, X., Zhao, S. and Che, C. (2001). Triterpenes from *Gymnema sylvestre* growing in China. *Biochemical Systematic Ecology,* **29**: 1193-1195.

Ye, W.C., Zhang, Q., Liu, X., Che, C. and Zhao, S. (2000). Oleanane saponins from *Gymnema sylvestre. Phytochemistry,* **53**: 893-899.

Yoshikawa, K., Amimoto, K., Arihara, S. and Matsura, K. (1989a). Structure studies of new anti-sweet constituents from *Gymnema sylvestre. Tetrahedron Letter,* **30**: 1103-1106.

Yoshikawa, K., Kondo, Y., Arihara, S. and Matsura, K. (1989b). Gymnemic acids V, VI and VII from the leaves of *Gymnema sylvestre. Chemical Pharmaceutical Bulletin,* **37**: 852-854.

Yoshikawa, K., Arihara, S., Matsura, K. and Toshio, T. (1992). Dammarane saponins from *Gymnema sylvestre. Phytochemistry,* **31**: 237-247.

Yoshikawa, K., Kondo, Y., Arihara, S. and Matsura, K. (1993). Antisweet natural products. IX. Structures of gymnemic acids XV–XVIII from *Gymnema sylvestre. Chemical Pharmaceutical Bulletin,* **41**: 1730-1732.

Yoshikawa, K., Nakagawa, M., Yamamoto, R., Arihara, S. and Matsuura, K. (1992). Antisweet natural products V. Structures of gymnemic acids VIII-XII from *Gymnema sylvestre. Chemical Pharmaceutical Bulletin,* **40**: 1779-1782.

Yoshikawa, K., Shigenobu, A. and Kouji, M. (1991). A new type of anti sweet principles occuring in *Gymnema sylvestre. Tetrahedron Letter,* **32**: 789-792.

Yoshikawa, M., Murakami, T. and Matsuda, H. (1997). Medicinal food stuffs X. structure of new triterpene glycosides, Gymnemosides -c, -d, -e and -f from the leaves of *Gymnema sylvestre,* influence of *Gymnema* glycoside on glucose uptake in rats small intestinal fragments. *Chemical Pharmaceutical Bulletin,* **45**: 2034-2038.

"This page is Intentionally Left Blank"

4

Andirobas of the State of Acre (Brazil): Chemical and Biological Aspects Associated with the Use and Management

ANA CLAUDIA F. AMARAL[1,*], JOSÉ LUIZ P. FERREIRA[1,4], SILVIA L. BASSO[2] AND JEFFERSON ROCHA DE A. SILVA[3]

ABSTRACT

Carapa guianensis (Meliaceae) and Fevillea cordifolia (Cucurbitaceae), best and popularly known as "andiroba" and "andiroba-de-rama", respectively, are two species found in the Brazilian Amazon region, which are used by the local population for various medicinal and economic purposes, especially due to the large amount of fixed oil that can be obtained from their seeds. The oils extracted showed to be a good alternative of participatory management due to the low costs involved and the large availability of the species in the Amazon region. The knowledge of their specific characteristics like: geographical distribution, morphological and chemical aspects as well as their respective medicinal uses helps improving the valorisation of the products through the sustainable application of a promising trade. Owing to the increasing use of these species, this work intends to present an

1. Laboratório de Plantas Medicinais e Derivados, Depto de Produtos Naturais, Farmanguinhos – FIOCRUZ – Rua Sizenando Nabuco, 100 – Manguinhos/RJ, 21041-250, Brasil.
2. Laboratório de Produtos Naturais, Fundação de Tecnologia do Estado do Acre (FUNTAC) Av. das Acácias, Lote 01, zona A - Distrito Industrial, Rio Branco/AC, 69917-100, Brasil.
3. Laboratório de Cromatografia - Departamento de Química – Universidade Federal do Amazonas - (UFAM) - Mini-Campus/Estrada do Contorno, 3000 - Japiim - Manaus/AM, 69077-000, Brasil.
4. Faculdade de Farmácia, Laboratório de Farmacognosia – MAF-Universidade Federal Fluminense (UFF). Viana, 523. Santa Rasa, Niteroi/RJ 24241- 000, Brasil.

* *Corresponding author* : E-mail : acamaral@fiocruz.br ; jrocha_01@yahoo.com.br

overview of existing publications as well as the results of the researches and works.

Key words : *Carapa guianensis, Fevillea cordifolia*, andiroba, botanic aspects, seed oil, chemical constituents, applications

INTRODUCTION

Since long, Amazonia has made part of the vision of the European settlers as a mysterious place, almost heavenly: as the legendary Amazons land or else as the lost Eden. Presently, the world's eyes turn towards Amazonia, yet, this time, attracted by its biodiversity, its huge genetic bank and its titanic diversity of fauna, flora and minerals. In this natural laboratory, the people of the forest have been using the Amazonian flora and fauna, mainly for subsistence and healing purposes among others. These costumes, which have been transmitted consecutively through generations, are being perpetuated as folk tradition. Nowadays, their knowledge, be it botanical or pharmacological, serves as a guide in the search for new and better products for humanity's welfare. Principally in the tropics, various species and their potential applications are being studied (Amaral *et al.*, 2005) as it is the case of the oils extracted of *Carapa* and *Fevillea* genus seeds and its possible application in the segments of production of medicines, cosmetics, repellents and insecticides. In this context, biodiversity should be considered a commodity and, thus, bring forth wealth wherever it shows off. On the other hand, this same biodiversity is becoming a scarce resource, being, nevertheless, essential not only for the conservation of the world ecological balance but also as a strategic heritage for the development and growth of the economic activities of this wealth holders, as it has been developed towards the communities in the State of Acre. In this sense, the scientific and technological researches play a major role in the knowledge enhancement and the economic application of the biologic diversity aiming to improve and innovate the initiatives of the communities, with direct impact on the families' income increase and life quality. *C. guianensis* (Meliaceae) and *F. cordifolia* (Cucurbitaceae), best and popularly known as "andiroba" and "andiroba-de-rama", respectively, are two species found in the State of Acre, which are used by the local population for various purposes, especially due to the large amount of fixed oil that can be obtained from their seeds. In Acre, the extraction process of these oils showed to be a good alternative of participatory management, thanks to the low costs involved and the large availability of the species in the Amazon region. Owing to the increasing use of these species, this work intends to present an overview of existing publications, as well as the results of the researches and works carried out with the communities using these spontaneous species that occur in the State of Acre in Brazil (border region with Bolivia and Peru).

BOTANIC ASPECTS

Synonyms

Carapa guianensis

Carapa nicaraguensis C. DC.; *C. slateri* Standley; *C. latifolia*; *C. macrocarpa* Ducke; *Amapa guianensis* (Aublet) Steud.; *Granatum guianensis* (Aublet) Kuntze; *G. nicaraguense* (C. DC.) Kuntze; *Guarea mucronulata* C. DC.; *Persoonia guareoides* Willd; *Xylocarpus carapa* Spreng. (Pennington *et al.*, 1981; Andrade *et al.*, 2001).

Fevillea cordifolia

Fevillea punctata Poir in Lam.; *F. javilla* H.B.K.; *F. karstenii* Cogn.; *Nhandiroba cordifolia* (L.) Kuntze; *Siolmatra mexiae* Standl. (http:// mobot.mobot.org/cgi-bin/search_vast).

Common Names

Carapa guianensis

Bastard mahogany, carapa, crabwood, Brazillian mahogany, white karaba, Roba mahogany (English); Andiroba, bois rouge, carepa, cabirma de Guinea, cahipon, bois caille, carapa (French); Andiroba, cabrima de Guiana, caobilla, cedro macho, masábalo, najeri, guino, tángare, figuraueroa, cedro bateo, krappa (Spanish); Andiroba, andiroba branca, andirobeira, mandiroba, carapa, inandiroba, iandirova, nandiroba, andiroba camacari, randiroba, andiroba de igapó, andiroba vermelha, yandiroba, purga de Santo Inácio, comaçari, caropá (Brazil); (Pio Corrêa, 1931; Revilla, 2000; Sampaio, 2000).

Fevillea cordifolia

Antidote-vine, antidote caccoon, antidote bush (Jamaica, English); ti concombre (French); jabilla, abilla, nacha, chichimora, ayamo, jayamo, jallamo (Spanish); mukula, sequa, mula, antidote beans (Nicarágua), cobalonga, habilla (Peru); andiroba de rama, fevilea (Brazil) Gentry & Wettach, 1986; Galy *et al.*, 2000; Balick *et al.*, 2000; Coe & Anderson, 2005; Ticktin & Dalle, 2005; Coe, 2008).

Taxonomy

Carapa guianensis

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Sapindales

Family: Meliaceae

Genus: *Carapa* Aubl.

Species: *C. guianensis* Aubl.

Fevillea cordifolia

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Dilleniidae

Superorder: Violanae

Order: Cucurbitales

Family: Cucurbitaceae

Subfamily: Zanonioideae

Tribe: Zanonieae

Subtribe: Fevilleinae

Genus: *Fevillea*

Species: *F. cordifolia* L.

Geographic Distribution

Carapa guianensis

Native: Brazil (throughout the Amazon region), Colombia, Costa Rica, Dominican Republic, Ecuador, French Guiana, Guadeloupe, Guyana, Haiti, Honduras, Panama, Peru, Puerto Rico, Surinam, Trinidad and Tobago, Venezuela. Exotic: Indonesia, Malaysia, Singapore.

Fevillea cordifolia

Native: Mexico; Costa Rica, Nicaragua, Panamá; Dominican Republic, Haiti, Jamaica, Puerto Rico; Venezuela, Brazil (Acre, Amazonas, Pará, Rondônia); Bolivia; Colombia; Ecuador; Peru (Robinson & Wunderlin, 2005).

Habitat

Carapa guianensis

A tree commonly establishes itself on rich soils, along streams and in the periodically inundated swamp forest and uplands forests along the rivers

(Prates-Clark *et al.*, 2008). It has been observed in altitudes ranging from 0 to 1.200 meters, in areas with mean annual rainfall 1500-3200 mm and a mean annual temperature of 24-26°C (Prates-Clark *et al.*, 2008).

Fevillea cordifolia

This species is apparently restricted to the seasonally inundated varzea forests, a habitat in which water dispersal is prevalent and is a perennial herb (Gentry & Wettach, 1986). *Fevillea* typically occurs along river banks, along the edge of tropical primary or secondary forests, and along the edge of seasonally inundated riverine forests, occasionally climbing to heights of 35 m in forest canopy openings. It is also found in forest clearings and along roadsides. It occurs at elevations from near sea level to about 500 m, less commonly up to 1.700 meters (Robinson & Wunderlin, 2005).

Botanical Description

Carapa guianensis

C. guianensis is a deciduous or semi-evergreen, monoecious, medium-sized to large trees 35-55 m tall; bole straight and cylindrical; branchless up to 20-30 m; diameter 100-200 cm, sometimes fluted, with short buttresses up to 2 m high. Bark surface flaking into squarish scales or in horizontal strips, light grey to greyish brown, dark brown or sometimes reddish; inner bark fibrous, red or pinkish brown. Young plants produce taproots but the trees tend to become surface rooted. Leaves alternate, paripinnate with a dormant glandular leaflet at the apex, exstipulate; leaflets opposite, entire. It shows gigantic leaves in the monocaulous juvenile stage, decreasing in size when branching is initiated (Figs 1a, b). Flowers small, white, borne in a large, axillary or subterminal thyrse; unisexual but with well-developed vestiges

Fig 1. *Carapa guianensis* (*a*) Tree; (*b*) Leaves; (*c*) Fruit; (*d*) Seeds

of the opposite sex; tetramerous to pentamerous (max. sextamerous); calyx lobed almost to the base; petals slightly contorted. Fruit dehiscent, 4-lobed, pendulous, subglobose, woody capsule containing 2-4 seeds in each lobe. Seeds smooth, pale brown, angular, with woody sarcotesta (Figs 1c, d). (Winner & Losers, 2003).

Fevillea cordifolia

Fevillea is a member of the relatively small subfamily Zanonioideae, which is distinguished from the rest of the Cucurbitaceae by stamens inserted on a disc rather than on the receptacle, 3 rather than 1, styles and tendrils that are bifid near the apex and coilboth above and below the point of bifurcation. This species is a riparian liana, generally found in groups by the riverside and lakeside (it seldom occurs in isolated form). With simple alternate leaves, with foliar blades of orbicular contour, acuminate apex, reentrant base and entire margin. Its consistency is coriaceous and its venation of palmatinerved type (Figs 2a, b). Petiole is straight and cylindric, with the presence of large amounts of tendrils. The inflorescence is thyrso-branched with flowers of about 5 mm, cream-colored flowers having five stamens. Within this group, genus is outstanding in having very large multiseeded fruits, a trait shared with its closest neotropical relative *Siolmatra*. That genus differs in having small winged dehiscent fruits. *Fevillea* seeds are the largest among the family Cucurbitaceae, probably as a result of adaptations for water dispersal of the individual seeds after the fruit breaks open. The thick, lenticular seeds of *Fevillea*, filled in large indehiscent fruits have large oil-rich cotyledons and are covered by a rather thick fibrous-corky seed coat (Figs 2c, d). (Gentry & Wettach, 1986; Robinson & Wunderlin, 2005).

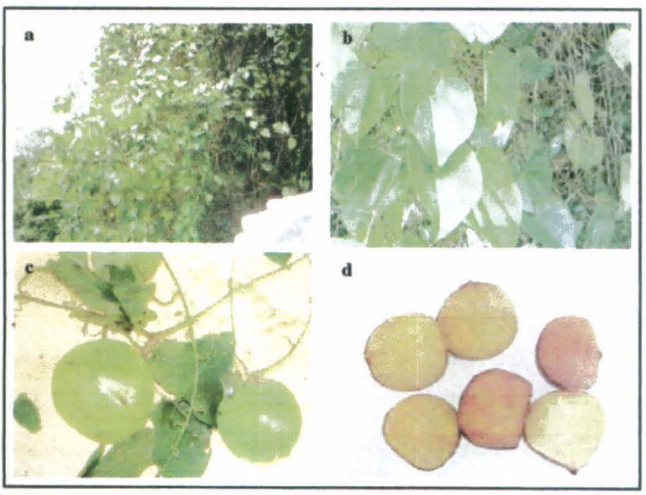


Fig 2. *Fevillea cordifolia* (*a*) Tree; (*b*) Leaves; (*c*) Fruit; (*d*) Seeds

Both species collected from the State of Acre could be differentiated on the basis of external morphology of leaves, fruits, flowers and seeds (Table 1).

Table 1. The main external characteristics of *C. guianensis* and *F. cordifolia*

***Carapa guianensis* (Acre, Brazil)**	***Fevillea cordifolia* (Acre, Brazil)**
Tree that can reach 35 m in height, trunk is straight and cylindric, with up to 200 cm thickness	Medium-sized Liana genus that can reach 3 m in height and is often found in groups
Compound, alternate, even-pinnate leaves; opposite leaflets, with oblong-elliptic contour, peninerved, entire, acute or acuminate apex and subacute base	Simple, alternate leaves, with orbicular contour, palmatinerved, acuminate apex, reentrant base and entire margin
Thyrso inflorescence with small white unisexual flowers, yet with vestiges of the other reproductive organ, eight to ten stamens, concrescent in tube, lobed calyx, slightly coiled petals	Thyrso inflorescence with small cream-colored flowers, with five free stamens, calyx deeply cupular, the lobes suborbicular, pustulate. Petals oblong.
Dehiscent capsule-typed fruits, tetralobe, subglobose	Indehiscent capsule-typed fruits subglobose, gourd-like, the surface pustulate, mottled green, zonate above the middle with the hypanthium lip scar and marked at the apex by a raised triradiate line.
Smooth seeds of pale-brown, poliedric contour, with a curved face and round extremities with ligneous aril	Smooth lenticular seeds, of pale-brown, flattened, compacted with fibrous peel.

ANATOMICAL ASPECTS

Microscopic Analyses

Leaves

Carapa guianensis

In a cross section of the foliar blade of a specimen collected in Mâncio Lima County (AC) city, an epidermis coating was observed with a thick cuticle on the adaxial surface. Dorsiventral mesophyll presented two palisade parenchyma layers with numerous calcium oxalate druses and spongy parenchyma with vascular bundles with fibrous elements (Figs 3a, b). The

Fig 3. *Carapa guianensis* (*a*); (*b*) Transverse section of dorsiventral mesophyll showing calcium oxalate druses (100 μm and 50 μm;); (*c*) Transverse section of midrib - adaxial region (50 μm)

midrib had in the fundamental parenchyma, next to the vascular region, a sclerenchymatic cells ring and at the innermost region, a fiber tissue surrounding the xylematic vessels, radially disposed, immerse in a fibrous matrix. The central region was taken by parenchymatic tissue (Fig 3c).

Fevillea cordifolia

In a cross section of the foliar blade collected in Mâncio Lima, a county in state of Acre, the presence of a dorsiventral mesophyll was observed with two palisade parechyma layers and five to seven spongy parenchyma, with the detached presence of a parenchymatic sheath surrounding the vascular bundles. The epidermic tissue of the adaxial surface is adjacent to a hypodermic cells layer (Fig 4a). The midrib presents subepidermic angular chollenchyma, sclerenchymatic cells in the fundamental parenchyma and a fibrous sheath neighbor to the vascular bundles. These are collateral, arc-disposed and take the central region (Figs 4b, c).

Fig 4. *Fevillea cordifolia* (*a*) Transverse section of the foliar mesophyll region (100 μm); (*b*) Overview of the transverse section of the foliar blade, showing the midrib and the appendage mesophyll (200 μm); (*c*) Transverse section of the midrib (50 μm)

Seeds

The reserve tissue of both seeds contained deposit of fixed oil (Figs 5a, b).

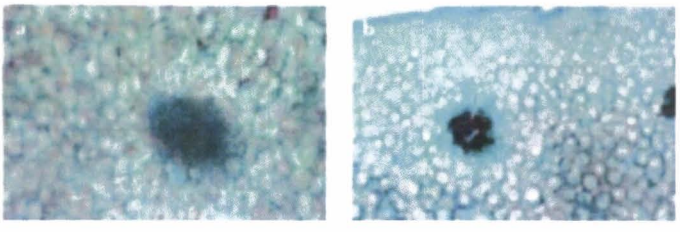


Fig 5. (*a*) Reserve parenchyma of the seed of the *F. cordifolia* with fixed oil (50 μm); (*b*) Vascular bundles placed concentrically into reserve parenchyma of the seed of the *C. guianensis* (50 μm)

Main Differences in the Morphology of the Two Species

An evaluation of the results achieved in the light of a microscope lamina of the cross-sections of *C. guianensis* and *F. cordifolia,* after four seasonal collections, showed the existence of anatomic characteristics that helped to

differentiate the two species. It was observed the presence of numerous calcium oxalate druses and vascular bundles with fibrous elements along the mesophyll of *C. guianensis*, while *F. cordifolia* had a parenchymatic sheath surrounding the bundles, with no druses. On the adaxial surface of *F. cordifolia* existence of hypodermis was observed which was not identified in the foliar blade of *C. guianensis*. The midrib of this latter species shows, next to the vascular region, a sclerenchymatic cells ring and the fiber tissue surrounding the xylematic elements, radially disposed, with fibers. The central region was taken by parenchyma. On its turn, *F. cordifolia* presented a set of these sclerenchymatic cells along the parenchymatic tissue and a fibrous sheath neighbor to the vascular bundles, which, being arc-disposed, took the central region (Amaral *et al.*, 2006).

POPULAR USES

Carapa guianensis has been used in the Amazon region as an insect repellent and for treatment of arthritis, throat inflammation, ear infections, uterine cancer, diarrhoea and diabetes. The local communities make a kind of soap using the seed oil for treatment of skin diseases, rheumatism, to heal wounds and bruises and as an insect repellent (Hammer & Johns, 1993; Klimas *et al.*, 2007). They also drink the oil to treat coughs and convulsions (Duke & Vasquez, 1994; Branch & Silva, 1983). Based on the traditional use, in general, 2 ml of the seed oil mixed in a small glass of warm water is taken twice or three times daily to expel worms, prevent tumours and reduce inflammation among other uses (http://rain-tree.com/andiroba.htm). Several seed oil products in many dosage forms such as cream, compound syrup, oily capsules and, more recently, gel are recommended for many respiratory infections, including pharyngitis, laryngitis, cough, flu, pneumonia and bronchitis as well as muscle and joint injuries and skin diseases (Orellana *et al.*, 2004). As to the domestic use of the oil from *C. guianensis*, it is employed as a natural lamp fuel, as it burns well, produces little smoke as well as repels mosquitoes, flies and other insects. In the early 1800s, the street lamps of Belém, a city of Brazil, were fuelled with andiroba oil (http://rain-tree.com/andiroba.htm).

Fevillea cordifolia is used in the folk medicine as purgative, antidote for many kinds of poisoning, leprosy, hair fall, dandruff, treatment of the side effects caused by snakebite and to treat numerous diseases (Gentry & Wettach, 1986; Robinson & Wunderlin, 2005) such as uterine fibroids (benign connective tissue tumours) (Balick *et al.*, 2000), also to dress wounds, with emetic and purgative potential (Mitchell and Ahmad, 2006). In 1870, Lindley and Moore had already reported the use of *Fevillea* seeds as candles by the Peruvians (Robinson & Wunderlin, 2005).

BOTANICAL MATERIAL

Vouchers

The two botanical materials were collected in Mâncio Lima, a county in the State of Acre, under IBAMA authorization number 028/2006.

Carapa guianensis and *Fevillea cordifolia*

The species vouchers were deposited in the Herbarium of Federal University of Acre, under number 18.384 and 18.385, respectively.

Geographic Area

Carapa guianensis

Material were collected on the Japin river margins, a tributary of the Moa river, on March 18, 2006, coordinates: S 07°24'01.9", 073°10'31.5" W - 18M – UTM= 721.223 and 9.168.887. The trees were fertile with fruits and in the surroundings, there were observed species like: "breu vermelho" (*Tetragastris cerradicola* Daly), "açai" (*Euterpe precatoria* Mart.), "patauá" (*Oenocarpus bataua* Mart.), "sumauma" (*Ceiba pentandra* L.), "buriti" (*Mauritia flexuosa* L), "paxiubinha" (*Socratea exorrhiza* Wendl.), "paxiubão" (*Iriartea deltoidea* Ruiz & Pavón) among others.

Fevillea cordifolia

This species was collected on the "São Pedro" stream margins, on March 18, 2006, coordinates: S 07°30'51.5", W 072°59'42.8" - 18M – UTM= 721.223 and 9.168.887. The lianas were fertile with fruits and in the surroundings predominant species were: "araticum" – (*Annona* sp), "ingá" (*Inga* sp) among others.

EXTRACTION METHODS OF THE SEEDS OIL

Alternative approaches are employed to the extraction of the oils but irrespective of the area or which tribe or group of persons is involved in its production, the extraction methods of the oils are noticeably similar (Winners & Losers, 2003). The seeds oils of the species were obtained using two different methods: hot and room temperature extractions, according to local communities of Acre.

Carapa guianensis

Hot Extraction: Firstly, the fruits passed by a cleaning process, then the seeds were smashed in a mortar and then boiled at 80° to 100°C, for approximately 1 h. During the heating process, the oil separates from the gross mass and flows out of the paste to the top of the bowl. With the help of a scoop, the delivered oil was taken from the bowl. After cooking, the mixture was left to rest at room temperature until cooling. The liquid was then leached and the small quantity of oil left was extracted by pressing.

Yield: each 100 g of seeds result in about 40 g of oil (each fruit contains from 8 to 15 seeds).

In the case of *Fevillea cordifolia* species, the extraction at room temperature was applied. Firstly, the fruits passed by a cleaning process, the seeds are broken and then the cotyledons were ground in a mortar. The remaining oil was extracted by pressing the pulp. *Yield*: each 100 g of seeds result in about 48 g of oil (each fruit contains from 10 to 20 seeds).

Characteristics

Carapa guianensis and *Fevillea cordifolia*

Both oils taste very bitter. The recent extracted liquids were transparent, of pale-yellow in color as to *C. guianensis* and dark-yellow as to *F. cordifolia* (Fig 6).

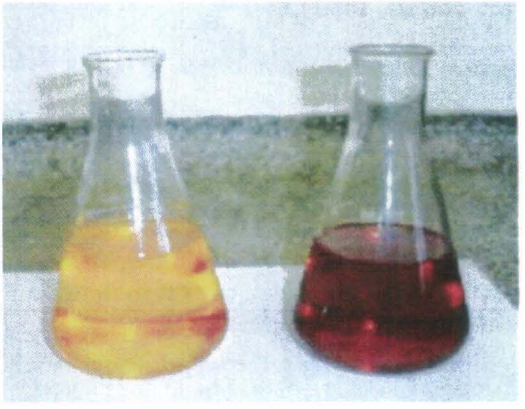


Fig 6. Oils of *C. guianensis* (left bottle) and *F. cordifolia* (right bottle)

After refrigeration storage, the oil from *C. guianensis* presented a white precipitate that increased in volume as days passed by. Being again put at room temperature, it did not show the same original characteristics of transparent oil. This behavior was also observed as to the *C. guianensis* and *F. cordifolia* stored oils, which created a white and solid fat layer.

PHYSICAL CHEMISTRY ANALYSES

The values found in the analyses of samples (Table 2), obtained by the extraction methods described above, to acidity, saponification index, refraction index, moisture and iodine index data are within the reported limits of *Carapa guianensis* and *Fevillea cordifolia* of other regions. (Winners & Losers, 2003; Gentry & Wettach, 1986; Souza *et al.*, 2006).

Table 2. Physical chemistry data

Sample (oil)	Viscosity (30°C, cSt)	Acidity mg/KOH/g	Saponification index mg/KOH/g	RI (25°C)	Moisture (%)	Iodine Index (%)
C. guianensis	85.6	28.9	186.5	1.467	9.98	69.2
F. cordifolia	155	22.2	177.9	1.465	8.16	70.1

RI = Refraction Index

Chromatographic Analyses

The chromatographic profiles of *C. guianensis* and *F. cordifolia* oils were obtained using Gas Chromatography – Mass Spectrometer (GC-MS) and Thin Layer Chromatography (TLC).

GC-MS

Table 3 presents a comparison of the esters of fatty acids found in the specimens of the seed oils extracted of *C. guianensis* and *F. cordifolia* from different regions.

The fatty acids common to all specimens were: oleic, palmitic and stearic. The analyses of the oils of the *C. guianensis* from Guyana and from Brazil presented a large amount of unsaturated fatty acids, mainly the oleic, with values between 60.8% and 63.4%. On the other hand, the oils from *F. cordifolia* have a significant amount of saturated fatty acids, 56.7% (Brazil) and 63% (Peru) and 58.2% (Costa Rica), with a predominance of stearic acid. Both species contain the stearic and palmitic acids, considering that the first one is more significant in *F. cordifolia*, while the latter is found in greater concentration in *C. guianensis*. These are possibly the constituents responsible for the use of the seeds of these species in the production of candles (Gentry & Wettach, 1986).

Other unidentified conjugated acids, as the α-eleostearic acid detected in *F. cordifolia*, represent a differential constituent when compared to the specimens of *C. guianensis*, and that might be characteristic of the genus (Gentry & Wettach, 1986). As to the large amount of oleic acid found in the specimens of *C. guianensis*, it is important to call the attention to the investigations that are being carried out to use the species as source for biodiesel production, which is part of a great impact program of the Brazilian Government (Pinto *et al.*, 2005). Biodiesel properties are strongly influenced by the properties of the individual fatty esters. It therefore appears reasonable to enrich it with certain fatty esters with desirable properties in the fuel in order to improve the properties of the whole fuel (Knothe, 2005). It may be possible in the future to improve the properties of biodiesel by means of genetic engineering of the parent oils, which could eventually lead to a fuel enriched with certain fatty acids that exhibit a

Table 3. Esters of fatty acids of *C. guianensis* and *F. cordifolia* oils

Species	Methyl esters of the oils (%)										
	Lauric 12:0	Miristic 14:0	Palmitic 16:0	Margarinic 17:0	Stearic 18:0	Araquidic 20:0	Palmitoleic 16:1	Oleic 18:1	Linoleic 18:2	α-Eleostearic 18:3	Linolenic 18:3
F. cordifolia - Costa Rica (Achenbach *et al.*, 1992)	-	-	4.2	0.1	53	0.9	-	4.7	4.3	31	1.8
F.cordifolia - Peru (Gentry & Wettach, 1986)	-	-	21	-	42	12[a]	-	14	7	12[a]	-
F. cordifolia - Brazil (Acre) (Basso *et al.*, 2006)	8.7	3.1	19.9	-	25.0	-	-	22.8	-	-	-
C. guianensis - Brazil (Acre) (Basso *et al.*, 2006)	-	-	27.5	-	7.3	-	-	63.4	-	-	-
C. guianensis - Guyana (Winners & Losers, 2003)	-	-	28.0	-	8.1	1.2	1.0	50.5	9.0	-	0.3

[a] Sum of C_{20} and $C_{18:3}$

combination of improved fuel properties (Knothe, 2005). *F. cordifolia* species is also being investigated for biodiesel source purposes (Pinto *et al.*, 2005; Souza *et al.*, 2006).

TLC

The TLC analyses were carried out on precoated silica gel 60 F_{254} (Merck) TLC aluminium sheet using toluene: ethyl acetate (9:1) as developing system. Plates were dried and the spots were visualized by spraying with detection reagents (sulphuric vanillin – VS and Liberman Burchard - LB), followed by heating at 110°C. These derivatisation reagents are useful for detecting terpenoids and other compounds (Wagner *et al.*, 1984) after heating through a characteristic colour reaction.

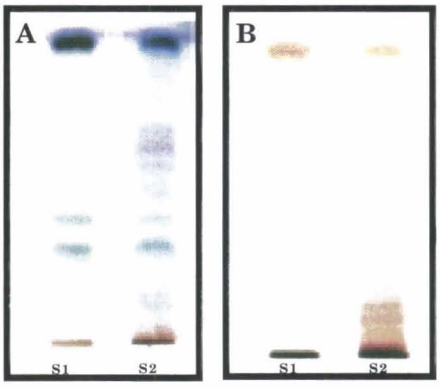


Fig 7. TLC – silica gel (toluene: EtOAc, 9:1) of the (S1): oil of *C. guianensis* and (S2): oil of *F. cordifolia*; chromatograms (A) and (B) treatment with derivatisation reagents VS and LB, respectively

The two chromatograms showed, after the revelation process, the terpenoidic nature of the compounds of both oils. The TLC plate treated with derivatisation reagent VS (chromatogram A) showed two major bluish-coloured spots with Rfs inferior to the half of the chromatogram and other intense spot in the solvent front in *C. guianensis* (S1). The oil of *F. cordifolia* (S2) showed the same two spots as S1, and six others displayed in a purplish-blue colour between the middle of the chromatogram and the solvent front. Next to origin, there were observed two extra dark spots in the profile of S2, which did not show any correspondence to the oil of S1. The evaluation of the chromatographic profiles has indicated a smaller number of spots in S1. The TLC plate treated with derivatisation reagent LB (chromatogram B) showed two major brown-coloured spots with Rfs close to the half of the chromatogram and a third yellow-coloured spot in the solvent front in S1, similar to the one observed with the other revelation reagent (chromatogram A). The oil of S2 showed eight brown to brownish-coloured spots, three of which showed correspondence in S1. The comparison of the samples in the

chromatogram B showed simplified chromatographic profiles with a smaller number of spots in the *C. guianensis* sample. When comparing the chromatographic profiles of the TLC plates eluted with toluene: ethyl acetate (9:1) after being treated with derivatisation reagent VS or LB, it was observed that it is possible to apply this technique to differentiate the two oils.

CHEMICAL CONSTITUENTS, USES AND PHARMACOLOGICAL ACTIVITIES

The Table 4 shows the substances related to the vegetables organs already studied, uses and pharmacological activities of *C. guianensis* and *F. cordifolia*.

ADMINISTRATION AND PROCESSING OF THE OILS OF *C. guianensis* AND *F. cordifolia*

In Amazonia, the survival of the "forest people", denomination given to the indigenous people, the riverside dwellers, fisherfolk, quilombolas[1], rubbertappers and family farmers, is intimately linked to the extractivism of forest products. According to Eloy (2001), extractivism is defined as a set of exploration systems of the forest products, which must be integrated to the market economy in a regional, national and international level.

In order to keep the sustainable extractivism in Amazonia, it is necessary to deploy management programs, either in conservation units or legal reserve areas. The term sustainable management of the forest is defined as the forest resources management aimed at social and economical benefits by keeping the respect for the ecosystem sustainability mechanisms (Silva, 2001; Decree no. 1.282 of Oct. 19, 1995), which being adopted leads to the achievement of said benefits. For the deployment of the forest management in Conservation Units, there must be adopted a series of actions and activities required to achieve the conservation objectives, considering the related activities like: protection, recreation, education, research as well as the administration or management activities. The sustainable management may be applied to timber and non-timber products and can be of three different types: Enterprise Management Plan, Community-Based Forest Management Plan and Multi-Use Management Plan. On a common point of view, the latter two plans are widely used when applied to non-timber forest products, since it results in obtaining socio-economic benefits, considering the respect for the ecosystem sustainability mechanisms.

The Community-Based Forest Management is one of the alternatives to make use of the resources of the legal reserve area in the family units, with little impact to the forest biodiversity. These timber and non-timber

[1] Descendants of slaves who escaped slave plantations

Table 4. Chemical constituents, uses and pharmacological activities

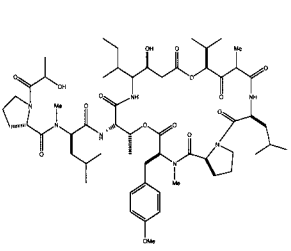
Species	Part of the Plant	Substances	Uses and Pharmacological activities
C. guianensis	Seeds	6-α-11-β-diacetoxygedunin, 11-β-acetoxygedunin, andirobin, 7-deacetoxy-7-oxogedunin (Connolly *et al.*, 1966), gedunin (Akisanya *et al.*, 1961) 6-α-acetoxy-epoxyazadiradione (Mulholland *et al.*, 1994), methyl angolensate (Bevan *et al.*, 1967), 1,3-di-benzene carbon amine-2-octadecylic acidglyceride, hexacosanoic acid-2,3-dihydroxy-glyceride, ursolic acid, naringenin, scopoletin, 3,4-dihydroxymethylbenzoate,2,6-dihydroxymethylbenzoate, tetratriacontanoic acid, triacontanoic acid (Qi *et al.*, 2004), epoxyazadiradione, 6-α-hydroxygedunin (Lavie *et al.*, 1972), 1,2-dihydro-3β-hydroxy-7-deacetoxy-7-oxogedunin, xyloccensin k (Ambrozin *et al.*, 2006).	*Candles* (Gilbert *et al.*, 1999). Discrete repellent effect against bites of *Aedes* sp., Miot (2004) and significant insecticide activity when used on *Aedes aegypti* (Mendonça *et al.*, 2005). Antiinflammatory, antiallergic and anti-hyperalgesic effects of natural tetranortriterpenoids (Penido *et al.*, 2005; Penido *et al.*, 2006). *C. guianensis* seed oil did not cause toxic effects on pregnancy of Wistar rats. However the increases in the ALT serum level and weight of the liver may indicate a possible hepatic toxicity (Costa-Silva *et al.*, 2007; Costa-Silva *et al.*, 2008).
		Miristic, palmitic, oleic, linoleic (Pinto, 1956; Teske and Trentini, 1997; Costa-Silva *et al.*, 2007), stearic and arachidic acids (Costa-Silva *et al.*, 2007).	
	Leaves	Bicyclogermacrene, germacrene D, α-humulene, β-caryophyllene (main) (Andrade *et al.*, 2001).	
	Flowers	Germacrene B, germacrene D, α-humulene, β-caryophyllene (main) (Andrade *et al.*, 2001).	
	Twigs	Photogedunin, ursolic acid (Qi *et al.*, 2004).	
		(-)-epicatechin-3-O-(3",5"-di-O-methyl) gallate, (-)-catechin, sciadopitysin, cleomiscosin B, chisocheton compound F, odoratone, 1,3-di-benzene carbon amine-2-octadecylic acid-glyceride, hexacosanoic acid-2,3-dihydroxy-glyceride, naringenin, scopoletin, 3,4-dihydroxymethylbenzoate, 2,6-dihydroxymethylbenzoate (Qi *et al.*, 2003; Qi *et al.*, 2004).	

Table 4. *Contd.*

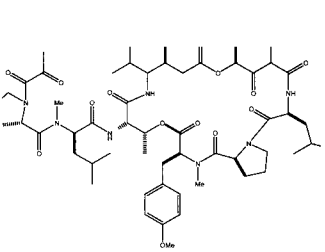
Species	Part of the Plant	Substances	Uses and Pharmacological activities
F. cordifolia	Seeds	Fevicordin A and its glucoside and gentiobioside; fevicordin B and its glucoside and gentiobioside; fevicordin C glucoside and gentiobioside; fevicordin D glucoside and gentiobioside; fevicordin E glucoside and gentiobioside; fevicordin F gentiobioside (Achenbach *et al.*, 1987; Achenbach *et al.*, 1993). cordifolin A and its glycoside;cordifolins B and C (Johnson *et al.*, 1989; Johnson *et al.*, 1991).	In the carrageenan-induced rat paw edema test, fevicordin A and its glucoside exhibited a cortisone-like anti-inflammatory activity even when administered in low doses. For fevicordin A, the anti-inflammatory effect observed was proportional to the dose applied (10, 5 and 1 mg/kg inhibitions of 96, 52 and 8.5%); in the animal test system the toxic dose for fevicordin A glucoside was found to be close to an effective therapeutic dose (50% to 10 mg/kg) (Achenbach *et al.*, 1993).
		Lauric, margarinic, palmitic, oleic, linoleic, linolenic, stearic, arachidic, α-eleostearic and pinicic acids (Achenbach *et al.*, 1992; Gentry & Wettach, 1986).	Tsuzuki *et al.* (2004) showed suppression tumor growth by α-eleostearic acid, via lipid peroxidation.

products guarantee income increase, life quality enhancement and maintenance of the natural resources for several generations, which means it is an economically-sustainable, ecologically-correct and socially-fair

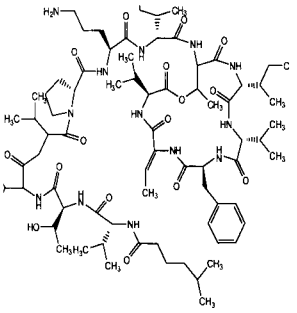
MAIN CHEMICAL STRUCTURES



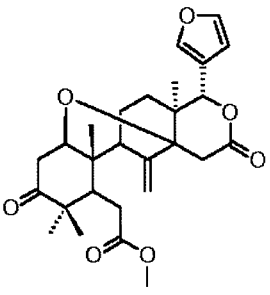
6α-acetoxygedunin



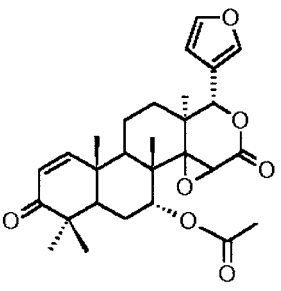
7-deacetoxy-7-oxogedunin



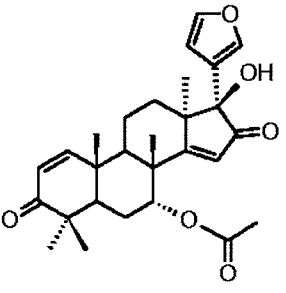
andirobin



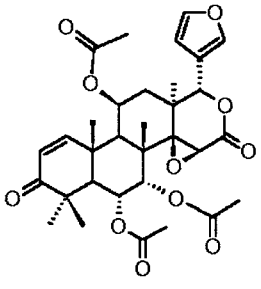
methyl angolensate



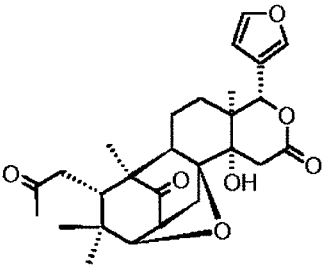
gedunin



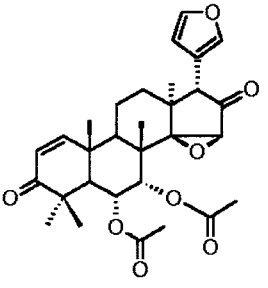
17β-hydroxyazadiradione



6α, 11β-diacetoxygedumin

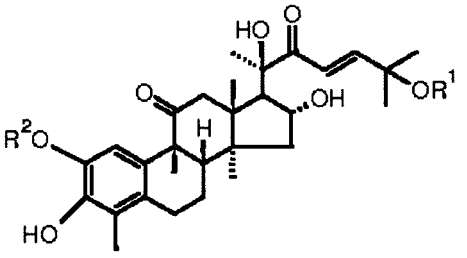


Xyloccensin k



6α-acetoxyepoxyazadiradione

Fig 8. Main chemical structures of *C. guianensis*



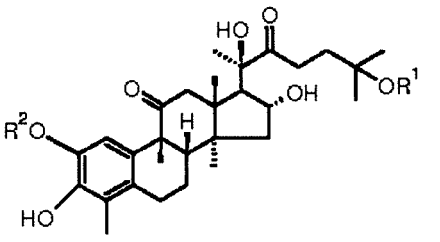
Fevicordin A glucoside R^1 = Ac, R^2 = β-D-glycopyranosil

Fevicordin R^1 = Ac, R^2 = H

Fevicordin C glucoside R^1 = H, R^2 = β-D-glycopyranosil

Fevicordin C gentiobioside R^1 = Ac, R^2 = β-D-gentiobiosyl

Fevicordin C gentiobioside R^1 = H, R^2 = β-D-gentiobiosyl



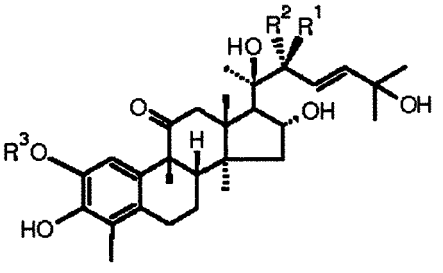
Fevicordin B glucoside R^1 = Ac, R^2 = β-D-glucopyranosil

Fevicordin B R^1 = Ac, R^2 = H

Fevicordin D glucoside R^1 = H, R^2 = β-D-glucopyranosil

Fevicordin B gentiobioside R^1 = Ac, R^2 = β-D-gentiobiosyl

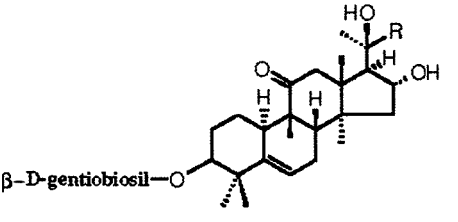
Fevicordin D gentiobioside R^1 = H, R^2 = β-D-gentiobiosyl



Fevicordin E glucoside R^1 = R^2 = H, R^3 = β-D-glucopyranosil

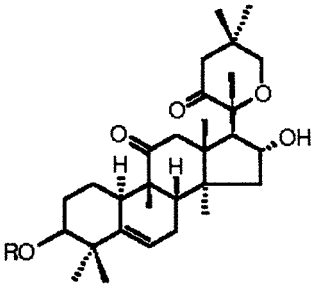
Fevicordin E gentiobioside R^1 = R^2 H, R^3 = β-D-gentiobiosyl

Fevicordin F gentiobioside R^1 = OH, R^2 = H, R^3 = β-D-gentiobiosyl



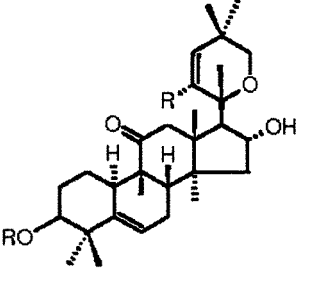
Cucurbitacin U gentiobioside R =

Cucurbitacin V gentiobioside R =



Cordifolin A R = H

Cordifolin A gentiobioside R = β-D-gentiobiosyl



Cordifolin B R = OH

Cordifolin C R = H

Fig 9. Main chemical structures of *F. cordifolia*

activity. Yet, success shall only be efficiently attained with the products from a managed forest, whose producers organize themselves and/or search for management advice (Amaral & Neto, 2002; Oliveira, 2008).

According to Ribeiro and collaborators (2005), the Community-Based Forest Management means to know how to work in the forest without harming it, with the objective to deal with all resources that nature provides in a rational way, respecting the fauna and flora, avoiding the irresponsible deforestation, which often provokes pasture formation and forest destruction.

For the licensing of Non-Timber Forest Products Management – NTFPM, it was enacted the Institutional Act no. 001 of August 12, 2004, in force in the State of Acre, which establishes the simplified administrative procedure for the economic exploration of non-timber forest products that does not consider individuals suppression in the Conservation Units of Sustainable Use, in the indigenous lands, in rural properties and in areas of legitimate owners of rural lands of up to 500 ha in the State of Acre, including the legal reserve areas, except for the vacant areas of the Country, State and City. The Act covers only the Non-Timber Forest Products – NTFP, whose extraction and collection do not promote the suppression (death) of the matrices. Considering the concepts and stated definitions, it might be added that the management of non-timber species has been strengthened in Amazonia in the last years, being that the management of *C. guianensis* (andiroba) carried out in agroextractive communities and in legal reserves has been developed since long ago when compared to *F. cordifolia* (andiroba de rama) and, then, showing more results.

As a management way, it is important to note that *C. guianensis* has also potential for the agroforest systems and for the enhancement of secondary forest areas (Shanley & Medina, 2005), as it produces excellent timber and vegetal oil. Germination begins after 6 days and ends in 2 to 3 months with 85% to 90% germination rates. Growth is fast, even in degraded areas, either sunny or shadowy. Although there exist more species in the lowlands, it can also be planted in uplands; nevertheless more studies on adequate spacing and shade are deemed necessary. In some places, the plant begins its development in the shade, notwithstanding the sun light is important for its fast growth (Shanley & Medina, 2005). The commercialization of the vegetal oil of *C. guianensis* (Cg) is very common at the city markets of the State of Acre, which, sometimes, comes from the communities located in the State of Amazonas and bordering the State of Acre, where there is a larger concentration of trees of this species. Several communities are engaged in a craftwork on the vegetal oil extraction without using presses, for example, the riverine families at the Esperança stream, in Tarauacá city, in the State of Acre, extract the oil from the andiroba (Cg) seeds, using it for self-consumption and for commercialization in the local city. The partnership formed among the government of the State of Acre, the Organization of Yawanawá Extractivist Farmers of the Gregório River

[2] Cosmetic products company

- OYEFGR and AVEDA[2] was established aiming to build up a plant for andiroba oil processing and improvement in Tarauacá city, which set, consequently, the beginning of the andiroba management process. The Andiroba Project implementation presented important results, which proved, *a priori*, to be an ecologically-sustainable alternative for the Yawanawá community, especially due to the insertion of non-indigenous people in the collection and processing activities. In this scenario, the extractivists and riverbank dwellers, who lived on their own, had a new life perspective with the implementation of this project. In other states in the Northern region, like Pará and Amazonas, there are several areas where the *C. guianensis* management process is already deployed or being deployed. In this context, it is worth mentioning that in Amazonas, in 2000, more precisely, in the Tapajós National Forest, there were initiated the deployment activities of the Pilot Project of Forest Management of the andiroba and copaiba vegetal oil of Intercommunity Association of Mini and Small Producers along Piquiatuba - Revolta, Tapajós River Right-Bank, where the Multi-Use Sustainable Forest Management is being developed, mainly, using the vegetal oil from andiroba and copaiba. The preliminary management study is divided in topics: information from the management area, characterization of the physical, biological and socio-economic environment, forest management (ecology and management, management location, methodology with GPS mapping, production batch separation, measurement and evaluations, production potential estimates), silvicultural treatment, crops annual plan. In 2003, the Brazilian Institute for the Environment and Renewable Natural Resources - IBAMA, through a legal act, approved the multi-use management plan. These activities go on being carried out in a sustainable way (Macedo *et al.*, 2000; Amaral & Neto, 2002).

Another model, which is in progress since 1997, is located in the Southwest Pará and considers the community of the "Praialta Piranheira Agroextractivist Settlement Project", in Nova Ipixuna city. This settlement develops joint activities with the Socio-Agronomic Laboratory of Tocantins of the Federal University of Pará - LASAT/ Federal University of Pará for the deployment of the Community-Based Management, where *C. guianensis*, an abundant species in this area, is inserted (Menezes, 2002).

In 2007 the communitarians together with the research agents – LASAT/Federal Univerty of Pará action – implemented the community management of andiroba, taking into consideration the following phases: activities discussion and planning, inventory activities (used to get the quantitative data referring to andiroba occurrence), planning of use and commercialization, extraction method and enhancement, works on the awareness of the species environmental conservation. The work on environmental education led to the publication of the leaflet: "Conhecendo os caminhos da Andiroba" (Knowing Andiroba's Path), elaborated by the children and teachers of the Municipal Schools: Chico Mendes and Boa

Esperança. The andiroba management construction in the Agroextractivist Settlement Project was consolidated, beginning with the union of community members (farmers) and the agents of the research-action (researchers) (Oliveira, 2008; Santos *et al.*, 2005).

As to the species *Fevillea cordifolia* (andiroba de rama), its identification and mapping in the São Salvador Sustainable Development Project, Mâncio Lima city, in the State of Acre, refer to it as a species for potential consideration in the proposal for the management plan of the area. This species is a liana with a short life cycle, about 2 to 3 years and, as already known, may be inserted in agroforest systems and/or for secondary forests enhancement, thanks to its relatively fast growth. In the State of Acre, it was verified that this species occurs typically along the rivers and mainly in marshes with frequent flooding. According to Gentry & Wettach (1986), the fruits and seeds of the *F. cordifolia* are dispersed as they float mainly in rivers water. These authors report this dispersion form of the *F. cordifolia* in Amazonian Peru where there exists seasonal flooding, being that germination occurs in the rivers water, and is less feasible in the sea water. The species *F. cordifolia,* in the State of Acre, appears in the list of the species in the preliminary version of the Annual Operational Plan (APO) being forecasted to be included in the Multi-Use Management Plan of the São Salvador Sustainable Development Project, in Mâncio Lima city in Acre.

Thus, it may be concluded that the stated initiatives demonstrate that in the Northern region there exist interest and determination for the management of non-timber species, among which *C. guianensis* species has a multi-use and community-based plan in progress and quite functional. Thanks to its easy breeding, *F. cordifolia* already appears in the list of managed species, mainly in several communities where its occurrence was already confirmed, guaranteeing sustainability mechanisms, generating life quality enhancement for the people of Amazonia as well as promoting the local ecosystems conservation.

DEVELOPMENT OF PRODUCTS

All along the years the traditional communities in Amazonia have been engaged in the craftwork on the oil of the species *C. guianensis* and *F. cordifolia.* These oils are used in the production of laundry soaps, toilet soaps, repellents and several other products for personal use and for commercialization, thus adding value to the forestry material in a sustainable way. In 2003, "Fundação de Tecnologia do Estado do Acre" (FUNTAC) participated and helped to carry out five participatory workshops addressed to different types of communitarians: riverside dwellers, indigenous people, rubber tappers, dwellers of the forest and its surroundings.

During these workshops there arose the interest in the development and production of laundry soaps, toilet soaps, body lotions, triphasic oils,

creams, shampoos, gels, ointments among other products using forestry oils and vegetable extracts (Fig 10), with quality and good manufacturing practices as it is the case of a gel against concussions with *C. guianensis* oil, which is of great interest of several communities.

A relevant point to mention is the fact that the communitarian that participated in the workshop from its beginning turns to be a facilitator in different areas, multiplying and transmitting the acquired knowledge. In this sense, the repercussion of these actions could be evidenced when communitarians from the State of Acre met others from other States in a Participatory Workshop in the Agroextractivist Settlement Project in Novo Ipixuna city. Ten products containing andiroba oil, a raw material abounding in the State of Pará, were developed in this workshop.

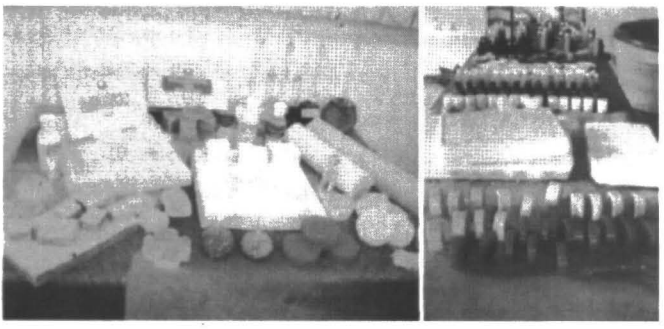


Fig 10. Products elaborated with *C. guianensis* and *F. cordifolia* oils

For years, the oil from the seeds of *F. cordifolia* (andiroba de rama) has been traditionally used by local people in the State of Acre, albeit there are limited scientific studies of this species. The riverside communities and the indigenous people extract this oil from the seeds in a craft way, using the method applied for extracting the oil from the andiroba seeds (*C. guianensis*), and use it to manufacture laundry and toilet soaps. Scientific studies made at the Natural Product Laboratory (NPL) of FUNTAC confirm the high saponification property of this oil, qualifying it as capable of intense foam formation with better quality. The products developed in the NPL, after related studies, are passed on to the community, as it happened with the andiroba-based repellent that is used by local people and by researchers during forest expeditions.

The utilization of oils from the seeds of *C. guianensis* and *F. cordifolia* are of great interest of the communitarians of the State of Acre and others. The elaboration of craft products using vegetable oils meets the demand for the improvement of life quality of the communities of the Brazilian Amazon region, specially the one in the State of Acre, as it facilitates the access to natural resources for personal hygiene. Additionally, there occurred changes in the income of these families, for instance, the ones that control

Xapuri soap factory increased their income with the production and commercialization of soaps, mainly after enhancing the production techniques with the sustainable utilization of the forest resources by means of the commercialization with the extractivists groups that became conscious of the importance of the controlled extraction. In this context, the minimization of the environmental impacts induced by the inappropriate land use and the added value to forest products are essential to insure the continuity of the success of these activities.

Production and Marketing

In this millennium there appears a new meaning for the use of traditional natural products – from popular consumer products to refined products with the appeal of being ecologically and socially-sustainable. Andiroba vegetal oil perfectly illustrates this tendency as, after being widely used by the laundry bar soap industry, in the 40's, it became the raw material for the "boundaries" products launched by renowned national and international cosmetics industries (Enríquez *et al.*, 2003). In this context the Amazon community has been answering an increasing demand for the collection of seeds of *C. guianensis* for the production of oil by the industries that commercialize the enhanced vegetable material to the cosmetics companies. The added value to natural products is the best way to increase and hold income in the region where they are processed. Additionally, the government investments made in the State of Acre aim to increase the quality control of some forest products in order to get the certification for the local products, thus increasing the income of the communitarians and helping to attain an outstanding position for the material from the State.

In the 20th century, from the 30's to the 80's, a lot has already been heard about the production and commercialization of the andiroba oil in the State of Amazonas. In those years production estimates came to 3 to 4 tons of oil per year, corresponding to the collection of 90 to 120 tons of seeds (Salgado, 2000). In the State of Pará, in the 40's, it was verified a large production of andiroba oil, which was used as raw material in the local laundry bar soap industry. In the State of Acre there have always happened the production and commercialization of the oil of *C. guianensis* in an informal way, just for using as home-made medicine and production of toilet and laundry soaps, yet never in large scale. Among the craft-producers of phytocosmetics and phytotherapics, the species *F. cordifolia* has been recently integrated in the local commercialization. In the search for alternative energy sources, the oil of this species is being studied for biodiesel production, mainly due to its breeding short cycle. (Souza *et al.*, 2006).

As a trend in Amazonia, partnerships are formed with large companies and industries that use the forest resources in different segments like: cosmetics, pharmaceuticals, food and automotive. The enterprises that do business locally vary in size and origin. The cosmetics industry is the leader,

due to the widespread use of materials from natural sources, vegetable products and built-in social responsibilities practices (Enríquez, 2001). In the State of Acre, in Juruá region, there are 2 so-called success examples: one is Tawaya, a Brazilian company that manufactures artisan soaps, using several species from Amazonia, including *C. guianensis*-based soaps, and exports them to Germany (Morsello, 2006). The other is the plant for andiroba oil extraction and improvement in Tarauacá city (Acre), resulting from the partnership formed among the government of the State of Acre, the Yawanawá Community and AVEDA3.

Important to stress that in the case of these two quoted species, specially as per *C. guianensis,* the production and commercialization process, which is carried out by the communitarians that extract the oil in most of the Northern states, begin with the seeds collection, going through the resale of oil to intermediaries and seeds to industries, and, finally, with the commercialization of oil and byproducts. Thus, the great challenge is to consolidate the market, adding value to products so as the productive communities have a better financial return. Besides, only the valorization of the extractive collection of seeds may turn it economically feasible a non-predatory exploration of andiroba (*C. guianensis*), towards the possibility of the resource impoverishment resulting from lumber exploration. The oil production seems to be an interesting perspective for the small-scale producer, since there exists a potential market (Salgado, 2000). It is, then, also urgent and necessary to have new investments in market studies to ensure the acquisition of these products. This market growth is associated to a greater sensibility of people as to natural products and, specially, the Amazon's. Notwithstanding the essential oils are the raw materials for the cosmetics industry since ancient times, the new techniques in bioprospection are allowing the production costs to drop. It is also worth mentioning the cosmetics industries that use natural products, either from the forest or from the indigenous tradition, as a marketing resource, as it happens with "Natura Ekos Line" and the British company "The Body Shop" (Enríquez, 2001). Other enterprises in the Northern Region stand out with investments in phytocosmetics, as it is the case of "Chamma da Amazônia", a typical company from the State of Pará that participates in the benefiting chain from Amazon biodiversity in the case of andiroba oil and high quality finished products, with an average annual revenue about R$ 2 milhões (Homma, 2001). "Brasmazon", located in Belém (State of Pará), is another company that was created in 1995 and is considered the leading Brazilian exporter of Amazon oils *in natura*. In 2001 it produced 80 tons of andiroba oil, besides developing innovating products as the andiroba oil powder (Enríquez, 2001). After the partnership formed with Beraca Ingredients, its growth was accelerated, reaching 90% market share of the Amazon natural oils, then becoming the supplier for the French Group Yves Rocher and for the Brazilian Natura. The agreement signed with Yves Rocher, as an example, sets a year remittance of 30 tons of

andiroba oil (Homma, 2001). The national cosmetics industry is contributing for reviewing a widespread idea in Amazonia: producers earn nothing to keep the forest alive. This review follows the increment of the beauty products lines made from Amazon herbs, and benefits the communities in the region. The new discoveries for the utilization of Amazon forest species are being spread worldwide by means of improvements and chemical modifications, obtaining oil derivatives, seeds, extracts, natural dyes, among others. There remains the search and expectation to find a tripod that perfectly defines how the relation enterprise/productive community must work: a socially-fair, ecologically-correct and economically-feasible business.

ACKNOWLEDGEMENTS

The authors are grateful to FDCT/CNPq, FUNTAC, FIOCRUZ for financial support of this work.

REFERENCES

Achenbach, H., Hefter-Buebl, U. and Constenla, M.A. (1987). Fevicordin A and fevicordin A glucoside, novel norcucurbitacins from *Fevillea cordifolia*. *Journal of the Chemical Society, Chemical Communications,* **6**: 441-442.

Achenbach, H., Hefter-Buebl, U., Waibel, R., Constenla, M.A. and Hahn, H. (1992). Gaschromatographische Analyse des Samenfettes der mittelamerikanischen *Fevillea cordifolia* (Cucurbitaceae). *Fat Science Technology*, **94**: 294-297.

Achenbach, H., Waibel, R., Hefter-Buebl, U. and Constenla, M.A. (1993). Constituents of *Fevilllea cordifolia:* new norcucurbitacin and cucurbitacin glycosides. *Journal of Natural Products*, **56**: 1506-1519.

Akisanya, A., Bevan, C.W.L., Halsall, T.G., Powell, J. and Taylor, D.A. (1961). West African timber. Part IV. Some reactions of gedinin. *Journal of the Chemical Society,* pp. 3705-3708.

Amaral, A.C.F., Simões, E.V. and Ferreira, J.L.P. (2005). Coletânea Científica de Plantas de Uso Medicinal. 1ª edição, Rio de Janeiro – Fiocruz.

Amaral, A.C.F., Basso, S.L., Ferreira, J.L.P., Simas, N.K., Simões, E.V. and Silva, J.R.A. (2006). Avaliação Microscópica das Lâminas Foliares de *Carapa guianensis* e *Fevillea cordifolia*. *LVIII Congresso Brasileiro de Botânica* – Gramado – Rio Grande do Sul.

Amaral, P. and Neto, M.A. (2002). Manejo Florestal Comunitário na Amazônia Brasileira: Situação Atual, Desafios e Perpectivas. Instituto Internacional de Educação do Brasil-IIEB, Brasília.

Ambrozin, A.R.P., Leite, A.C., Bueno, F.C., Vieira, P.C., Fernandes, J.B., Bueno, O.C., Silva, M.F.F., Pagnocca, F.C., Hebling, M.J.A. and Bacci Jr, M. (2006). Limonoids from Andiroba oil and *Cedrela fissilis* and their insecticidal activity. *Journal of the Brazilian Chemical Society,* **17**: 542-547.

Andrade, E.H., Zoghbi, M.G. and Maia, J.G. (2001). Volatiles from the leaves and flowers of *Carapa guianensis* Aubl. *Journal of Essential Oil Research*, **13**: 436-438.

Balick, M.J., Kronenberg, F., Ososki, A.L., Reiff, M., Fugh-Berman, A., O'Connor, B., Roble, M., Lohr, P. and Atha, D. (2000). Medicinal plants used by latino healers for women's health conditions in New York city. *Economic Botany*, **54**: 344-357.

Basso, S.L., Amaral, A.C.F., Ferreira, J.L.P., Simas, N.K. and Silva, J.R.A. (2006). Comparação Cromatográfica do Óleo das Sementes de Andiroba de Rama no XIX Simpósio de Plantas Medicinais do Brasil, Salvador – Bahia.

Bevan, C.W.L., Powell, J.W., Taylor, D.A.H., Halsall, T.G., Toft, P. and Welford, W. (1967). The structure of methyl angolensate. *Journal of Chemical Society* (C), pp. 163-170.

Branch, L.C. and Silva, M.F. (1983). Folk medicine of Alter do Chão, Pará, Brazil. *Acta Amazônica*, **13**: 737-797.

Coe, F.G. and Anderson, G.J. (2005). Snakebite ethnopharmacopoeia of eastern Nicaragua. *Journal of Ethnopharmacology*, **96**: 303-323.

Coe, F.G. (2008). Ethnobotany of the Rama of southeastern nicaragua and comparisons with miskitu plant lore. *Economic Botany*, **62**: 40-59.

Connolly, J.D., McCrindle, R., Overton, K.H. and Feney, J. (1966). Tetranortriterpenoids – II. Heartwood constituents of *Carapa guianensis* Aubl. *Tetrahedron*, **22**: 891-896.

Costa-Silva, J.H., Lyra, M.M.A., Lima, C.R., Arruda, V.M., Araújo, A.V., Ribeiro, A.R., Arruda, A.C., Fraga, M.C.C.A., Lafayette, S.S.L. and Wanderley, A.G. (2007). A toxicological evaluation of the effect of *Carapa guianensis* Aublet on pregnancy in Wistar rats. *Journal of Ethnopharmacology*, **112**: 122-126.

Costa-Silva, J.H., Lima, C.R., Silva, E.J.R., Araújo, A.V., Fraga, M.C.C.A., Ribeiro, A.R., Arruda, A.C., Lafayette, S.S.L. and Wanderley, A.G. (2008). Acute and subacute toxicity of the *Carapa guianensis* Aublet (Meliaceae) seed oil. *Journal of Ethnopharmacology*, **116**: 495-500.

Duke, J.A. and Vasquez, R. (1994). Amazonian Ethnobotanical Dictionary. CRC Press, Boca Raton, Florida.

Eloy, L. (2001). Valorização da produção dos óleos fixo no Bailique, Mazagão e Maruanum. Amapá, Brasil.

Enríquez, G. (2001). A Trajetória Tecnológica dos Produtos Naturais e Biotecnológicos Derivados na Amazônia. Belém: NUMA/UFPA.

Enríquez, G., Silva, M.A. and Cabral, E. (2003). Biodiversidade da Amazônia: usos e potencialidades dos mais importantes produtos naturais do Pará. Belém: NUMA/UFPA.

Galy, S., Rengifo, E. and Hay, Y.O. (2000). Factores De La Organización Del Mercado De Las Plantas Medicinales En Iquitos - Amazonía Peruana. *Folia Amazónica*, **11**: 139-158.

Gentry, A.H. and Wettach, R.H. (1986). *Fevillea* – A new oil seed from Amazonian Peru. *Economic Botany*, **40**: 177-185.

Gilbert, B., Teixeira, D.F., Carvalho, E.S., De Paula, A.E.S., Pereira, J.F.G., Ferreira, J.L.P., Almeida, M.B.S., Machado, R.S. and Cascon, V. (1999). Activities of the Pharmaceutical Technology Institute of the Oswaldo Cruz Foundation with medicinal, insecticidal and insect repellent plants. *Anais da Academia Brasileira de Ciências*, **71**: 265-271.

Instituto Adolfo Lutz. (1985). Normas analíticas do Instituto Adolfo Lutz. São Paulo-IMESP.

Hammer, M.L.A. and Johns, E.A. (1993). Tapping an Amazon plethora: four medicinal plants of Marajó island, Pará (Brazil). *Journal of Ethnopharmacology*, **40**: 53-75.

Homma, A.K.O. (2001). O desenvolvimento da agroindústria no Estado do Pará. *Ciências Exatas e Tecnologia*, **3**: 47-76.

Johnson, L.B.N., Reese, P.B., Roberts, E.V., Lam, L.K.P. and Vederas, J.C. (1989). Structure elucidation of cordifolin A, a novel cucurbitacin from *Fevillea cordifolia*, using one and two dimensional NMR techniques. *Journal of the Chemical Society Perkin Transactions* **1**: 2111-2116.

Johnson, L.B.N., Griffiths, W.J., Roberts, E.V., Lam, L.K.P., Vederas, J.C., Reid, C.J. and Ballantine, J.A. (1991). A mass spectral analysis of some cucurbitacins isolated from *Fevillea cordifolia*. *Journal of the Chemical Society Perkin Transactions* **1**: 2583-2588.

Klimas, C.A., Kainer, K.A. and Wadt, L.H.O. (2007). "Population structure of *Carapa guianensis* in two florest types in the southwestern Brasilian Amazon. *Florest Ecology and management*, **250**: 256-265.

Knothe, G. (2005). Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. *Fuel Process Tech.*, **86**: 1059-1070.

Lavie, D., Levy, E.C. and Zelnik, R. (1972). The constituents of *Carapa guianensis* Aubl. and their biogenetic relationship. *Bioorganic Chemistry*, **2**: 59-64.

Macedo, D. (2000). Projeto Piloto de Manejo Florestal Sustentável de óleo de Andiroba e Copaíba - Projeto de Apoio ao Manejo Florestal Sustentável na Amazônia – Promanejo-Floresta Nacional Tapajós. Associação Intercomunitária de Mini e Pequenos Produtores Rurais da Margem Direita do Tapajós de Piquiatuba a Revolta – ASMIPRUT. Santarém/PA.

Mendonça, F.A.C., Silva, K.F.S., Santos, K.K., Ribeiro Júnior, K.A.L. and Sant'Ana, A.E.G. (2005). Activities of some Brazilian plants against larvae of the mosquito *Aedes aegypti*. *Fitoterapia*, **76**: 629-636.

Menezes, A.J.E.A. (2002). Análise econômica da "produção invisível" nos estabelecimentos agrícolas familiares no projeto de assentamento agroextrativista Praialta e Piranheira, município de nova Ipixuna, Pará. Dissertação de Mestrado. Universidade Federal do Pará, Pará.

Miot, H.A., Batistella, R.F., Batista, K.A., Volpato, D.E.C., Augusto, L.S.T., Madeira, N.G., Haddad Jr., V. and Miot, L.D.B. (2004). Comparative study of the topical effectiveness of the andiroba oil (*Carapa guianensis*) and Deet 50% as repellent for *Aedes* sp. *Revista do Instituto de Medicina Tropical de São Paulo*, **46**: 253-256.

Mitchell, S.A. and Ahmad, M.H. (2006). A review of medicinal plant research at the University of the West Indies, Jamaica, 1948-2001. *West Indian Med J.*, **55**: 243-269.

Morsello, C. (2006). Company community non-timber forest product deals in the Brazilian Amazon: A review of opportunities and problems. *Forest Policy and Economics*, **8**: 485-494.

Mulholland, D.A., Osborne, R., Roberts, S.L. and Taylor, D.A.H. (1994). Limonoids and triterpenoid acids from the bark of *Entandrophragma delevoyi*. *Phytochemistry*, **37**: 1417-1420.

Oliveira, M.G. (2008). "Manejo Florestal comunitário da Andiroba - *Carapa guianensis*, Aublet": Integrando o conhecimento local à Pesquisa-ação no sudeste do Pará. TCC de conclusão da faculdade de agronomia. UFPA.

Orellana, B.J.P., Kobayashi, E.S. and Lourenço, G.M. (2004). Terapia alternativa através do uso da andiroba. *Lato & Sensu*, **5**: 136-141.

Penido, C., Costa, K.A., Pennaforte, R.J., Costa, M.F.S., Pereira, J.F.G., Siani, A.C. and Henriques, M.G.M.O. (2005). Anti-allergic effects of natural tetranortriterpenoids isolated from *Carapa guianensis* Aublet on allergeninduced vascular permeability and hyperalgesia. *Inflammation Research*, **54**: 295-303.

Penido, C., Conte, F.P., Chagas, M.S.S., Rodrigues, C.A.B., Pereira, J.F.G. and Henriques, M.G.M.O. (2006). Antiinflammatory effects of natural tetranortriterpenoids isolated from *Carapa guianensis* Aublet on zymosan-induced arthritis in mice. *Inflammation Research*, **55**: 457-464.

Pennington, T.D., Styles, B.D. and Taylor, D.A.H. (1981). Meliaceae. *Flora Neotropica Monograph*, **28**: 235-244.

Pinto, G.P. (1956). Contribuicão ao estudo químico do óleo de Andiroba. *Boletim Técnico do Instituto Agronômico do Norte*, **31**: 195-206.

Pinto, A.C., Guarieiro, L.L.N., Rezende, M.J.C., Ribeiro, N.M., Torres, E.A., Lopes, W.A., Pereira, P.A.P. and Andrade, J.B. (2005). Biodiesel: An Overview. *Journal of the Brazilian Chemical Society*, **16**: 1313-1330.

Pio Corrêa, M. (1931). Dicionário de plantas úteis do Brasil e das exóticas cultivadas. Rio de Janeiro: Imprensa Nacional, v. 4.

Prates-Clark, C.C., Saatchi, S.S. and Agosti, D. (2008). Predicting geographical distribution models of high-value timber trees in the Amazon Basin using remotely sensed data. *Ecological Modelling*, **211**: 309-323.

Qi, S.H., Wu, D.G., Ma, Y.B. and Luo, X.D. (2003). A novel flavane from *Carapa guianensis*. *Acta Botanica Sinica*, **45**: 1129-1133.

Qi, S.H., Wu, D.G., Zhang, S. and Luo, X.D. (2004). Constituents of *Carapa guianensis* Aubl. (Meliaceae). *Pharmazie*, **59**: 488-490.

Revilla, J. (2000). Plantas da Amazônia: oportunidades econômicas e sustentáveis. Manaus: Programa de Desenvolvimento Empresarial e Tecnológico.

Ribeiro, B.F., Araújo, C.S. and Nachtergaele, M.F. (2005). Manejo florestal comunitário, conhecendo e aprendendo para conviver com a floresta. Execução Grupo de Apoio a Agricultura Familiar de Região de fronteiras – GRAAL/LASAT. Marabá-Pará.

Robinson, G.L. and Wunderlin, R.P. (2005). Revision of *Fevillea* (Cucurbitaceae: Zanonieae). *SIDA*, **21**: 1971-1996.

Salgado, L. (2000). A Floresta em jogo: o extrativismo na Amazônia Central. São Paulo: UNESP.

Sampaio, P. de T.B. (2000). Andiroba (*Carapa guianensis*). *In*: Biodiversidade Amazônica: exemplos e estratégias de utilização. *Ed.* Clay, J.W., Sampaio, P. de T.B. and Clement, C. R. Manaus: Programa de Desenvolvimento Empresarial e Tecnológico, Brasil, pp. 243-251.

Santos, C.S., Sampaio, L.S., Nachtergaele, M.F. and Oliveira, M.G. (2005). Conhecendo os caminhos da andiroba. Execução Grupo de Apoio a Agricultura Familiar de Região de fronteiras – GRAAL/LASAT. Marabá-Pará.

Shanley, P. and Medina, G. (2005). Frutíferas e Plantas Úteis na Vida Amazônica. Belém : CIFOR, Imazon.

Silva, J.N.M. (2001). Manejo Florestal. EMBRAPA Amazona Oriental (Belém - Pará) Brasília.

Souza, T.M.H., Assis, L.B., Regiani, A.M., Graebner, I.B., Sartori, R.A., Pantoja, N.V., Albuquerque, S.R.S. and Ferreira, E.J.L. (2006). Estudo do potencial do óleo de andiroba-de-rama (*Fevillea cordifolia*) para produção de biodiesel através da reação de transesterificação. *29ª Reunião anual – SBQ*. Águas de Lindóia – São Paulo.

Teske, M. and Trentini, A.M.M. (1997). Herbarium: Compêndio de Fitoterapia. Herbarium Laboratório Botânico. Paraná.

Ticktin, T. and Dalle, S. (2005). Medicinal plant use in the practice of midwifery in rural Honduras. *Journal of Ethnopharmacology*, **96**: 233-248.

Tsuzuki, T., Tokuyama, Y., Igarashi, M., Nakagawa, K., Ohsaki, Y., Komai, M. and Miyazawa, T. (2004). α-Eleostearic Acid (9Z11E13E-18:3) is quickly converted to conjugated linoleic acid (9Z11E-18:2) in Rats. *The Journal of Nutrition*, **134**: 2634-2639.

Wagner, H., Bladt, S. and Zgainski, E.M. (1984). Plant Drug Analysis, Springer-Verlag.

Winners and Losers, (2003). *Final Technical Report*, FRP R7795, pp. 7.

"This page is Intentionally Left Blank"

5

Natural Bioresource of the North Himalayan Region as a Source of Promising Radiation Countermeasure Agents: Lessons from *Podophyllum hexandrum*

RAJESH ARORA[1*], A.S. DHAKER[1], J. SHARMA[1], M. ADHIKARI[1], D. GUPTA[1], R. CHAWLA[1], R. KUMAR[1], A. SHARMA[1], R.K. SHARMA[1], S. SULTANA[2], G.N. QAZI[3], P.S. AHUJA [4] AND R.P. TRIPATHI[1]

ABSTRACT

In recent years, natural bioresources are being viewed as a source of potential radiation countermeasure agents. Podophyllum hexandrum is an important medicinal plant species of the North Himalayan region that finds diverse applications in the traditional as well as modern systems of medicine. The plant has survived and evolved in harsh environments of the high altitude over several millions of years and synthesizes a number of secondary metabolites, mainly aryltetralin lignans, several of which are useful in view of their bioactive nature. The anticancer drugs viz., etoposide and teniposide are widely employed as a part of the chemotherapeutic regimen, are derived from the plant. Podophyllum has been used in the clinic for a variety of other medical purposes too. The first report on the radioprotective efficacy

1. Division of Radiation Biology and Radioprotection, Institute of Nuclear Medicine and Allied Sciences, Defence Research and Development Organization (DRDO), Brig. S.K. Mazumdar Marg, Delhi - 110 054, India.
2. Department of Medical Elementology and Toxicology, Jamia Hamdard, New Delhi - 110 062, India.
3. Indian Institute of Integrative Medicine (Formerly Regional Research Laboratory), Canal Road, Jammu Tawi - 180 001, India. Presently: Vice Chancellor, Jamia Hamdard, New Delhi - 110 062, India.
4. Institute of Himalayan Biosource Technology, Palampur - 176 061, Himachal Pradesh, India.

* *Corresponding author* : E-mail : rajesharoradr@rediffmail.com

of Podophyllum emanated from our group. This article discusses the immense potential of the plant being used as a radioprotector during planned and unplanned radiation exposures for alleviating radiation-mediated damage. The promise of this plant finding application as a multipurpose, multiscenario radiation countermeasure agent in coming years is high and hence the need to intensify research on this important plant, which has almost reached a state of extinction. In India, several such propositions are discussed in the chapter.

Key words : Bioresource, *Podophyllum hexandrum*, ionizing-radiation, free radicals, radioprotection, radiation countermeasure agent

INTRODUCTION

Nature has bestowed upon the Indian subcontinent several unique features. India is uniquely placed on the world map in such a position that a plethora of endemic flora and fauna have evolved in this region and several of the herbs that grow here have immense potential for diverse medical applications (Arora *et al.*, 2000, 2004a, b, 2006a, b, c, d, 2007b, 2008a, b, c; Sharma & Arora, 2006). India is one of the twelve mega biodiversity centers and is characterized by immense phytodiversity that has been tapped since ages for treating various human ailments in the Ayurvedic system of medicine. It is only in recent years that renewed interest has been exhibited. The Indian subcontinent is blessed with a climate that ranges from sub-zero temperatures in the North and North East to the extremely high temperatures of the West and humid and moist regions in other parts. Such unique features have resulted in the region becoming a biodiversity hotspot. Natural plant bioresource is an invaluable treasure and should be sustainably harvested to achieve pivotal leads in agriculture and medicinal plant cultivation. Phytobioresources exhibit dramatic diversity of type, number and distribution, which can be attributed mainly to the environment. The phytobioresource of the Himalayan region exhibits intricate intrinsic features and functions that have evolved through natural selection over a period of several million years. Amongst the whole lot of species of plants found in the Indian subcontinent, each is different and unique in its own way. There is an urgent need to judiciously utilize the phytobioresource, that can be considered as biological wealth, for the benefit of the humans. A number of plants are endemic to India and several of them have been utilized for various medicinal purposes.

In recent years, considerable interest has been generated to develop potential drugs of plant origin for the modification of radiation effects. Plants hold immense promise as radioprotectors, mitigators, recovery and therapeutic agents since the molecular drugs currently available have not yielded the desired results. Plant extracts such as *Allium sativum*, *Panax ginseng*, *Aloe vera*, *Ocimum sanctum*, *Spirulina*, chlorococcal algae

(Ivastimul) and *Mentha* etc. have been found to have radioprotective effects in various model systems, including mammals (see Arora *et al.*, 2005d; 2006a,b,c; Arora 2008; Vacek *et al.*, 1990). Plant products appear to have an advantage over the synthetic compounds in terms of low/no toxicity at the effective dose with minimum/no side effects. However, the use of medicinal plants in modern medicine suffers from the fact that though several plants are used in the world to prevent or to cure diseases, evidence-based scientific data are lacking in most cases. With this in mind, attempts have been made to obtain scientifically validated data–a glimpse of which is provided in this chapter.

An important medicinal plant that we considered worth investigating for biological activity is *Podophyllum hexandrum* (Himalayan Mayapple).

Podophyllum hexandrum Royle (Himalayan Mayapple): The Promising Radioprotective Herb

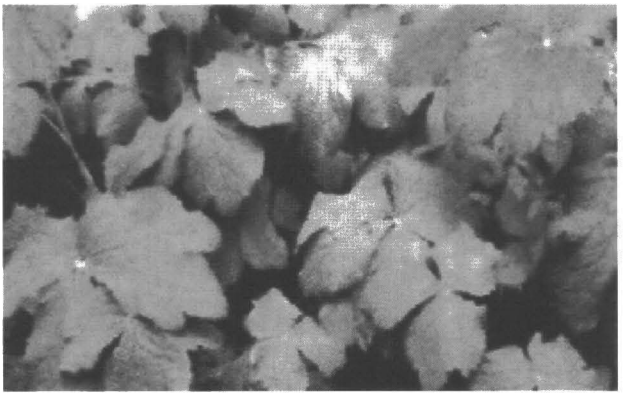


Fig 1. Plants of *Podophyllum hexandrum* growing in their natural habitat in the North Himalayan region.

Podophyllum hexandrum (Fig 1) has been widely used in both traditional and modern systems of medicine for a wide variety of medicinal uses. The plant was evaluated by our group for the first time for radioprotective efficacy and was found to mitigate the harmful effects of ionizing radiation (Arora & Goel, 2000; Arora *et al.*, 2005d, 2006b, c & Table 1). The radioprotective efficacy has been validated *in vitro*, *ex vivo* and *in vivo* and the mechanism of action has also been elucidated to a great extent (Gupta *et al.*, 2002a, b, 2003a, b, 2004; Chawla *et al.*, 2005a, 2006c; Kumar *et al.*, 2008).

Table 1. Radioprotective studies* carried out on Mayapple (*Podophyllum*)

Radioprotective activity	Extract (used)	Study
Iron-chelation	Aqueous extract	Extract exhibited chelation of Fe^{2+} more efficiently than Fe^{3+} and also modulated Fe^{2+}/Fe^{3+} ratio.
Anti-lipid peroxidation against radiation stress alone	Aqueous extract	Maximal inhibition (92%) reported at 1000 micrograms/ml concentration.
Gastroprotection	Aqueous extract	Increased the number of surviving crypts in the jejunum by a factor of 3.0 and villi cellularity by 2.7 ($p < 0.05$) fold in comparison to irradiated control attributed to ability to arrest cell division, which protects clonogenic cells against radiation.
Protection to reproductive system	RP-1	Prophylactic administration caused significant increase in the testis weight, repo-pulating tubules, resting primary spermatocytes, stem cell survival index, sperm counts and reduction inabnormalities of sperm morphology.
	RP-1	Countered radiation-induced decrease in the activity of glutathione peroxidase, Glutathione reductase and glutathione-S-transferase enzymes, enhanced protein content, protection to DNA and reducing lipid hydroperoxides and increased thiol content as compared to irradiated control in testicular system.
Protection from the radiation induced delay of postnatal appearance of reflexes and physiological markers	Rhizome extract	Intraperitoneal administration of extract mitigated radiation (2 Gy)- induced postnatal physiological alterations like pinna detachment, incisors and eruption etc.
Protection against neuronal damage *in utero*	Aqueous extract	Prophylactic treatment prevent the (2 Gy) radiation-induced significant reduction in dendritic branching and intersections of CA1 neurons of the hippocampal region of rats *in utero* (day-17 of gestation).

Table 1. *Contd.*

Radioprotective activity	Extract (used)	Study
Protection to hepatic cells (*in vitro* study)	RP-1	Decreased gamma radiation-induced leakage of electrons from electron transport chain and also significantly reduced both ROS and NO generation and enhanced glutathione levels, thereby inhibiting lipid peroxidation in Hep G2 cells.
Cytoprotection and Immuno-modulation	Aqueous extract	Significantly countered the radiation-induced loss of splenocyte proliferation. Irradiation decreased plasma antioxidant status. Extract augmented radiation-induced G2 delay and elicited significant recovery in S-phase fraction in bone marrow cells.
Modulation of gamma radiation-induced immunosuppression inBalb/c mice	RP-1	Enhanced macrophage survival; countered the decrease in CD4+ and CD8+ T cells populations and CGM-CFU and also countered radiation- induced decrease in the titre of IL-1, IL-3 and IgG's in the serum of mice indicating its immuno-stimulatory potential against radiation-induced oxidative stress.
Anti-inflammatory potential	Aqueous extract	Inhibited lipopolysaccharide (5 microg/ml)-induced nitrite generation to 37% and IFN-gamma secretion to 5%, IL-6 secretion to 50% and TNF-alpha secretion to 50% of LPS treated control values. This attributed to the 78% survival exhibited by the extract.
Tumorocidal activity in U87 cell lines	RP-1	Doses above 0.5 microg/ml reduced colonogenic survival (maximum reduction of 62% at 10 microg/ml) and increased the free radical generation, G2/M fraction and apoptotic frequency. Mitochondrial anti-apoptotic proteins Bcl-2 and HSP-70 levels were also reduced by RP-1. Such activities attributed to the cytotoxic behaviour of the extract at specific dose.

Table 1. *Contd.*

Radioprotective activity	Extract (used)	Study
Modulation of expression of proteins associated with apoptosis induced by radiation stress (*in vivo* study)	Aqueous extract	Studies at molecular level have revealed interesting data *e.g.*, anti-inflammatory activity [reduction of interferon-gamma, interleukin-6 and tumour necrosis factor-alpha secretion in lipopolysaccharide-induced inflammation in isolated macrophages]; [enhanced MAPKAP (mitogen-activated protein kinase-activated protein) kinase-2 activation along with HSF-1 (heat-shock transcription factor-1), leading to up-regulation of HSP-70 (heat-shock protein-70), with concomitant strong inhibition of AIF (apoptosis-inducing factor) expression, DNA degradation and translocation of free NF-κB (nuclear factor kappa beta) from cytoplasm to nucleus, leading to decreased expression of tumour suppressor protein p53 and simultaneous increase in Bcl-2 (B-cell chronic lymphocytic lymphoma 2), Ras-GAP (Ras-GTPase-activating protein) and PCNA (proliferating cell nuclear antigen), hence improved survival status).
Protection against radiation-induced damage to mitochondrial system (*in vivo* study)	Aqueous extract	Reduced the radiation-induced superoxide ion generation, formation of lipid peroxides and protein carbonyl there by increased the levels of glutathione within an hour; enhanced levels of glutathione-induced increase in complex I, complex I/III complex II/III activity. It significantly inhibited the radiation-induced decrease in mitochondrial membrane potential.
Antioxidant activity in aqueous phase (comparative analysis)	Five fractionated extracts [n-hexane (HE), chloroform	AE exhibited maximum antioxidant potential (lowest absorption unit value: 0.0389 +/- 0.00717) as compared to other fractions.
Anti-lipid peroxidation activity against combined stress (supra-lethal + iron/ascorbate system)	(CE), alcohol (AE), hydro-alcohol (HA) and water(WE)]	HA exhibited higher percentage of inhibition (93.05%) as compared to other fractions.
DNA protection (against supra-lethal radiation stress)		CE exhibited maximum protection to plasmid (pBR322) DNA in the plasmid relaxation assay (68.09% of SC form retention).

Table 1. *Contd.*

Radioprotective activity	Extract (used)	Study
Bioactivity-guided fractionation and isolation of novel flavonoid	Aquo-ethanolic(AEE) fractionated extract	AEE is protective against radiation, Fe^{2+} and Cu^{2+}-induced linoleic acid degradation, dose-dependant hydroxyl scavenging. Protein protection potential established. The novel 3-O-beta-D-galactoside of quercetin was isolated and characterized.
Identification and characterization of novel podophyllotoxin derivative	High-altitude *Podophyllum hexandrum*	Two epimers of a novel and minor constituent, podophyllotoxin-4-O-(D)-6-acetyl-glucopyraniside from high-altitude *Podophyllum hexandrum*. The method can be as employed as reference standards (marker) and is particularly useful in view of the scarcity of standards.
Anti-oxidant activities related to radiation protection	Low-altitude*Podophyllum hexandrum* (LAPH) extract	42.20% of ferrous chelation potential as compared to quercetin (34.9%), significantly higher anti-oxidant potential in aqueous phase (absorption unit value = 0.041+/-0.06), dose-dependent free radical scavenging potential and exhibits DNA protection ability at 30 microg/mL.
Protection of hemopoietic system against lethal radiation stress	*Podophyllum hexandrum* extract	Hemopoietic system stimulation by restoration of hemoglobin content (14.73 +/- 0.33) and total leukocyte count (TLC) (4166.66 +/- 0.02) in lethally (10 Gy) gamma-irradiated mice on the 15^{th} day in comparison to the radiation control supported Up-regulation of heme-oxygenase-1 and the prosurvival multidomain protein Bcl-2.
	REC-2001 (semi-purified fraction with 3.25% of podophyllotoxin only)	Modulation of levels of total and differential leucocytes, restoration of levels of haemoglobin attributed to significant antioxidant status of semi-purified fraction.
Dose reduction factor and survival analysis	REC-2001 (semi-purified fraction with 3.25% of podophyllotoxin only)	10-75 mg/body weight exhibited non-toxic range with > 90% survival at specific dose regimen; the dose reduction factor = 1.62 attributed to its ability to enhance the counts of colony forming units in spleen (thereby lies in consensus with its ability to avert radiation-induced oxidative stress in hemopoietic system).

Table 1. *Contd.*

Radioprotective activity	Extract (used)	Study
	REC-2006 (with higher % of podophyllotoxin content as compared to REC-2001)	6-45 mg/body weight exhibited non-toxic range with > 90% survival at specific dose regimen; the dose reduction factor = 1.62 attributed to its ability to modulation of antioxidant enzyme and enhanced level of endogenous colony formation units of spleen.
Identification of aryl-tetralin lignans as contributing factors to radiation protection	Two fractionated fractions of *Podophyllum hexandrum* [methanolic (S1) and chloroform fractions (S2)] with varying levels of lignans	Comparable peroxyl ion scavenging and nitrite radical scavenging potential of both the fractions. S1 exhibited higher electron donation potential, which could be attributed to the higher content of specific aryl-tetralin lignans.

* Based on the studies (n = 22) indexed in Pubmed.

Taxonomical Classification

The taxonomical classification of the plant is as follows:

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Superdivision	:	Spermatophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Magnoliidae
Order	:	Ranunculales
Family	:	Podophyllaceae
Genus	:	*Podophyllum*
Species	:	*hexandrum* Royle

The plant has several common names. Since the plant species is usually found in the high altitude (2600-4500 m) North Himalayan region of India (mainly Jammu & Kashmir, Uttarakhand and Himachal Pradesh and Sikkim), China, Pakistan and Afghanistan. Common names are very few. Some common names of the plant are given below:

1. English	:	Himalayan Mayapple
2. Hindi	:	Bankakri, Papra
3. Kashmiri	:	Banwangan
4. Ladakhi	:	Demobkusu or ol-mo-se
5. Marathi	:	Patvel
6. Punjabi	:	Bankakri

Distinctive Botanical Features of *Podophyllum hexandrum*

Flower	:	Solitary in the fork of stem, on a round, nodding peduncle (1-2″ long), white, large (2″ in diameter) and somewhat fragrant, cup shaped.
Fruit	:	Berry, reddish colored upon maturity with orange to red colored seeds and containing many seeds embedded in the red pulp, 2-5 cm in diameter.
Leaves	:	usually 2, long, stout, stalked, orbicular-reniform, palmate, peltate with lobed segments.

Plant	:	Erect, glabrous, succulent scapigerous herb, Reaching upto 30-60 cm high.
Root	:	Long, jointed, dark to light brown in colour
Stem	:	Simple, round, smooth erect dividing at the top into two petioles, modifies into creeping rhizome bearing numerous roots.
Flowering & Fruiting	:	April to August; Flowers are white in colour and berries are red to orange red in colour

Habitat

Podophyllum hexandrum is usually found growing on the high slopes of the mountainous region of the North/Central/North East Himalayas (Fig 2), mostly under moist humid conditions, as undergrowth in the forest rich in humus and decayed organic matters. *Podophyllum* flourishes at altitudes between 2,600-4,500 m in the Zanskar and Suru valleys of Ladakh in Jammu and Kashmir and the Lahaul and Spiti valleys, Kangra, Chamba, Kinnaur region of Himachal Pradesh, the high altitude regions of the Uttarakhand region (Central Himalayas) and in the North East Himalayas to some extent.

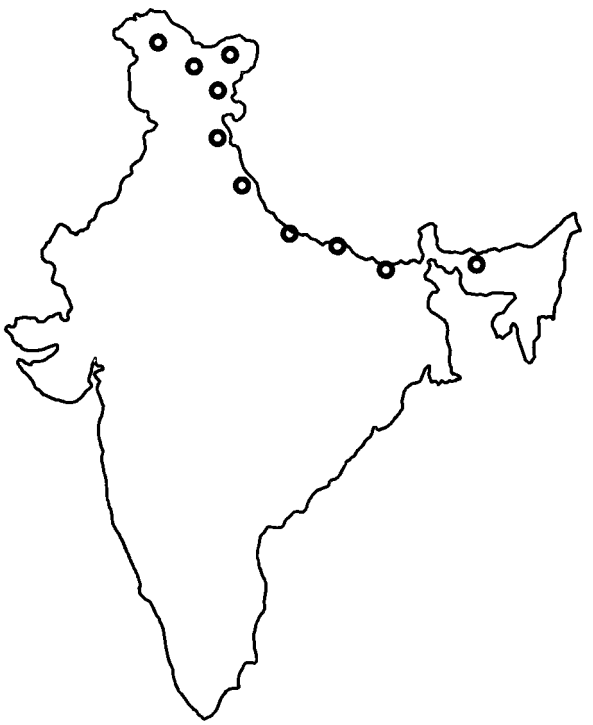


Fig 2. Areas in India where *Podophyllum hexandrum* is found in its natural habitat

There are several species of *Podophyllum* that are found in other parts of the world (Table 2). The leaves of *Podophyllum* are quite soft making the plant sensitive to hot, direct sunlight, at least in the dry season. The aerial parts of the plant are usually fragile. However, the lower underground portions on the contrary are very strong. In order to establish itself firmly, the deep growing root system needs sufficiently deep soil to reach the ground water table. The sensitivity to frost and the late sprouting in spring are the characteristics which *Podophyllum hexandrum* shares with other plants that prefer sites with an early, high and long lasting snow cover. The plant is able to tolerate fairly low temperatures.

Table 2. Some common species of *Podophyllum* found in various parts of the world

***Podophyllum* species**	**Common name**	**Geographical distribution**
P. hexandrum	Himalayan Mapple	Alpine regions of the North Central and East Himalayas
P. sikkimensis	Mayapple	North East Himalayas
P. peltatum	Mayapple or Western Mandrake	Eastern North America
P. veitchii	*Dysoma veitchii*	Western China
P. verisipelle, and *P. pleianthum*	Mayapple	China

Harnessing Genetic Diversity: A Sustainable Approach is Imperative

The genus Podophyllum is represented by three species in the Himalayan zones of India *viz.*, *P. hexandrum*, *P. peltatum*, *P. sikkimensis* (Arora *et al.*, 2008b). The plant material of *Podophyllum hexandrum*, used for radioprotection studies by us at the Institute of Nuclear Medicine and Allied Sciences, Delhi was procured mainly from Suru and Zanskar valley of Ladakh with the help of scientists at Defence Institute of High Altitutde Research (DIHAR), Leh formerly Field Research Laboratory (FRL), Leh, which is a premier laboratory of the Defence Research and Development Organization. Plant material was also procured from various other regions of Jammu and Kashmir like Sonamarg, Yousmarg, Gulmarg, Khilanmarg, Shopain, Yousmarg, Khrew, Pahalgam, Verrinag, Gurez etc. with the help of Indian Institute of Integrated Medicine, Jammu and also from Lahaul valley and Kinnaur region of Himachal Pradesh. *Podophyllum hexandrum* exhibits wide diversity and a fairly high range of variation is observed in plant height, shape, number of leaves, fruit size and colour of seeds in plants occurring in the various regions. It has been rougly estimated that more than two dozen variants of *Podophyllum hexandrum* are found in the Leh and Ladakh region and several of them have been documented by FRL, Leh. On the basis of leaf number, four morphotypes *viz.*, single-leaved, double-leaved, triple-leaved, four-leaved have been identified and collected from Suru and Zanskar valleys of Ladakh by FRL, Leh.

The higher reaches of the Himalayan region in Jammu and Kashmir, Himachal Pradesh and Uttarakhand are mostly inaccessible to humans and, therefore, floristic diversity blooms with every propitious season in this region. From a conservation point of view, it is higly desirable that such areas are kept unscathed from human interventions. The natural processes of selection keep operating in such untouched lands and form the basis for evolution of elite strains and varieties by coaxing the plants to synthesize a plethora of secondary metabolites-all in an effort to survive!

Biotechnological Interventions in India for Conservation of Genetic Resource of *Podophyllum hexandrum*

The Indian Institute of Integrative Medicine (formerly RRL, Jammu) and Institute of Himalayan Bioresource Technology, Palampur and G.B. Pant Institute of Himalayan Environment and Development, Kosi-Katarmal, Almorah, Uttarakhand are currently actively engaged in *in situ* as well as *ex situ* conservation of the genetic diversity of Indian *Podophyllum hexandrum*. Biotechnological interventions employing *Podophyllum* have been on-going at several biotechnology centers in India, including the Central Institute of Medicinal and Aromatic Plants, Lucknow and University of Delhi, since long with a view to conserving elite genotypes and reducing the dependence on natural populations. Within the Defence Research and Development Organization (DRDO), endeavours are being made to conserve the rich naturally-occurring biodiversity in the region in the form of several elite genotypes in a Gene Bank at Defence Institute of High Altitude Research (DIHAR) Leh, formerly FRL, Leh, while development of micropropagation protocols are also going on at Defence Institute of Bioenergy Research (DIBER), Haldcons (formerly Defence Agricultural Research Laboratory, Pithoragarh, Uttarakhand). The Indian Institute of Technology (IIT), Delhi has attempted to develop techniques to upscale the production of the bioactive constituents, mainly aryltetralin lignans, with a view to reducing the reliance on dwindling natural populations of *Podophyllum*.

Major Bioactive Chemical Constituents

The main biologically active chemical constituents present in *Podophyllum,* several of which have a contributory role to play in rendering protection against lethal ionizing radiation (Arora *et al.,* 2005c, d, e, 2006b, c, 2008a, b, c; Chawla *et al.,* 2005b, 2006c, d; Puri *et al.,* 2005, 2006a, b) include:

- Picropodophyllotoxin
- Podophyllotoxin glucoside
- Podophyllotoxin
- Podophyllotoxone

- Epi-Podophyllotoxin
- Podophyllotoxin-β-D-galactopyranoside
- 4'-Demethyl podophyllotoxin
- 4 – demethylpodophyllotoxin glucoside
- Quercetin-3-O-β-D-galactopyranoside
- Kaempferol-3-O-glucoside
- Deoxypodophyllotoxin
- Podophyllin
- Isopicrophyllone
- Peltatins (α and β)
- α-peltatin glucoside
- β-peltatin glucoside
- Picropodophyllone
- Quercetin
- Kaempferol
- Astragalin
- Kaempferol-3-glucoside

Use of *Podophyllum* in Holistic System of Medicine and Western Medicine

The plant was known to the ancient Ayurvedic Physicians and has been mentioned as 'Aindri'- a divine drug in ancient literature, including the Charak Samhita (Singh & Shah, 1994; Arora *et al.*, 2008*c*; Chawla *et al.*, 2008). *Podophyllum hexandrum* plays a very important role in traditional systems of medicines *viz.* Ayurveda, Unani and Amchi system. All the parts *viz.*, rhizomes, roots, leaves, fruits possess medicinal properties (Fig 3). *Podophyllum hexandrum* has been known as a cure for allergic and inflammatory conditions of the skin, biliary fever, burning sensation, cold, constipation, cancer of brain, bladder and lung, erysipelas, Hodgkin's disease, insect bites, mental disorders, monocytoid leukemia, non-Hodgkin's lymphoma, rheumatism, septic wounds plague, and venereal warts since ages, (Arora *et al.,* 2008b; Singh & Shah, 1994; Beutner & Von Krogh, 1990). The Amchi name of the plant *'ol-mo-se* refers to its ability to improve the assimilation and circulation of the blood, correct menstrual irregularities and gynecological diseases, including diseases of the uterus. It is also used to clear out the placenta and to ease childbirth.

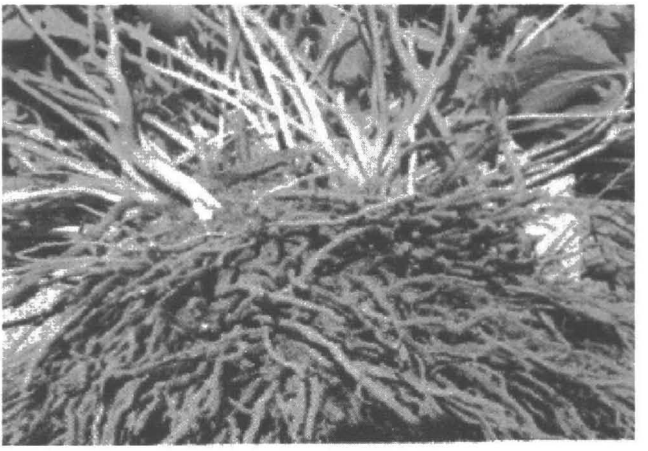


Fig 3. The medicinally useful aerial and underground portions of *Podophyllum hexandrum* also form the starting source for isolation of radioprotective molecules

Traditionally the plant is known for its cholagogue and purative effects and is often used to cure indigestion. They are also useful for treatment of diarrhoea. The root paste is applied in ulcers, cuts and wounds and also for curing skin diseases and tumorous growth. In Nepal a paste prepared from the rhizome is applied to treat vaginal infections. The fruits are edible and are sometimes used against cough. Fruits of *Podophyllum peltatum* when ripe have a sub acid, sweetish, peculiar taste and are sometimes eaten; they are also said to be preserved. Birds appear to relish the fruit of *Podophyllum*.

Podophyllum hexandrum is an endangered medicinal plant species of the Himalayas and is the commercial source of podophyllotoxin and other aryltetralin lignans and several other bioactive constituents (Foster, 1993; Singh & Shah, 1994; Bedir *et al.,* 2002). The semi-synthetic derivatives of the naturally occurring podophyllotoxin *viz.* teniposide (obtained by addition of the carbohydrate moiety β-D-thenylidene glucoside to podophyllotoxin), and later etoposide (obtained by addition of β-D-ethylidene glucoside to podophyllotoxin) have proved to be excellent antitumour agents). These drugs are used for the treatment of hepatoma, lung cancer, testicular cancer, neuroblastoma and other tumours. Clinical trials on teniposide started in 1967, and etoposide was introduced in clinical trials in 1973. Etoposide is quite effective as an anticancer agent and is used in the treatment of several malignancies like refractory testicular, lymphoid and myeloid leukemia, stomach, ovarian, brain, breast, pancreatic, and small and large cell lung cancer. Etoposide, therapeutically administered either orally or intravenously and due to its poor water solubility is formulated with Tween 80, polyethylene glycol and ethanol. Both teniposide and etoposide have

comparable distribution within the body. Etopophos (etoposide 4'-phosphate), launched in 1996 by Bristol-Myers Squib, is a water-soluble phosphate ester prodrug of etoposide and gets rapidly converted to the parent compound by endogenously present phosphates. Etopophos has the advantage that it can be given orally also. Teniposide has been shown to possess similar anticancer properties. 4'-demethylepipodophyllotoxin and related compounds inhibit the enzyme topoisomerase II, preventing DNA synthesis and replication. Another successful synthetic anti-cancer compound based on the podophyllotoxin scaffold is Azatoxin. These drugs and other derivatives, based on podophyllotoxin, find use for the treatment of leukemias, lung and testicular cancers, dermatological disorders like warts, rheumatoid arthritis, psoriasis and malaria (Leander & Rosen, 1988; Lerndal & Svensson, 2000; Beutner & von Krogh, 1990).

P. hexandrum as a Potential Radiation Countermeasure (RCM) Agent

The radioprotective effect of *P. hexandrum* rhizome extract was reported *in vivo* by our group for the first time. Elucidation of the mode of action provides crucial information for better drug design and clinical studies. Keeping this in mind, we studied the mechanistic aspects some of which have been discussed in this chapter.

This plant possesses immense potential as a natural bioresource and can be utilized to manage ionizing radiation related hazards (Arora *et al.*, 2005b, 2003b, 2004c, 2007a, b, c, 2008a, b, c, d, e, f, g, h, i; Chawla *et al.*, 2004, 2005, 2008). However, the plant is currently an endangered species and hence there is a need to manage this bioresource in a sustainable manner at the same time utilizing its immense medicinal potential.

Deleterious impact of ionizing radiation, especially with low LET on biological systems is well documented. Molecular oxygen, due to its biradical nature is the most important electron acceptor in the biosphere. It plays an important role in accepting unpaired electron, giving rise to a series of partially reduced species. In addition to this, radiolytic product of water, *e.g.* -OH and -H also react with oxygen and generate various ROS. These reactive species can induce damage in cellular macromolecules. Both direct and indirect effects of ionizing radiation damage to cellular DNA. In indirect effect, DNA damage is mainly induced by abstraction of the H-atom from the C'-4 position of the deoxyribose or by attack of the bases via the hydroxyl radicals produced by the radiolysis of water (Spotheim-Maurizot *et al.*, 1992). Activated oxygen species such as hydrogen peroxide, superoxide anions, singlet oxygen and hydroxyl radical can be formed in cells not only during ionizing radiation, but also during aerobic metabolism of either endogenous or exogenous substances. If free radical production and scavenger system somehow becomes imbalanced, cells are exposed to oxidative damage resulting in cell injury.

Lipid peroxidation increases with increase in radiation dose in heart, liver mitochondria, microsomes and splenic lymphocytes (Bloor *et al.,* 2000; Santosh Kumar *et al.,* 2004; Priyadarshini *et al.,* 2003; Gupta *et al.,* 2002a, 2003a). Lipid radicals (L·) are believed to be formed by the reaction of ·OH radicals generated by ionizing radiation with polyunsaturated fatty acids (LH), which reacts with oxygen to form lipid peroxyl radical (LOO') after undergoing molecular rearrangement of conjugation in double bonds and eventually a chain reaction is set up on irradiation in oxygenated condition. Further, lipid peroxidation products such as malondialdehyde forms adduct with cellular DNA.

To maintain the redox balance, in order to protect themselves from these free radicals action, the living cells have evolved endogenous antioxidant defence mechanism which include non-enzymatic entities like glutathione, ascorbic acid and also enzymes like catalase, superoxide dismutase, glutathione peroxidase etc. (Mittal *et al.,* 2001). Radiation exposure can alter the balance of endogenous protective systems such as glutathione and antioxidant enzyme systems and can even alter mitochondrial functioning (Fridovich, 1978). Therefore, to understand the mode of action of antioxidant compounds in *Podophyllum* as a radioprotector it was necessary to investigate its effect on radiation induced DNA damage, lipid peroxidation with reference to alterations in the endogenous antioxidant defense mechanism (Gupta *et al.,* 2002a, 2003b, 2004; Kumar *et al.,* 2004, 2005a, b, 2006). A number of agents like cysteine, cysteamine, dextran, vitamins, Ca^{2+} channel blockers like diltiazem, amifostine, cytokines, leucotrienes, DNA binding ligands, such as bibenzimidazole in *in vitro* and *in vivo* models and interleukins (IL-1, IL-4 and IL-6) have been reported to render radioprotective effects (Bump & Malaker, 1997). However, two main factors- the toxicity of molecular agents at effective doses and the low margin of safety have necessitated the search for more effective, yet less toxic agents (Arora *et al.,* 2005, 2006b, c). Study on plant extracts and phytochemicals as modifiers of radiation effects is a new area of research. It is necessary to assess the protective action of phytochemicals and exploit their possible application in radiation therapy of cancer as an alternative source of non-toxic radioprotectors. Some reports are available regarding plant extracts and phytochemicals on radiation induced cellular damage in model as well as in animal systems (Shimoi *et al.,* 1996; Sagar *et al.,* 2003; Lata *et al.,* 2007).

In recent years, numerous plants, including *Podophyllum hexandrum,* have been reported to exert significant radioprotective effect without much toxicity and acting via multifactorial mechanisms (Arora *et al.,* 2007b; Chawla *et al.,* 2004, 2006a, b; Kumar *et al.*, 2009; Singh *et al.*, 2009). Several mechanisms, such as prevention of damage through inhibition of free radical

generation or their intensified scavenging, enhancement of DNA and membrane repair, replenishment of dead hemopoietic (Arora *et al.*, 2006e; 2007a, c; Chaudhary *et al.*, 2004; Sagar *et al.*, 2006) and other cells and stimulation of immune cell activity have been considered important for radioprotection (Arora *et al.*, 2005b; Sagar *et al.*, 2006; Singh *et al.*, 2009). *Podophyllum hexandrum* has been extensively exploited in traditional systems of medicine for the treatment of a number of diseases including bacterial and viral infections, cancers of the brain, bladder, lung etc., venereal warts (Singh & Shah, 1994; Singh *et al.*, 2008, 2009). The immunostimulatory effect of *Podophyllum hexandrum* has also been reported (Pugh *et al.*, 2001; Shukla *et al.*, 2004). The plant contains a number of bioactive constituents, molecules that include aryltetralin lignans (podophyllotoxone, α and β-peltatins, 4-demethylpodophyllotoxin glycoside, podophyllotoxin glycoside, podorhizol-β-D-glycoside, deoxypodophyllotoxin and podophyllotoxin) and flavonoids like quercetin, and kaempferol (Arora *et al.*, 2003a; Singh & Shah, 1994; Sagar *et al.*, 2006) which render potent antioxidant, immunostimulant, and radioprotective activity *in vivo* and have been specifically shown to mitigate lethal ionizing radiation-induced toxicity (Arora *et al.*, 2002a; Arora *et al.*, 2005d).

CONCLUSIONS

This chapter has discussed some of the promising activities, mainly the radioprotective potential of *Podophyllum hexandrum* and its ability to mitigate the hazardous effects of radiation in biological systems, though the plant offers immense scope in other areas too (Fig 4). The plant can be taken forward to accomplish radioprotection in higher model systems, including primates. Clinical studies and elucidation of mechanism of action at cellular and molecular level of the individual chemical moieties, along with pharmacokinetic/dynamic studies, will help in realizing the ultimate goal radioprotection for human welfare. In view of its multifarious mode of action at molecular, cellular, tissue, organ and whole-body level, *Podophyllum* as a phytobioresource holds immense potential for use as a radiation countermeasure agent and could be utilized for defence during possible chemical, biological, radiological/nuclear terror incidents and in future space missions for protecting astronauts, besides having applications in other areas. There is, therefore, an urgent need to conserve this important phytobioresource in its natural habitat especially in areas where its population is fast dwindling. Conserving genetic diversity and developing alternative biotechnological production methods will greatly help harness the true potential of this plant. Some attempts in this direction are summarized in Table 3. Endeavours in this direction will help restore the natural ecosystem and conserve this important medicinal plant which has tremendous scope in alleviating human sufferings in the future.

Table 3. Some studies reported by various workers related to genetic diversity and biodiversity conservation of endangered species of *Podophyllum* and proposed alternative methods for sustained production of bioactive constituents

Objective	Extracts/Cultures	Study
Assessment of diversity in *Podophyllum hexandrum* by genetic and phytochemical markers	Twelve accessions grown in gene repository were subjected to RAPD analysis	Morphological characteristics, phytochemical variations and random amplified polymorphic DNA (RAPD) profiles among various accessions with fewer exceptions in some of accessions revealing genetic diversity. Individual regressions of podophyllotoxin and podophyllotoxin-D-glycoside by RAPD analysis against HPLC values exhibited linearity requisite for quality control. Strong correlation and association of values of phytochemical variables with DNA polymorphism reported.
Levels of podophyllotoxin varies with altitudinal shift	P1 to P11 extracts of varying altitude	Plant height, stem diameter and leaf area were, in general, negatively correlated with an increase in the altitude. Maximum above ground and below ground biomass values were compared revealing that performance was better at the lower altitudes. The podophyllotoxin content of rhizomes ranged between 0.36-1.08% (on dry wt. basis) in different populations, and a positive correlation was observed between podophyllotoxin content and an increase in the altitude.
Efficient methods of vegetative propagation	Seeds of *Podophyllum hexandrum*: Vegetative propagation	Germination of seeds of *P. hexandrum* (while they still retained moisture) in sand at 25°C and GA(3) at 200 ppm of 1-week seedlings can alleviate hypocotyl dormancy besides reducing the time taken for true or functional leaf emergence, which can help in establishment of large-scale seedling plants.
Podophyllotoxin production in plant cell cultures of *Podophyllum hexandrum* in bioreactor	Plant cell cultures of *P. hexandrum*	Submerged cultivation resulted in an overall podophyllotoxin productivity of 0.19 mg/(l.d), which represented an increase of 27% in comparison to its productivity in a shake flask. Podophyllotoxin production was found to be a combined growth-associated and non-growth associated process.

Table 3. *Contd.*

Objective	Extracts/Cultures	Study
Isolation, identification, and characterization of a novel fungal endophyte that produces aryltetralin lignans	Rhizomes of *P. hexandrum* associated endophytic fungus	*Trametes hirsuta* has been established as novel endophyte which can act as a renewable and constant source of aryl-tetralin lignans which exhibit potent antioxidant, anticancer and radioprotective properties.
Endophytic fungus for production of podophyllotoxin	Rhizomes of *P. peltatum* associated endophytic fungus	Two endophytic fungi isolated, both strains of *Phialocephala fortinii*, from rhizomes of the plant *Podophyllum peltatum* with the yield of the compound ranging from 0.5 to 189 microg/L in 4 weeks of culture.

* Based on the studies (n= 6) indexed in Pubmed.

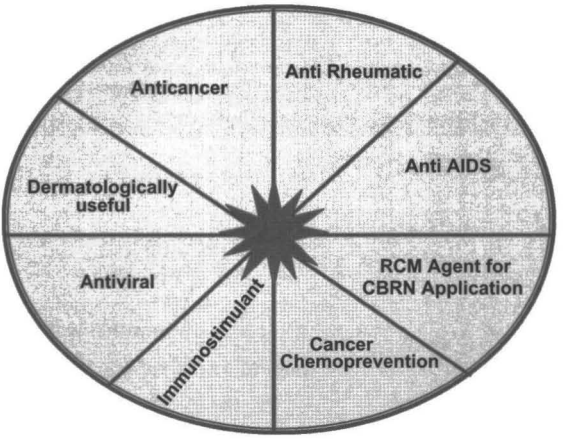


Fig 4. *P. hexandrum* as a prophylactic/therapeutic agent holds immense potential in some of these areas

ACKNOWLEDGEMENTS

Research grants received from the Defence Research and Development Organization (DRDO) in the form of two major R&D projects *viz.*, RD-P1-98/INM-285 and RD-P1/DIP-233 under the CHARAK *'Herbs for Health of Armed Forces'* program are duly acknowledged. A.S. Dhaker are grateful to the University Grants Commission (UGC), Government of India and Indian Council of Medical Research (ICMR) respectively for the award of research fellowships. Manish Adhikari and Jyoti Sharma are thankful to DRDO for grant of a research fellowship. Thanks are due to Ms. Manjeet Kaur and Ms. Reema Sardana for going through the manuscript.

REFERENCES

Arora, R., Chawla, R., Puari, S.C. *et al.* (2005e). Radioprotective and antioxidant properties of low altitude *Podophyllum hexandrum* (LAPH). *Journal of Environmental Pathology Toxicology and Oncology*, **24(4)**: 253-269.

Arora, R., Gupta, D., Chawla, R., Prasad, J., Singh, S., Sharma, A.K., Kumar, R., Sagar, R.K., Samanta, N. and Sharma, R.K. (2005d). Radioprotection by plant products: Present status and future prospects. *Phytotherapy Research*, **19**: 1-22.

Arora, R. (2006a). Botanicals as Potential Radioprotective and Radiorecovery Agents: Current Status and Emerging Directions for Clinical Applications. *Proceedings of the World Ayurveda Conference (in press)*, November 5-12, 2006 (Pune).

Arora, R. and Goel, H.C. (2000). Herbal Radioprotectors, *Proceedings of the International Conference on Radiation Biology (Radiobiology 2000)* organized under the aegis of Indian Society for Radiation Biology by Regional Cancer Centre (RCC), Trivandrum, Thiruvananthapuram. 17-19 Feb 2000 (India).

Arora, R. and Sharma, R.K. (2004a). Development of Herbal Agents for Biological Radiation Protection. *Proceedings of the DRDO Life Sciences Scientist's Conference*, Defence Food Research Laboratory, Mysore. 15-16 April 2004 (India).

Arora, R., Chawla, R., Chaudhary, P., Sharma, R.K., Puri, S.C., Gupta, V.K., Handa, G.

and Qazi, G.N. (2004b). Antioxidant activities of fractionated extracts of *Podophyllum hexandrum* Royale. *Proceedings of the International Conference on Chemistry Biology Interface: Synergistic New Frontiers*, University of Delhi, Delhi, India, 21-26 November, 2004, pp. 25-46.

Arora, R., Chawla, R., Puri, S.C., Sagar, R.K., Singh, S., Kumar, R., Sharma, A.K., Prasad, J., Singh, S., Kaur, G., Chaudhary, P., Qazi, G.N. and Sharma, R.K. (2005b). Radioprotective activity of low altitude *Podophyllum hexandrum*. *Journal of Environmental Pathology, Toxicology and Oncology,* **24(4)**: 299-314.

Arora, R., Chawla, R., Sagar, R.K., Singh, S., Kumar, R., Sharma, A.K., Singh, S., Prasad, J., Puri, S.C., Gupta, M.L. and Sharma, R.K. (2005c). Low Aryl-Tetralin Lignans containing *Podophyllum hexandrum* extract protects against radiation mediated lethal damage. *Proceedings of the Conference "Nanobiotechnology the Road Ahead" held at National Botanical Research Institute,* Lucknow, India, 17-19 November 2005, pp. 29-30.

Arora, R., Chawla, R., Singh, S., Sagar, R.K., Kumar, R., Sharma, A., Prasad, J., Singh, S., Gurudatta, G.U. and Sharma, R.K. (2006b). Bioprospection for Radioprotective Molecules from Indigenous Flora. *In*: Recent Progress in Medicinal Plants. *Editor*: JN Govil, Studium Press, USA; at **16**: 179-219.

Arora, R., Chawla, R., Singh, Shikha., Sagar, R.K., Kumar, Raj at, Sharma, A.K., Singh, S., Prasad, J., Sharma, R.K. and Tripathi, R.P. (2006c). Radioprotection by Himalayan High-Altitude Region Plants. *In*: 'Herbal Drugs: A twenty first Century Perspective', *Editors*: Sharma R.K. and Arora, R. Jaypee Brothers Medical Publishers (P) Ltd., Delhi, India, pp. 301-325.

Arora, R., Gupta, D., Garg, A.P. and Goel, H.C. (2002a). Effect of *Podophyllum hexandrum* on mitochondria and its role in affording radiation protection, *Proceedings* of the International Conference on Radiation Damage and its Modification, organized under the aegis of Indian Society for Radiation Biology at Laser Science and Technology Centre (LASTEC), Delhi. 13-15 November 2002, 47 (India).

Arora, R., Gupta, D., Prasad, J., Sharma, A.K., Singh, S., Sagar, R., Samanta, N., Kumar, R. and Goel, H.C. (2003a). Bioprospection of Himalayan High-altitude Region Flora for Radioprotective Biomolecules. *Proceedings* of the National Seminar on New Millennium Strategies for Quality, Safety and GMPs for Herbal Drugs/Products, held at National Botanical Research Institute, Lucknow. 11-13 November 2003, 51 (India).

Arora, R., Gupta, D., Prasad, J., Singh, S., Sharma, A.K., Sagar, R., Samanta, N., Kumar, R. and Goel, H.C. (2003b). Screening of Indigenous Medicinal and Aromatic Plants for their Radioprotective Potential: Some Considerations. *Proceedings* of the XXIV Annual Conference of Indian Association of Biomedical Scientists. Held at Laser Science and Technology Centre and Institute of Nuclear Medicine and Allied Sciences, Delhi, India from 1-4 Nov 2003, pp. 86-87.

Arora, R., Gupta, D., Sharma, A.K., Sagar, R.K., Prasad, J., Singh, S., Samanta, N., Kumar, R., Goel, H.C., Sinha, A.K., Chawla, R. and Sharma, R.K. (2004c). Modification of radiation-induced damage in mammalian model systems by natural plant products: implications for radiation protection. *Proceedings of the IUPAC Sponsored International Conference on Biodiversity and Natural Products held at Delhi University*, Delhi, India. 26-31 January 2004, pp. 56.

Arora, R., Puri, S.C., Chawla, R., Sharma, A.K., Kumar, R., Sharma, R.K., Qazi, G.N. and Tripathi, R.P. (2008a). ^{1}H AND ^{13}C NMR Studies on a novel quercetin derivative (with radioprotective properties) isolated from *Podophyllum hexandrum* Royle. *Proceedings of the Special Symposium on Advanced MR Applications and 14th National Magnetic Resonance Society Meeting,* Institute of Nuclear Medicine and Allied Sciences, Delhi, p. 154.

Arora, R., Sagar, R.K., Singh, S., Kumar, R., Singh, S., Prasad, J., Puri, S.C., Qazi, G.N., Krishan, B., Sharma, R.K. and Tripathi, R.P. (2007a). Hemoprotective effect of *Podophyllum hexandrum* against γ-radiation: *Role of heme oxygenase-1 and BCl-2. Integrative Cancer Therapies,* **6(1)**: 54-65.

Arora, R., Singh, S., Sagar, R., Chawla, R., Kumar, R., Sharma, A., Gupta, M.L., Lahiri, S.S. and Tripathi, R.P. (2007b). Radioprotection by high-altitude flora and phyto-constituents therefrom: Potential for Armed Forces. *Proceedings of the International Conference on Frontiers of Radiation and Photochemistry,* Mahatma Gandhi University, Kottayam, India, pp. 190.

Arora, R., Singh, S., Sagar, R.K., Kumar, R., Sharma, A., Puri, S.C., Tripathi, R.P. and Qazi, G.N. (2007c). Role of antioxidant and DNA protective activity of a promising fraction of *Podophyllum hexandrum* (REC-2006) in providing protection against γ-radiation, *Proceedings of the International Conference on Frontiers of Radiation and Photochemistry*, Mahatma Gandhi University, Kottayam, India, pp.78.

Arora, R., Singh, S., Puri, S.C. and Sharma, R.K. (2008b). Himalayan Mayapple (*Podophyllum hexandrum* Royle): Traditional Uses, Clinical Indications and Future Prospects. *Editors*: Ronald Ross Watson and Victor R. Preedy. *In*: Botanical Medicine in Clinical Practice, CABI Publishing, Wallingford, Oxon, UK, pp. 71-84.

Arora, R., Kumar, R., Sharma, A. and Tripathi, R.P. (2008c). Radiomodulatory Compounds of Herbal Origin for New Frontiers in Medicine, Homeland Security, Management of Radiological Incidents and Space Applications. *In*: Herbal Radiomodulators: Applications in Medicine, Homeland Defence and Space. (*Editor*: Rajesh Arora), CABI Publishing, Oxon United Kingdom, pp. 1-22.

Arora, R., Singh, S., Sagar, R., Kumar, R., Sharma, A., Gupta, M. and Tripathi, R.P. (2006d). Protection of hemopoietic system against ionising radiation induced damage by *Podophyllum hexandrum*: Role of heme oxygenase-1 and Bcl-2. *Indian Journal of Radiation Research*, **3(4)**: pp. 253-254.

Arora, R. (2008a). (ed) Herbal Radiomodulators: Applications in Medicine, Homeland Defence and Space. CABI Publishing, Wallingford, Oxon, UK. pp. 332.

Arora R., Dhaker, A.S., Chawla. R., Gupta. D., Adhikari. M., Kumar. R., Sharma. A., Jain. S., Sharma R.K. and Tripathi, R.P. (2008d) Critical issues in development of novel radiation countermeasures from natural sources for nuclear and radiological emergencies. *Indian Journal of Radiation Research,* **5(3-4)**: 108.

Arora, R., Chawla, R., Puri, S.C., Dhaker, A.S., Adhikari, M., Gupta, D., Sharma, A.K., Kumar, R., Sharma, R.K., Qazi G.N. and Tripathi R.P. (2008e). Fractionation, isolation and chemical characterization of 3-O-β-D galactopyranoside of quercetin from *Podophyllum hexandrum* Royle and evaluation of its biological properties *vis-à-vis* radioprotection. *Indian Journal of Radiation Research,* **5(3-4)**: 108-109.

Arora, R., Sharma, A., Kumar, R. and Tripathi R.P. and (2008f). Herbal Radiation Countermeasure Agents: Promising Role in the Management of Radiological/Nuclear Exigencies. *In* Radiation Protection and Environment. 28th IARP National conference. Focal Theme: Management of Nuclear and Radiological Emergencies. 19-21 November, 2008, Dr. S.N. Medical College and Defence Laboratory (DRDO), Jodhpur. 304-306.

Arora, R., Singh, S., Chawla, R., Sharma, A.K., Kumar, R., Sharma, R.K,. Puri, S.C., Qazi, G.N. and Tripathi, R.P.. Ionizing radiation-induced free radical mediated oxidative stress can be effectively mitigated by *Podophyllum hexandrum*. International Conference on Advances in Free Radical Research and 8th Annual Meeting of the Society for Free Radical Research-India. Department of Biochemistry, Chhatrapati Shahuji Maharaj Medical University (formerly King George's Medical University), Lucknow, India and Era's Lucknow Medical College, Lucknow, India, March 19-21, (2009), pp. 118.

Arora, R., Dhaker, A.S., Adhikari, M., Sharma, J., Chawla, R., Kumar, R., Sharma, A., Gupta, D., Prasad, J., Singh, S., Sinha, AK., Puri, S.C., Qazi, G.N., Ahuja, P.S. and Tripathi, R.P. (2008g). Strengthening the armamentarium of Indian Defence Forces for Radiological/Nuclear Preparedness: Potential of Radiation Countermeasure Agents of Natural Origin. *In*: In Service of the Soldier: Life Sciences Perspective (*Editor* Manas K. Mandal; Associate Editors: K. Ramachandran and A. Gupta). (Macmillan Advanced Research Series), Macmillan, New Delhi, India, pp. 72-96.

Arora, R., Gupta, D., Goenka, S., Adhikari, M., Sharma, J., Dhaker, A.S., Goyal, V., Chawla, R., Kumar, R., Sharma, A., Sharma, R.K. and Tripathi, R.P. (2009). Antioxidant, anticancer, cytoprotective and radioprotective properties of Indian *Podophyllum hexandrum*. *In*: Herbal Drugs: A Cancer chemopreventive and therapeutic perspective. *Editor* Rajesh Arora, Jaypee Brothers Medical Publishers (P) Ltd., Delhi, India, pp. 72-96.

Arora, R. (2008h) The Fascinating World of Plants: A Peep Reveals A Storehouse of Radioprotective Molecules. Proceedings of the First Structured Workshop on New Frontiers in Radiation Sciences, Institute of Nuclear Medicine and Allied Sciences, Delhi and University of Delhi, Delhi, India, June 16-4 July, 2008, pp. 7-17.

Arora, R., Kumar, R., Sharma, A., Prasad, J., Singh, S., Gupta, D., Sharma, R.K. and Tripathi R.P. (2008i). Radiation Countermeasure Agents from Novel High-Altitude Himalayan Herbs: Promise and Prospects. *Proceedings* of the Indo-US Workshop on Medical Countermeasures for Radiation Injury: Current and Evolving Technologies. National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), USA and Institute of Nuclear Medicine and Allied Sciences Defence Research and Development Organization (DRDO), Delhi, India, August 17-20, 2008, pp. 57-60.

Arora, R. (2008c). Radiation Countermeasure Agents from Medicinal and Aromatic Plants: Leads for Today and Tomorrow. *Proceedings* of the Indo-US Symposium on Indian Systems of Medicine and Botanicals. Indian Institute of Integrative Medicine and National Center for Natural Product Research (NCNPR), University of Mississippi, USA, 15-16 October, 2008, pp. 47-49.

Arora, R. (2008b) Development of Safe and Efficient Radiomodifiers Utilizing Metabolomic Approach: Challenges and Future Perspectives. *Proceedings* of the National Interactive Meet on Scope and Opportunities in Research and Business of Medicinal and Aromatic Plants: Meeting the Global Competition and Quality Standards. Central Institute of Medicinal and Aromatic Plants, Lucknow, India. 29-30 November, 2008, pp. 3-4.

Bedir and E., Khan, I and Moraes, R.M. (2002). Bioprospecting of podophyllotoxin. Trends in New Crops and New Uses; Herbs, Medicinal, and Aromatics, ASHS Press, Alexandria, VA, USA.

Beutner, K.R. and Von Krogh, G. (1990). Current status of podophyllotoxin for the treatment of genital warts. *Semin Dermatol*, **9**: 148.

Bloor, K.K., Kamat, and J.P. and Devasagayam. (2000). Chlorophillin as a protector of mitochondrial membranes against γ-radiation and photosensitization. *Toxicology*, **155**: 63-71.

Bump and Malaker, (1997). The mechanism of radiation protection by non-protein sulfhydryls: Glutathione, Cysteine and Cysteamine *In:* Radioprotectors Chemical, Biological, and Clinical Perspectives, *Eds*. Edward A. CRC Press, Boca Raton, Bostan New York Washington, D.C. London, pp. 25-52

Chaudhary, P., Puri, S.C., Shukla, S.K., Sagar, R.K., Arora, R. and Sharma, R.K. (2004). Prevention of radiation-induced damage to pBR322 plasmid DNA by fractions of *Podophyllum hexandrum*. *Indian J. Rad. Res.*, **1(2)**: 41-42.

Chawla. R., Arora, R., Kumar, A. *et al.,* (2005). Antioxidant activity of fractionated extracts of rhizomes of high-altitude *Podophyllum hexandrum*: Role in Radiation Protection. *Molecular and Cellular Biochemistry*, **273**: 193–208.

Chawla, R., Arora, R. and Sharma, R.K. (2008). Radioprotective activity of Himalayan Seabuckthorn and Aindri: Role of secondary metabolites. *Proceedings of the National Seminar on Physiological and Biotechnological Approaches to Improve Plant Productivity Centre for Plant Biotechnology,* CCS Haryana Agricultural University, Hissar, India, pp. 93-94.

Chawla, R., Arora, R., Kumar, R., Sharma, A.K., Prasad, J., Singh, S., Sagar, R.K., Chaudhary, P., Shukla, S., Kaur, G., Sharma, R.K., Puri, S.C., Handa, G., Gupta, V.K. and Qazi, G.N. (2005a). Antioxidant activities of fractionated extracts of high altitude *Podophyllum hexandrum* in radiation protection. *Molecular and Cellular Biochemistry*, **273**: 193-208.

Chawla, R., Arora, R., Puri, S.C., Kumar, R., Sharma, A., Prasad, J., Singh, S., Sagar, R., Kaur, G. and Sharma, R.K. (2004). A comparative study of the antioxidant potential of various fractions of *Podophyllum hexandrum* vis-à-vis radiation protection. *Indian J. Rad. Res.*, **1(2)**: 40-41.

Chawla, R., Arora, R., Puri, S.C., Singh, S., Sagar, R.K., Kumar, R., Sharma, A.K., Singh, S., Prasad, J., Gupta, M.L., Sharma, R.K., Tripathi, R.P. and Qazi, G.N. (2006a). Protection against radiation mediated damage by *Podophyllum hexandrum*. *Proceedings of the IUPAC Second International Symposium on Green/Sustainable Chemistry,* Organized by University of Delhi and New Jersey Institute of Technology, USA, 10-13 January, 2006.

Chawla, R., Arora, R., Sagar, R.K., Singh, S., Puri, S.C., Kumar, R., Singh, S., Sharma, A.K., Prasad, J., Khan, H.A., Sharma, R.K., Dhar, K.L., Spiteller, M. and Qazi, G.N. (2005b). 3-O-β-D-galactopyranoside of quercetin as an active principle from high-altitude *Podophyllum hexandrum* and evaluation of its radioprotective properties. Z. Naturforschung 60C (9/10), pp. 728-738.

Chawla, R., Arora, R., Singh, S., Kumar, R., Puri, S.C. and Sharma, R.K. (2006b). Aryltetralin lignans of *Podophyllum hexandrum* exhibit lipoprotective effect and thereby render protection against ionizing radiation. *Indian Journal of Radiation Research*, **3(4)**: 162.

Chawla, R., Arora, R., Singh, S., Sagar, R., Sharma, R.K., Puri, S.C., Khan, H.A., Shakir, M., Shawl, A.S., Kumar, R., Sharma, A., Singh, S., Prasad, J., Tripathi, R.P. and Qazi, G.N. (2006c). Variation in aryltetralin lignan content of *Podophyllum hexandrum* influences radioprotective efficacy. *Evidence Based Complement Alternat Med.*, **3(4)**: 503-511.

Chawla, R., Singh, S., Arora, R., Sharma, R.K., Puri, S.C. and Bhardwaj, J.R. (2006d). Effect of altitudinal variation on radioprotective constituents of Indian *Podophyllum*. *Proceedings of the Plant Physiology Conference,* 28-30 November, 2006, Thrissur, Kerala, India.

Foster, S. (1993). Medicinal plant conservation and genetic resources: examples from the temperate Northern hemisphere. *Acta Hort.*, **330**: 67-73.

Fridovich, I. (1978). The biology of oxygen radicals. *Science,* **201**: 875-880.

Gupta, D., Arora, R. and Goel, H.C. (2002a). Effect of Himalayan Mayapple on mitochondria: Implications in radiation protection. *Indian Journal of Nuclear Medicine*, **17(4)**: 19-20.

Gupta, D., Arora, R. and Goel, H.C. (2002b). Radioprotection by *Podophyllum hexandrum* in hepatocarcinoma (HepG2) cells. *Proceedings of the National Convention on Current Trends in Herbal Drugs:* The Natural Alternative, KB Institute of Pharmaceutical Education and Research, Gandhinagar, India. 17-18 January 2003, 50.

Gupta, D., Arora, R., Garg, A.P. and Goel, H.C. (2003a). Radiation protection of HepG2 cells by *Podophyllum hexandrum* Royale. *Molecular and Cellular Biochemistry*, **247**: 25-40.

Gupta, D., Arora, R., Garg, A.P., Bala, M. and Goel, H.C. (2004). Modification of radiation damage to mitochondrial system *in vivo* by *Podophyllum hexandrum*: mechanistic aspects *Molecular and Cellular Biochemistry*, **266**: 65-77.

Gupta, D., Arora, R., Gupta, S., Gupta, S., Garg, A.P. and Goel, H.C. (2003b). Radiation-induced oxidative damage to mitochondria and its modification by endogenous antioxidants *in vivo*: role of some herbal preparations. *Indian Journal of Nuclear Medicine* **18(4)**: 14.

Kumar, A., Verma, S. and Kumar, M. (2002). Radiomodifying effects of Spirulina. *In*: Mosaddegh, M., Nagivhi, F. (*Eds.*), Traditional Medicine and Material Medica. TMRC, pp. 91-103.

Kumar, R., Singh, P.K., Arora, R., Chawla, R. and Sharma, R.K. (2009). Radioprotective activities of *Podophyllum hexandrum*: Current knowledge of the molecular mechanisms. *Trees for Life* (in press).

Kumar, R., Singh, P.K., Arora, R., Sharma, A., Prasad, J., Sagar, R.K., Singh, S. and Sharma, R.K. (2005a). Radioprotection by *Podophyllum hexandrum* in the liver of mice: A mechanistic approach. *Environ Toxicol Pharmacol*, **20**: 326-334.

Kumar, R., Singh, P.K., Arora, R., Sharma, A.K. and Sharma, R.K. (2006). Modulation of apoptosis-inducing protein in mouse kidney by *Podophyllum hexandrum*. *In*: Recent Progress in Medicinal Plants. *Editor*: J.N. Govil, Studium Press, USA. 16, pp. 236.

Kumar, R., Singh, P.K., Sharma, A., Prasad, J., Sagar, R., Singh, S., Arora, R. and Sharma, R.K. (2005b). *Podophyllum hexandrum* extract provides radioprotection by modulating the expression of proteins associated with apoptosis role in radioprotection. *Biotechnology and Applied Biochemistry*, **42(1)**: 81-92.

Kumar, R., Singh, P.K., Sharma, A., Prasad, J., Sagar, R.K., Singh, S., Arora, R. and Sharma, R.K. (2004). Modulation of expression of cytoprotective proteins in mouse kidney by a herbal preparation of *Podophyllum hexandrum*. *Proceedings of the Indo-Tunisian Workshop on Biotechnology in Medicine,* Delhi. 28-29 July 2004, pp. 56.

Lata, M., Prasad, J., Singh, S., Kumar, R., Singh, L., Choudhary, P., Arora, R., Chawla, R., Tyagi, S., Soni, N.L., Sagar, R.K., Devi, M., Sharma, R.K. and Puri, S.C. (2007). Whole-body protection against lethal ionizing radiation in mice by REC-2001: A semi-purified fraction of *Podophyllum hexandrum*. Phytomedicine (epub advance access; in press).

Leander, K. and Rosen, B. (1988). Medicinal uses for podophyllotoxin. US patent 4,788,216.

Lerndal, R. and Svensson, B. (2000). A clinical study of CPH 82 vs. methotrexate in early rheumatoid arthritis. *Rheumatology (Oxford).* **39**: 316.

Mittal, A., Pathania, V., Agarwala, P.K. *et al.* (2001). Influence of *Podophyllum hexandrum* on endogenous antioxidant defense system in mice: possible role in radioprotection. *J. Ethnopharmacol*, **76**: 253-262.

Pugh. N., Khan, I., Moraes, R.M. *et al.* (2001). Podophyllotoxin lignans enhance II-1b but suppress TNF-α mRNA expression in LPS-treated monocytes. Immunopharmacol. *Immune Toxicol,* **23**: 83-95.

Puri, S.C., Arora, R., Sharma, R.K. and Qazi, G.N. (2006a). Biotransformation of Lignans and Neolignans. *In*: Herbal Drugs: A Twenty First Century Perspective, *Editors*: Sharma, R.K. and Arora, R. Delhi, India. Jaypee Brothers Medical Publishers Pvt. Ltd, 108-116.

Puri, S.C., Nazir, A., Chawla, R., Arora, R., S, Riyaz-ul-Haq, Amna, T., Ahmed, B., Verma, V., Singh, S., Sagar, R.K., Sharma, A.K., Kumar, R., Sharma, R.K. and Qazi, G.N. (2006b). The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. *J. Biotechnol.*, **122**: 494-510.

Puri, S.C., Nazir, A., Gupta, V.K., Negi, J.S., Handa, G., Dhar, K.L., Qazi, G.N., Arora, R., Kumar, R., Sharma, A.K., Kumar, P., Prasad, J., Singh, S., Chawla, R., Sagar, R.K. and Sharma, R.K. (2005). A process for preparation of a radioprotective formulation containing aryltetralin lignans. Patent application filed with The Controller of Patents, Indian Patents Office, Delhi, India through CSIR (NF/235/2005).

Sagar, R.K., Chawla, R., Arora, R., Singh, S., Krishna, B., Sharma, R.K., Puri, S.C., Singh, P., Kumar, R., Sharma, A.K., Singh, S., Prasad, J., Gupta, B., Ahmed, B., Dhar, K.L., Khan, H.A., Gupta, M.L. and Qazi, G.N. (2006). Protection of hematopoietic system by *Podophyllum hexandrum*. *Planta Medica*, **72**: 114-120.

Sagar, R.K., Prasad, J., Singh, S., Arora, R. and Goel, H.C. (2003). Protection of Mammalian Hemopoietic System Against Lethal (10Gy) Radiation Damage By Certain Herbal Extracts: A Comparative Study. Presentation made during the XXIV Annual Conference of Indian Association of Biomedical Scientists (Theme: "Radiotracers in Drug Development") held at Laser Science and Technology Centre and Institute of Nuclear Medicine and Allied Sciences, Delhi. 1-4 Nov 2003, 87-88 (India).

Santosh Kumar, S., Shanker B. and Sainis, K.B. (2004). Effect of chlorophillin against oxidative stress in splenic lymphocytes *in vitro* and *in vivo*. *Biochem. Biophys. Acta*, **1672**: 100-111.

Sharma, R.K. and Arora, R. (*Editors*) (2006). Herbal Drugs: A Twenty First Century Perspective. Delhi, India. Jaypee Brothers Medical Publishers (P) Ltd. pp. 688.

Shimoi, K., Masuda, S., Shen, B. *et al.* (1996). Radioprotective effects of antioxidative plant flavonoids in mice. *Mutat. Res,* **350**: 153-161.

Shukla, S., Puri, S.C., Chaudhary, P., Chawla, R., Arora, R. and Sharma, R.K. (2004). Effect of a fractionated extract of *Podophyllum hexandrum* (REC-2001) on γ-radiation-induced programmed cell death in peritoneal macrophages. *Indian J. Rad Res,* **1(2)**: 39.

Singh, J. and Shah, N.C. (1994). *Podophyllum*: A Review. *Curr. Res. Med. Arom. Plants,* **16**: 53-83.

Singh, P.K., Kumar, R., Bansal, D.D., Patel, D.D., Mohammed, A., Sharma, A., Arora, R., Gupta, M., Lahiri, S.S., Jain, S.K., and Sharma, R.K. (2008). Role of p53 expression in radioprotection offered by lignan rich fraction of *Podophyllum hexandrum* (REC-2006) in human hepatoma HepG2 (p. 53^{++}) and Hep3B ($p53^{--}$) cells. *Proceedings of the International Conference on Free Radicals and Natural Products in Health and Seventh Annual Meeting of the Society for Free Radical Researh,* Jaipur, India.

Singh, P.K., Kumar, R., Sharma, A., Arora, R., Jain, S.K. and Sharma, R.K. (2009). Pifithrin-alpha decreases the radioprotective efficacy of *Podophyllum hexandrum* fraction (REC-2006) in HepG2 cells. *Biotechnol and Applied Biochem* (May 1 epub ahead of print).

Spotheim-Maurizot, M., Garnier, F., Sabattier, R. *et al.* (1992). Metal ions protect DNA against strand breakage induced by fast neutrons. *Int. J. Radiat. Biol.*, **62**: 659-666.

Vacek, A., Rotkovska, D. and Bartonickova, A. (1990). Radioprotection of hemopoiesis conferred by aqueous extract from chlorococcal algae (Ivastimul) administered to mice before irradiation. *Experimental Hematology*, **18(3)**: 234-237.

6

Red Ginseng as a Potential Anti-Obesity Agent

JI HYUN KIM[1] AND INSOP SHIM[2,*]

ABSTRACT

The present review focuses on the anti-obesity effects of natural products. The pharmacological effects of natural herbs are still not clear although it is proved to be effective against disease, which are also the major reason preventing natural products from being accepted worldwide. Panax ginseng is known to contain high level of bioactive saponins. Here we have studied that saponins isolated from Korean red ginseng (KRG) have the anti-obesity effects on the obese rat fed with high-fat diet. Crude saponin from KRG suppressed body weight, body fat, plasma total cholesterol and serum leptin in high-fat diet group. The mode by crude saponins inhibited weight gain was assessed as in vivo regulation of appetitive neuropeptides such as neuropeptide Y (NPY) and cholecystokinin (CCK) in the hypothalamus. NPY is known to stimulate appetite and decrease energy expenditure and CCK acts on reduction in food intake and creation of satiety. Crude saponin decreased hypothalamic NPY expression and increased hypothalamic CCK expression. Furthermore, we investigated and compared anti-obesity activity of the protopanaxadiol (PD)- and protopanaxatriol (PT)- type saponins, major active compounds isolated from crude saponin. Treatment with PD and PT in the high-fat diet group reduced the body weight, total food intake, fat contents, total plasma cholesterol and leptin to level equals or below the normal diet group. The hypothalamic expression of orexigenic NPY was significantly decreased with PD or PT treatment, whereas that of anorexigenic CCK was increased, compared to the high-fat diet group. Particularly, PD-type saponins have more potent anti-obesity properties

1. Immunology and cell Biology Core Lab., Catholic Research Institutes of Medical Science, The Catholic University of Korea, Seoul - 137 701, South Korea.
2. Department of Integrative Medicine, College of Medicine, The Catholic University of Korea, Seoul - 137 701, South Korea.
* *Corresponding author* : E-mail : ishim@catholic.ac.kr

than PT-type saponins, indicating that PD-type saponins are the major components contributing to anti-obesity activities of crude saponin. Our results suggest that anti-obesity action of KRG (crude saponin, PD- and PT-type saponins) in rat fed with high-fat diet may be mediated through inhibiting energy gain and normalizing serum biochemicals and hypothalamic neuropeptides related to the control of obesity. In addition, PD-type saponins of crude saponin from KRG may be useful ingredients in the treatment of obesity and related disorders as anti-obesity agents. The present review supports the possibility that medicinal herbs are good candidate compounds for the treatment of obesity.

Key words : Anti-obesity, cholecystokinin, crude saponin of Korean red ginseng, hypothalamus, leptin, neuropeptide Y, protopanaxadiol, protopanaxatriol

INTRODUCTION

The increasing prevalence of overweight and obesity has become a crucial worldwide health problem. The epidemic of obesity on an unchanging genetic background suggests that powerful environmental forces can affect energy balance. Diet is a major factor within current obesogenic environment, where energy-dense, high-fat, high sugar foods and beverages engage both homeostatic and hedonic regulatory systems. A number of different types of diet-induced obesity model have been employed to induce obesity and research characterizing the outbred SD rat has frequently employed a high energy diet (Levin & Keesey, 1998; Levin & Dunn-Meynell, 2002). The mechanism of high fat (HF) diet-induced obesity is still unclear, but long-term exposure to a HF diet can increase body weight and adiposity in human and animals (Astrup *et al.,* 1994; Portillo *et al.,* 1999). When rats fed a HF diet, rats predisposed to develop diet-induced obesity exhibit raised body weight and carcass adiposity and become hyperleptinemic and hyperinsulinemic compared with diet-resistant rats (Levin & Dunn-Meynell, 2000).

Understanding the neural mechanisms underlying the regulation of appetite and the control of energy expenditure by the central nervous system (CNS) is becoming increasingly important in obesity research because the neuronal system originating from various hypothalamic nuclei regulates feeding behavior and metabolic processes (Oomura, 1985). Specialized neurons in the hypothalamus and other brain areas control appetite and energy metabolism, as well as signals that circulate throughout the body. The hypothalamus including the arcuate nucleus (ARC), lateral hypothalamus (LH) and closely related paraventricular nucleus (PVN), serving as the feeding or hunger center, ventromedial hypothalamus (VMH), acting as the satiety center, and nucleus tractus solitarius (NTS) conveying the peripheral signals, particularly from the gut to the feeding centers, is

implicated in the control of appetitive behavior. Thus, the CSN receives (through NTS) numerous neural impulses and hormones from peripheral organs, especially from the gastrointestinal mucosa, and fat tissue that are involved in short and long-term coordination of feeding and energy expenditure in response to constantly altered energy balance (Konturek *et al.*, 2004). The gut peptides signaling to the hypothalamus act via the ARC to mediate the appetite-stimulating effect through the activation of neurons containing neuropeptide Y (NPY) and agouti-related peptide (AgRP) or appetite-inhibitory effect via neurons containing the preopiomelanocortin (POMC)-derived α-melanocyte stimulating hormone (α-MSH) and cocaine and amphetamine regulated transcript (CART) peptide to hunger centers in the LH, the satiety center in the medial hypothalamus (Blevins *et al.*, 2002). The role of many hypothalamic peptides such as NPY, AGRP, cholecystokinin (CCK), melanocortins, CART, melanin concentrating hormone (MCH), orexins and endocannabinoids has been characterized in rodent models (Flier, 2004; Harrold, 2004; Schwartz *et al.*, 2000). The pharmacological potential of several endogenous peripheral peptides released prior to, during and/or after feeding is also being explored. Short-term signal hormones, including CCK, NPY, ghrelin and glucagon-like peptide 1 (GLP-1), control the meal size via pathways converging on the hypothalamus. Long-term regulation is provided by the main circulating hormones leptin and insulin (Harrold, 2004). These systems, among others, have been implicated for hypothalamic appetite regulation, and they all provide potential therapeutic targets to treat obesity and eating disorders.

Although a multitude of pharmaceutical agents, such as sibutramine (Reductil), orlistat (Xenical) and rimonabant (Acomplia), are available for the treatment of obesity, the long-term persistence rate with medications is poor probably due to adverse effects (Chaput & Tremblay, 2006), modest efficacy, and expense. There is a need for better tolerated anti-obesity drugs expanding understanding of peripheral signals and CNS pathways involved in the regulation of adiposity to treat obesity in the near future (Weigle, 2003). In particular, many therapeutic herbs and nutrients have far fewer side effects and may provide an alternative treatment or can be used to enhance the effect of prescription medications. While extensive research has been conducted on the development of anti-obesity drugs (Schwartz *et al.*, 2000), the compounds in foods derived from plant sources, including caffeine in oolong tea, saponin in the roots of broad bellflower and capsiate in sweet pepper, for preventing and ameliorating obesity have been investigated (Birketvedt *et al.*, 2002; Han *et al.*, 1999; Han *et al.*, 2002; Ohnuki *et al.*, 2001; Zhao *et al.*, 2005).

Many researchers have investigated the mechanisms of medical ginseng (*Panax ginseng* C.A. Meyer) as a therapeutic medicine, and the findings from these studies are currently in clinical application. Pharmacological effects of ginseng have been demonstrated in the CNS

and in cardiovascular, endocrine, and immune systems. In addition, ginseng and its constituents have been ascribed to possess antineoplastic, antistress, and antioxidant activity (Attele *et al.*, 1999; Gillis, 1997; Han *et al.*, 1998; Inoue *et al.*, 1999; Shin *et al.*, 2000; Sotaniemi *et al.*, 1995). To preserve ginseng for an extended period of time, red ginseng (RG, *Ginseng Radix Rubra*) is made by steaming and drying the fresh ginseng, suggesting chemical transformation by heat (Park, 1996). RG and its saponin fraction have also shown a variety of efficacies such as anti-cancer, anti-hypertension, anti-diabetes, anti-nociception properties (Jung & Jin, 1996), anti-amnesic activity (Jin *et al.*, 1999; Park *et al.*, 1994) and neuroprotective effects against ischemia (Lim *et al.*, 1997).

A variety of pharmacological effects of ginseng were verified to be due to different types of ginsenosides. For example, the protopanaxadiol (PD)-type ginsenosides Rb1, Rb2, Rc and Rd have an antioxidant activity (Kim & Chang, 1998) whereas the protopanaxatriol (PT)-type ginsenosides Re, Rf and Rg1 have an efficacy on the improvement of learning and memory (Jaenicke *et al.*, 1991).

This study demonstrated that the crude saponin (CS) of RG had an anti-obesity effect and compared anti-obesity activity of the PD- and PT-type saponins, major active compounds isolated from CS in rats fed with an HF diet. We measured the changes in body weight, food consumption, the regional fat content and the concentration of serum lipid and leptin in the development of obesity after treatment of the CS, PD or PT. More specially, hypothalamic expression of orexigenic NPY and anorexigenic CCK was measured in order to evaluate neural mechanisms underlying anti-obesity effects of CS, PD or PT treatment.

MATERIALS AND METHODS

Animals and Diets

Male SD rats at 3 weeks of age from Orient Bio Inc. (Seoul, Korea) were fed a commercial rat chow for 1 week. Rats were rendered obese by HF diet, while control rat had access to normal (N) diet. After 5 weeks later, rats fed HF randomly assigned to the treatment of CS, PD or PT. Rats received daily intraperitoneal injections of CS at 200 mg/kg, PD/PT at 50 mg/kg or saline at 1 mL/kg for 3 weeks. They were maintained in a temperature controlled room on a 12:12 light-dark cycle and allowed free access to diets and tap water. The experimental diets contained either a normal fat (11.7% of calories as fat, AIN-76A diet # 100000, Dyets Inc., Bethlehem, PA, USA), or a HF (40% of calories as fat, ANI-76A diet # 100496, Dyets Inc., Bethlehem, PA, USA) (Table 1). To avoid auto-oxidation of the fat components, the feed was stored at –20°C and it was freshly prepared each day. The rats were divided into five groups: the N diet group, the HF diet group, the HF diet-CS group, the HF diet-PD group, and the

HF diet-PT group. All procedure relating to the animals and their care conformed to the International Guideline Principles of Laboratory Animals Care (NIH publication No. 85-123, revised 1985).

Table 1. Composition of the experimental diets (g/kg diet)

Ingredients	N diet[1)]	HF diet[2)]
Casein	200	200
DL-Methionine	3	3
Corn starch	150	150
Sucrose	500	345
Cellulose	50	50
Corn oil	50	-
Beef tallow	-	205
Salt mixture	35	35
Vitamin mixture	10	10
Choline bitartrate	2	2
Fat % (Calories)	11.7	40.0

[1)] Normal diet: AIN-76A diet #100000 (Dyets Inc., Bethlehem, PA, USA)
[2)] High fat diet: AIN-76A diet #100496 (Dyets Inc., Bethlehem, PA, USA)

Preparation of CS, PD and PT from KRG

CS, PD- and PT-type ginsenosides purified from KRG were kindly provided from the Korean Ginseng and Tobacco Research Institute (Daejon, Korea). KRG was manufactured by the Korean Ginseng and Tobacco Research Institute from the roots of a 6-year-old fresh *Panax ginseng* C.A. Meyer. Voucher specimens have been deposited in the laboratory of the KT & G Center Research Institute in Korea. The yields for CS from 100% ethanol extract of KRG were 4.76%, the main composition of CS was ginsenoside-Rb1: 20.14%, -Rb2: 10.19%, -Rc: 11.34%, -Rd, 4.63%, -Re, 12.27%, -Rf: 3.01%, -Rg1: 16.44%, -Rg2: 2.01%, -Rg3: 2.64%, and other minor ginsenosides and components (Ko *et al.*, 1992). The procedure of extraction from KRG is as follows. KRG were extracted four times with 80% ethanol (EtOH) under reflux at 80°C for 2 h, and then the KRG-CS was prepared by Diaion HP-20 (Mitsubishi Jasei, Kasei, Japan) adsorption chromatography (Kim *et al.*, 1998). CS was freeze-dried and ground into a fine powder. The powder of CS extraction was dissolved in water and then absorbed on benzene ethylene resin (Mitsubishi Kasei, Kasei, Japan, 2.8 × 20 cm) with 200 (v/w)-fold water of the CS weight. The total adsorbed saponins on the resin was washed with 10-fold water of the resin weight, and the nonsaponins were eluded with 8-fold 20% EtOH of the resin weight, and the PT or PD was eluted with 8-fold 30~80% EtOH of the resin weight. The product was identified as PD when it was eluded in above 45% EtOH and the product as PT below 40% EtOH. All analysis was performed on a Waters HPLC system, equipped with a Waters™ 510 pump, an automated gradient controller and a Waters 484 UV detector (MA, USA). Separation of PD and

PT was achieved on a column of Waters Nova-Pak C_{18} (3.9 × 150 mm, 5 μm) (MA, USA) and the mobile phase was a mixture of acetonitrile and water in the mode of gradient elution (Kim *et al.*, 1998).

Measurements and Sample Collections

Food intake and body weight were recorded twice a week. Food cups were removed at 8:00 am and returned to animals with fresh food at 5:00 pm. Rats were randomly collected in each group and weighed. At the end of the feeding period, blood was drawn from the heart under sodium pentobarbital (60 mg/kg, *i.p.*) anesthesia following 4-5 h deprivation of feed and centrifuged at 3000 g for 15 min at 4°C. After collecting blood samples, epididymal fat, perirenal fat and peritoneal fat pad were immediately excised, weighed and frozen in liquid N_2. All serum and tissue samples were frozen at –70°C to measure biochemical parameters.

Biochemical Analyses

The serum total cholesterol (TC), triglyceride (TG) and high density lipoprotein-cholesterol (HDL-C) were measured using commercial kits (Sigma Chemical, St Louis, MO). Serum leptin concentration was determined by ELISA kit (DueSet ELISA development system, R & D Systems, Inc., Minneapolis, MN) with sensitivity of detection level 7.8 pg/mL.

Immunohistochemistry of NPY or CCK

Rats under anesthesia were perfused transcardially with a 4% solution of formalin in 0.2 M phosphate buffer saline (PBS). The brains were removed, postfixed in the same fixative for 2 h at 4°C and then placed overnight at 4°C in PBS containing 20% sucrose, and cut in 30 μm-thick coronal sections through the hypothalamic areas by microtome (Leica, 1850, Germany). The sections were incubated with rabbit anti-NPY antibody (Immunostar, WI) at a dilution of 1:2000 or with rabbit anti-CCK antibody (Immunostar, WI) at a dilution of 1:500 for 72 h at 4°C with constant agitation. Following rinsing in PBS containing 0.3% Triton X-100 (PBST), sections were incubated for 2 h at room temperature in biotinylated goat anti-rabbit serum (Vector Lab., Burlingame, CA) diluted 1:200 in PBST containing 2% normal goat serum. The sections were placed in the Vectastain Elite ABC reagent (Vector Lab., Burlingame, CA) for 2 h at room temperature. Following further rinsing in PBS, the tissue was developed using a glucose oxidase-3, 3-diaminobenzidine as the chromogen with nickel intensification. Sections were mounted on gelatine-coated slides, air-dried and cover slipped for microscopic observation. Images were captured using Axio Vision 3.0 imaging system (Zeiss, Oberkochen, Germany) and processed in Adobe Photoshop. NPY or CCK-immunoreactivity was counted at 200 magnification using a microscope rectangle grid measuring 100 × 100 microns. For measuring the number of NPY or CCK-immuoreactive cells, the grid was

placed on the ARC, LH, VMH and PVN according to a stereotaxic atlas [Paxinos & Watson, 1986].

Statistical Analysis

The values of experimental results were expressed as mean ± S.E.M. Group differences were analyzed by analysis of variance (ANOVA) with or without repeated measures (time) as applicable. Individual comparisons among groups were analyzed by one-way ANOVA followed by the Tukey's post hoc test. In all instances, values of $p<0.05$ were considered to be significant. Analyses were performed using SPSS statistical software (version 13.0 for Windows).

RESULTS

Body Weight and Food Intake

Body weight, food intake and the food efficiency ratio of the rats that were fed experimental diets after treatment with CS, PD or PT are shown in Fig 1 and Table 2. The body weight was gradually increased with time, and the body weight was higher for the HF diet fed rats than for the N diet fed rats. The gain of body weight was lower in the rats treated with CS, PD or PT compared to the rats that were fed the HF diet alone [$F(4,25)=5.651$, $p<0.01$]. CS reduced about 20-30% body weight and food intake in rats fed HF diet. In the case of the HF diet-PD group, the body weight gain was 18% lower than that of the control HF diet group. The daily food intake was not significantly different between the N and HF diet groups, but the daily food intake of the CS, PD or PT treated groups continued to diverge

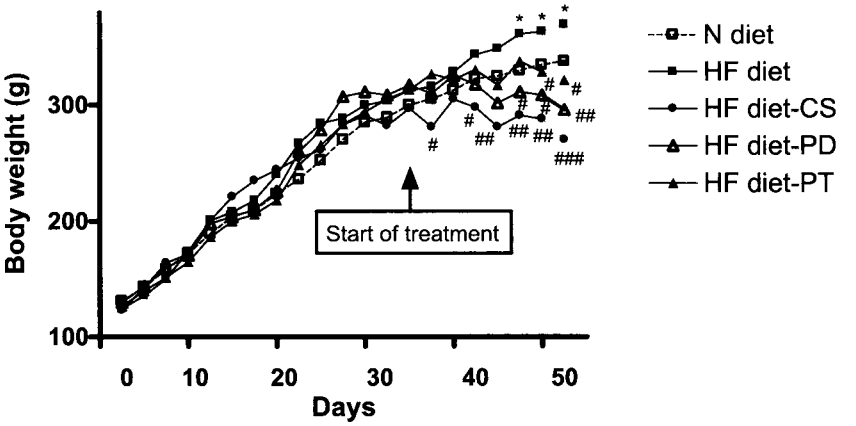


Fig 1. Change of body weight in the experimental groups. Values are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA with repeated measures followed by Tukey's post hoc test. $^{*}p<0.05$ vs. N diet group & $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. HF diet group respectively. N, normal; HF, high fat; CS, crude saponin; PD, protopanaxadiol; PT, protopanaxatriol

from the control group, for the HF diet [$F(4,25)=5.485$, $p<0.01$]. The total food consumption during the whole experimental period was significantly different among the groups [$F(4,25)=10.524$, $p<0.001$]. However, the food efficiency ratio was not different among the groups [$F(4,25)= 0.752$, $p>0.05$].

Table 2. Body weight, total dietary intake and food efficiency ratio of the experimental groups

Parameter	N diet	HF diet	HF diet-CS	HF diet-PD	HF diet-PT
Body weight (g)	338.3 ± 15.2	370.0 ± 9.2*	270.0 ± 11.3###	295.8 ± 4.7##	321.8 ± 7.9#
Total food intake (g)	799.4 ± 10.3	802.4 ± 9.8	620.5 ± 11.5###	671.8 ± 10.5###	721.1 ± 12.3#
FER[a]	43.6 ± 3.2	45.1 ± 2.3	43.5 ± 1.6	44.0 ± 1.9	44.6 ± 2.2

a FER food efficiency ratio = [body weight gain (g)/total food intake(g)] × 10^2.

Values are presented as means ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. *$p<0.05$ *vs.* N diet group & #$p<0.05$, ##$p<0.01$, ###$p<0.001$ vs. HF diet group respectively. N, normal; HF, high fat; CS, crude saponin; PD, protopanaxadiol; PT, protopanaxatriol

Fat Storage

The effects of CS, PD or PT on body fat accumulation are shown in Table 3. The mass of the various adipose tissues (epididymal, perirenal and peritoneal) is shown in terms of per body weight (kg). Parallel to the body weight change, the weights of the regional fat mass were higher in the HF diet group than in the N diet group. There were significant differences among the groups for the epididymal fat [$F(4,28)=3.874$, $p<0.05$], the perirenal [$F(4,28)=6.458$, $p<0.01$] and peritoneal fat [$F(4,28)=5.120$, $p<0.01$].

Table 3. Weight of the regional fat of the experimental groups (g/kg B.W.)

Parameter	N diet	HF diet	HF diet-CS	HF diet-PD	HF diet-PT
Epididymal fat	11.6 ± 0.5	17.9 ± 1.0	16.7 ± 1.8	8.6 ± 0.6##	10.9 ± 0.5#
Perirenal fat	12.5 ± 1.7	18.6 ± 1.7*	9.0 ± 1.4##	8.0 ± 1.8##	10.6 ± 1.1#
Peritoneal fat	14.5 ± 1.2	22.0 ± 2.1*	9.6 ± 2.5##	11.4 ± 2.3##	15.4 ± 2.7#

Values are presented as means ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. *$p<0.05$ vs. N diet group & #$p<0.05$, ##$p<0.01$ vs. HF diet group respectively. N, normal; HF, high fat; CS, crude saponin; PD, protopanaxadiol; PT, protopanaxatriol

Serum Lipid Levels

The results of serum lipids levels are shown in Table 4. The TG level was lower in N diet group than in the HF diet group and the TG levels of the HF diet-PD and HF diet-PT groups were different from that of the HF diet group, except HF diet-CS group [$F(4,28)=6.408$, $p<0.01$]. The TC level was also higher in the HF diet group than in the N diet group and the increase of TC in the HF diet group was suppressed by treatment with CS, PD or PT [$F(4,28)=7.057$, $p<0.01$]. Additionally, PT lowered the TC level compared to the level of the N diet group. On the other hand, the HDL-C level was significantly higher in the rats treated with PD, as compared to the rats fed the HF diet alone [$F(4,28)=3.879$, $p<0.05$]. The ratio of HDL-C/TC (HTR) was significantly higher in the HF diet-PD group and HF diet-PT group than in the HF diet group [$F(4,28)=4.002$, $p<0.05$].

Table 4. Serum parameters of the experimental groups

Parameter	N diet	HF diet	HF diet-CS	HF diet-PD	HF diet-PT
TG (mg/dl)	81.6 ± 5.5	127.6 ± 7.4$^{\#}$	142.7 ± 11.2	60.2 ± 6.6$^{\#\#}$	55.8 ± 3.6$^{\#\#}$
TC (mg/dl)	65.9 ± 2.1	78.6 ± 1.9*	56.7 ± 2.7$^{\#}$	57.2 ± 4.8$^{\#}$	48.2 ± 1.5$^{**,\#\#}$
HDL-C (mg/dl)	47.9 ± 2.0	38.9 ± 1.5	32.7 ± 6.0	49.2 ± 2.7#	42.0 ± 2.7
HTR[a]	0.73 ± 0.02	0.56 ± 0.04	0.60 ± 0.07	0.90 ± 0.11#	0.88 ± 0.03$^{\#}$

[a]HTR = HDL cholesterol/Total cholesterol ratio

Values are presented as means ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$ vs. N diet group & $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. HF diet group respectively. N, normal; HF, high fat; CS, crude saponin; PD, protopanaxadiol; PT, protopanaxatriol; TG, triglyceride; TC, total cholesterol; HDL-C, high density lipoprotein-cholesterol.

Serum Leptin

The results of serum leptin levels are shown in Fig 2. The serum leptin level in the HF diet group was significantly higher than that in the N diet group, suggesting the development of hyperleptinemia. In contrast to the HF fed rats, those treated with CS, PD or PT showed significantly suppressed elevation of the serum leptin concentration [$F(4,28)=12.053$, $p<0.001$].

Hypothalamic NPY and CCK Neurons

The number of hypothalamic NPY-immunoreactive neurons is shown in Fig 3. The NPY-immunoreactivity was significantly different among groups

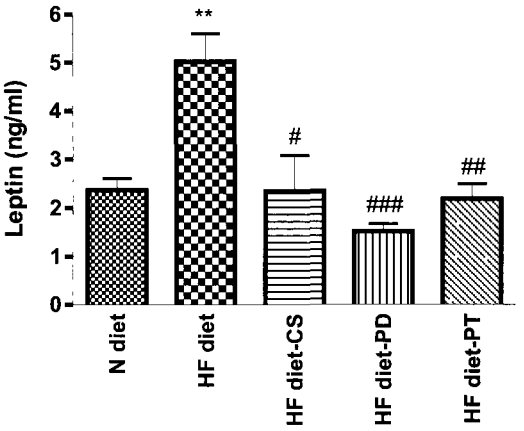


Fig 2. Levels of leptin in the serum of experimental groups. Data are presented as the mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. $^{**}p<0.01$ vs. N diet group & $^{\#}P<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. HF diet group respectively. N, normal; HF, high fat; CS, crude saponin; PD, protopanaxadiol; PT, protopanaxatriol

in the ARC [$F(4,51)=5.006$, $p<0.05$], LH [$F(4,49)=7.213$, $p<0.01$], VMH [$F(4,57)=13.402$, $p<0.01$], PVN [$F(4,50)=10.114$, $p<0.01$]. However, there was no difference between N-diet and HF-diet groups. NPY localized in these hypothalamic nuclei was not affected by the HF diet. The NPY-immunoreactive expressions with CS treatment in HF diet group were significantly reduced in all hypothalamic nuclei, relative to the HF diet control group. The PD treatment was significantly reduced NPY-immunoreactive expression in the PVN ($p<0.05$), but the PT treatment was not significantly different compared with HF diet group.

The number of hypothalamic CCK-immunoreactive neurons is shown in Fig 4. The CCK immunoreactivity was significantly different among groups in the ARC [$F(4,42)=9.035$, $p<0.05$], LH [$F(4,42)=10.092$, $p<0.01$], VMH [$F(4,41)=12.523$, $p<0.01$] and PVN [$F(4,38)=12.129$, $p<0.001$]. The CCK immunoreactivities were also unaffected after N or HF dietary intervention. The CCK expressions with PD treatment in the HF diet group were only significantly increased in all hypothalamic nuclei compared with the HF diet, but the CCK expressions of the HF diet-CS or -PT groups were not significantly different relative to the HF diet group.

DISCUSSION

The present study demonstrated that constituents of KRG *i.e.* CS, PD- and PT-type saponins, could have an effects on the HF diet-induced obesity. The weight of the whole body, food consumption, adipose tissues, the serum

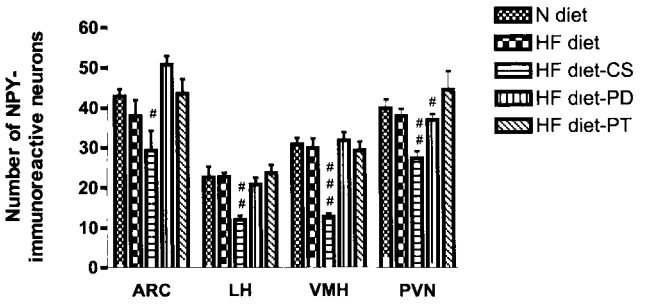


Fig 3. Expression of neuropeptide Y (NPY) neurons in the hypothalamus of the experimental groups. Data are presented as mean ± SEM of the NPY-immunoreactive neurons within the rectangular grid at 200×magnification. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. HF diet group respectively. N, normal; HF, high fat; CS, crude saponin; PD, protopanaxadiol; PT, protopanaxatriol, ARC, arcuate nucleus; LH, lateral hypothalamus; VMH, ventromedial hypothalamus; PVN, paraventricular nucleus

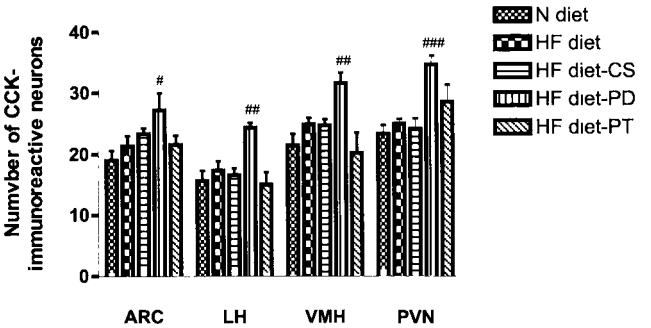


Fig 4. Expression of cholecystokinin (CCK) neurons in the hypothalamus of the experimental groups. Data are presented as mean ± SEM of the CCK-immunoreactive neurons within the rectangular grid at 200 × magnification. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. HF diet group respectively. N, normal; HF, high fat; CS, crude saponin; PD, protopanaxadiol; PT, protopanaxatriol, ARC, arcuate nucleus; LH, lateral hypothalamus; VMH, ventromedial hypothalamus; PVN, paraventricular nucleus

lipid and leptin levels and the change of appetitive hypothalamic peptides such as NPY and CCK were investigated in rats.

Compared with N diet control group, HF diet resulted in greater increase body weight in rats. Parallel to the change of the body weight, the weights of the regional (perirenal and peritoneal) fat masses and, plasma

levels of lipid and leptin were higher in the HF diet group than in the N diet group. The role of leptin in the pathogenesis of obesity can be inferred by measurement of plasma leptin. An increase of plasma leptin suggests that obesity in the result of resistance to leptin. Leptin synthesis occurs in proportion to adipose cell size and number and it is released into the blood and reaches its receptors in the brain via facilitated passage across blood-brain barrier. Activation of leptin receptors in the ARC initiates a cascade of leptin downstream events. These include the inhibition of neurons containing NPY, and orexigenic peptide, and the stimulation of neurons containing POMC, the precursor of α-MSH, an anorexigenic peptide (Spiegelman & Jeffery, 2001; Schwartz *et al.,* 2000). Leptin acts centrally to inhibit the effects of NPY by decreasing its synthesis in the ARC and its concentration in the PVN (Erickson *et al.,* 1996).

In this study, analysis of hypothalamic NPY- and CCK-immunoreactive neurons expression revealed that HF diet group had not alteration statistically. But there tended to be a lower NPY level and a higher CCK level in the HF diet group as compared to the N diet group for 8 weeks. The result that the expression of NPY-immunoreactive neurons in ARC was decreased in some degree suggested that there is an important interaction between ARC NPY expression and the plasma leptin for the control of the body weight in diet-induced obese rats. NPY and leptin are actually two of the most potent peptides involved in the regulation of food intake with their respective stimulatory and inhibitory actions. Intracerebroventricular (i.c.v.) injection of leptin inhibited NPY-induced feeding in the rat (Yokosuka *et al.*, 1998). Our result also demonstrated that the increase of leptin by HF could inhibit hypothalamic NPY expression. Other studies have been reported that diet-induced obesity (DIO)-prone rats had more ARC NPY mRNA expression than diet resistant (DR)-prone rats for up to 2 weeks after the HF diet is started, despite the increased leptin levels, and DIO-prone rats fail to increase their ARC NPY expression in a normal fashion with fasting or food restriction (Levin & Dunn-Meynell, 1997). However, after 12 weeks on the HF diet, DIO rats become quite obese with high leptin level. At that point, their ARC NPY expression was actually lower than the non-obese rats and it increased normally during food restriction (Levin, 1999).

Animals showed far more sensitive to the meal-suppressing action of CCK when leptin was administrated to animals (Barrachina *et al.,* 1997; Matson & Ritter, 1999). In this study, the reasons that a little increase of body weight gain in spite of hypothalamic CCK-immunoreactive neurons' increase in response to HF diet may be diminish sensitivity to CCK. Other study that CCK significantly suppressed food intake in low fat (LF)-fed rats but not HF-fed rats demonstrate that chronic ingestion of a HF diet leads to short-term overconsumption of a high-energy, high-fat food compared with LF-fed cohorts, which is associated with a decreased sensitivity to CCK (Savastono & Covasa, 2005).

The administration of CS from KRG (200 mg/kg, *i.p.*) for 3 weeks reduced the weight of the body, the parametrical adipose tissues and the levels of serum lipid and leptin in the HF diet group. It seems that the anti-obese effects of CS are due to the action of active compounds. Both the PD- and PT- type saponins, the representative compounds of CS, reduced the body weight, the body fats and the serum lipid and leptin levels in the HF diet-induced obesity rats. Our data support that ginseng may be a useful therapeutic agent in the management of obesity. HF diet feeding can induce metabolic disorders as well as obesity in rodents that resemble the human metabolic syndrome. HF diet promotes hyperglycemia and whole body insulin resistance and can be used to generate a valid rodent model for the metabolic syndrome (Oakes *et al.*, 1997). The HF feeding induced a significant increase in the levels of serum lipid (TG and TC) and leptin, and this represents adipocyte hypertrophy. It was shown that obesity-prone mice elevated their plasma leptin levels very gradually along with a significantly increased body fat mass in response to a HF diet (Surwit *et al.*, 1995). The important factor related to obesity is body-fat distribution, especially visceral adipose tissue. The association between abdominal obesity and metabolic syndrome is not clear, but one leading concept maintains that visceral adipose tissue has a higher rate of lipolysis, resulting in elevated portal non-esterified fatty acids that increase hepatic very-low-density lipoprotein production, increase hepatic glucose production, and impair peripheral insulin sensitivity (Klein, 2004). Therefore, the results of this study are worth in terms of that visceral adipose tissue was decreased after treatment of the PD-type saponin. Taken together, our data suggest that PD type saponin may be helpful in human in preventing diet-induced obesity and perhaps even metabolic syndrome.

The control systems that regulate body weight are numerous and include signals from fat that travel to the hypothalamus where cognitive and internal signals are integrated. The integration of these signals involves a complex array of neuropeptides, neurotransmitters and structural circuits. These circuits regulate appetite intake and energy expenditure (Konturek *et al.*, 2004; Kontuerk *et al.*, 2005). After a meal or when the body fat stores are replete, the levels of leptin rise, and this leads to inhibition of the NPY and AGRP neurons and it stimulates the CCK and CART neurons; in turn, a reduction of orexin and MCH occurs in the LH, and this is accompanied by increased production of corticotropin-releasing hormone (CRH) in the PVN (Barrachina *et al.*, 1997; Sotaniemi *et al.*, 1995). The role of the hypothalamic NPY and CCK in mediating the food intake in the HF diet obese rats after treatment of KRG constituents was examined in the present study. Hypothalamic NPY neurons potentially stimulate food intake (Billington *et al.*, 1994; Karla *et al.*, 1991), and CCK neurons have been reported to reduce appetite and inhibit food intake (Dourish *et al.*, 1989; Gibbs *et al.*, 1973; Smith & Gibbs, 1992). When CS was administered to HF diet obese rat, NPY-immunoreacive neurons in the hypothalamus were

reduced but CCK-immunoreactive neurons were not changed. However, PD-type saponin only reduced the NPY-immunoreactive neurons of the PVN, and it increased the CCK-immunoreactive neurons in the hypothalamus, as compared to the HF diet-group, but the PT-type saponins did not alter the NPY- and CCK-immunoreactive neurons in the hypothalamus. These results suggested that PD was more effective than PT for the anti-obesity effects related to the above factors. The result that reduced NPY immunoreactivity in the PVN, but not the ARC, after treatment with PD-type saponins may inhibit the release of NPY or its transport to the PVN, rather than NPY synthesis in the ARC. Lee *et al.* (2004) have reported the anorexic effect of capsaicin for the treatment of the obesity-related neurotransmitters/neuromodulators, NPY and CCK. Capsaicin appears to result in decreases in NPY expressions than in CCK expression to diminish food intake through the low expression NPY in hypothalamus.

The chosen dosage of PD or PT (50 mg/kg) in this study is a relatively standard one that has previously been demonstrated to produce the effect of anti-amnesia in another study (Jin *et al.*, 1999). Therefore, the long-term behavioral effects were examined with using one single dose in the present study. According to types of ginseng batches, species and preparation, the glycemic effects are variable, in which the PD/PT-ginsenoside ratio might be involved (Sievenpiper *et al.,* 2004). Therefore, anti-obesity effects of the different types of ginseng secondary to the variability in their composition would be further investigated.

The safety of available herbal products for weight reduction, as well as their efficacy, remains major issues that need to be resolved through research and education. The most wildly used herbal supplements for weight loss contain ephedra alkaloids, herbal forms of caffeine (guarana), catechins of green tea, synephrine from bitter orange (*Citrus aurantium*), and capsaicin from peppers. Ephedra alkaloids commonly are combined with caffeine or botanical sources of caffeine for weight loss (Boozer *et al.,* 2002). However, no long-term data (*i.e.*, greater than six months) on efficacy were available. Herbal products for weight reduction may ultimately prove to be helpful in overcoming clinically significant obesity.

In conclusion, CS, PD- and PT-type saponins from KRG have been shown to exert anti-obesity effects in the rats fed with a HF diet by reducing their body weight, their food consumption and their fat storage. They were also effective in the regulation of serum lipid and leptin and the hypothalamic NPY and CCK expressions. In addition, PD type saponins have more potent anti-obesity properties for the factors related to HF diet-induced obesity, indicating that PD-type saponins are the major components contributing to anti-obesity activities of ginseng CS. It is necessary to separate and purify the active ginsenosides from the PD-type saponins, and we need to carry out further study on their anti-obesity effects.

REFERENCES

Astrup, A., Buemann, B., Western, P., Toubro, S., Raden, A. and Christiensen, N.J. (1994). Obesity is an adaptation to a high-fat diet: evidence from a cross-sectional study. *The American Journal of Clinical Nutrition,* **59**: 350-355.

Attele, A.S., Wu, J.A. and Yuan, C.S. (1999). Ginseng pharmacology: multiful constituents and multiple actions. *Biochemical Pharmacology,* **58**: 1685-1693.

Barrachina, M.D., Martinez, V., Wang, L., Wei, J.Y. and Tache, Y. (1997). Synergistic interaction between leptin and cholecystokinin to reduced short-term food intake in lean mice. *Proceedings of the National Academy of Sciences of the United States of America,* **94**: 10455-10460.

Billington, C.J., Briggs, J.E., Harker, S., Grace, M. and Levine, A.S. (1994). Neuropeptide Y in hypothalamic paraventricular nucleus: a center coordinating energy metabolism. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology,* **266**: R1765-R1770.

Birketvedt, G.S., Travis, A., Langbakk, B. and Florholmen, J.R. (2002). Dietary supplementation with bean extract improves lipid profile in overweight and obese subjects. *Nutrition,* **18**: 729-733.

Blevins, J.E., Schwartz, M.W. and Baskin, D.G. (2002). Peptide signals regulating food intake and energy homeostasis. *Canadian Journal of Physiology and Pharmacology,* **80**: 396-406.

Boozer, C.N., Daly, P.A., Homel, P., Solomon, J.L., Blanchard, D., Nasser, J.A., Strauss, R. and Meredith, T. (2002). Herbal ephedra/caffeine for weight loss: a 6-month randomized safety and efficacy trial. *International Journal of Obesity and Related Metabolic Disorders,* **26**: 593-604.

Chaput, J.-P. and Tremblay, A. (2006). Current and novel approaches to the drug therapy of obesity. *European Journal of Clinical Pharmacology,* **62**: 793-803.

Dourish, C.T., Rycroft, W. and Iverson, S.D. (1989). Postponement of satiety by blockade of brain cholecystokinin (CCK-B) receptors. *Science,* **245**: 1509-1511.

Erickson, J.C., Hollopeter, G. and Palmiter, R.D. (1996). Attenuation of the obesity syndrome of ob/ob mice by the loss of neuropeptide Y. *Science,* **274**: 1704-1707.

Flier, J.S. (2004). Obesity wars: molecular progress confronts an expanding epidemic. *Cell,* **116**: 337-350.

Gibbs, J., Young, R.C. and Smith, G.P. (1973). Cholecystokinin decreases food intake in rats. *Journal of Comparative and Physiological Psychology,* **84**: 488-495.

Gillis, C.N. (1997). *Panax ginseng* pharmacology: a nitric oxide link. *Biochemical Pharmacology,* **54**: 1-8.

Grundy, S. (1998). Multifactorial causation of obesity: Implication for intervention. *The American Journal of Clinical Nutrition,* **67(Suppl)**: 563S-572S.

Han, K.H., Choe, S.C., Kim, H.S., Sohn, D.W., Nam, K.Y., Oh, B.H., Lee, M.M., Park, Y.B., Choi, Y.S., Seo, J.D. and Lee, Y.W. (1998). Effects of red ginseng on blood pressure in patients with essential hypertension and white coat hypertension. *The American Journal of Chinese Medicine,* **26(2)**: 199-209.

Han, L.K., Takaku, T., Li, J., Kimura, Y. and Okuda, H. (1999). Anti-obesity action of oolong tea. *International Journal of Obesity and Related Metabolic Disorders,* **23**: 98-105.

Han, L.K., Zheng, Y.H., Xu, B.J., Okuda, H. and Kimura, Y. (2002). Saponins from *Platycodi radix* ameliorate high fat diet-induced obesity in mice. *The Journal of Nutrition,* **132**: 2241-2245.

Harrold, J.A. (2004). Hypothalamic control of energy balance. *Current Drug Targets,* **5**: 207-219.

Inoue, M., Wu, C.Z., Dou, D.Q., Chen, Y.J. and Ogihara, Y. (1999). Lipoprotein lipase activity by red ginseng saponins in hyperlipidemia model animals. *Phytomedicine,* **6**: 257-265.

Jaenicke, B., Kim, E.J., Ahn, J.W. and Lee, H.S. (1991). Effect of *Panax ginseng* extract on passive avoidance retention in old rats. *Archives of Pharmacal Research,* **14**: 25-29.

Jin, S.H., Park, J.K., Nam, K.Y., Park, S.N. and Jung, N.P. (1999). Korean red ginseng saponins with low ratios of protopanaxadiol and protopanaxatriol saponin improve

scopolamine-induced learning disability and spatial working memory in mice. *Journal of Ethnopharmacology,* **66**: 123-129.

Jung, N.P. and Jin, S.H. (1996). Studies on the physiological and biochemical effects of Korean ginseng. *Korean Journal of Ginseng Science,* **20(4)**: 431-471.

Kalra, S.P., Dube, M.G., Sahu, A., Phepls, C.P. and Kalra, P.S. (1991). Neuropeptide Y secretion increases in the paraventricular nucleus in association with increased appetite for food. *Proceedings of the National Academy of Sciences of the United States of America,* **88**: 10931-10935.

Kim, C.S., Jeong, S.I. and Lee, Y.G. (1998). A rapid separation of an edible panaxadiol and panaxatriol in ginseng saponins by benzene ethylene resin absorption. *Journal of Ginseng Research,* **22**: 211-215.

Kim, D.Y. and Chang, J.C. (1998). Radioprotective effect of ginseng components on antioxidant enzymes, glutathione and lipid peroxidation of liver in γ - irradiated mice. *Korean Journal of Ginseng Science,* **22**: 1-10.

Kim, S.K., Kwak, Y.S., Kim, S.W., Hwang, S.Y., Ko, Y.S. and Yoo, C.M. (1998). Improved method for the preparation of crude ginseng saponin. *Journal of Ginseng Research,* **22**: 155-160.

Klein, S. (2004). The case of visceral fat: argument for the defense. *The Journal of Clinical Investigation,* **113**: 1530-1532.

Ko, S.R., Kim, S.C. and Choi, K.J. (1992). Extract yields and saponin contents of red ginseng extracts prepared with various concentrations of ethanol. *Korean Journal of Pharmacognosy,* **23(1)**: 24-28.

Konturek, P.C., Konturek, J.W., Cześnikeiwicz-Guzik, M., Brzozowski, T., Sito, E. and Konturek, S.J. (2005). Neuro-hormonal control of food intake; Basic mechanisms and clinical implications. *Journal of Physiology and Pharmacology,* **56(Suppl 6)**: 5-25.

Konturek, S.J., Konturek, J.W., Pawlik, Y. and Brzozowski, T. (2004). Brain-gut axis and its role in control of food intake. *Journal of Physiology and Pharmacology,* **55(Suppl 1)**: 137-154.

Lee, I.S., Nam, Y.S., Lee, C.H., Chung, D.W., Yoon, Y.S., Kim, J.S., Yi, S.J., Pai, T. and Lee, H.S. (2004). Expressional changes of neuropeptide Y and cholecystokinin in the arcuate and paraventricular nuclei after capsaicin administration. *Journal of Nutritional Science and Vitaminology,* **50**: 144-148.

Leonhardt, M., Hrupka, B. and Langhans, W. (1999). New approaches in the pharmacological treatment of obesity. *European Journal of Nutrition,* **38(1)**: 1-13.

Levin, B.E. (1999). Arcuate NPY neurons and energy homeostasis in diet-induced obese and resistant rats. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology,* **276**: R382-387.

Levin, B.E. and Dunn-Meynell, A.A. (1997). Dysregulation of arcuate nucleus preproneuropeptide Y mRNA in diet-induced obese rats. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology,* **272**: R1365-R1370.

Levin, B.E. and Dunn-Meynell, A.A. (2000). Defense of body weight against chronic restriction in obseity-prone and -resistant rats. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology,* **278**: R231-R237.

Levin, B.E. and Dunn-Meynell, A.A. (2002). Defense of body weight depends on dietary composition and palatability in rats with diet-induced obesity. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology,* **282**: R46-R54.

Levin, B.E. and Keesey, R.E. (1998). Defense of differing body weight set points in diet-induced obese and resistant rats. *The American Journal of Physiology,* **274**: R412-R419.

Levin, B.E., Triscari, J. and Sullivan, A.C. (1983). Altered sympathetic activity during development of diet-induced obesity in rat. *The American Journal of Physiology,* **244(3)**: R347-R355.

Lim, J.H., Wen, T.C., Matsuda, S., Tanaka, J., Maeda, N., Peng, H., Aburaya, J., Ishihara, K. and Sakanaka, M. (1997). Protection of ischemic hippocampal neurons by ginsenoside Rb1, a main ingredient of ginseng root. *Neuroscience Research,* **28(3)**: 191-200,

Matson, C.A. and Ritter, R.C. (1999). Long-term CCK-leptin synergy suggests a role for CCK in the regulation of body weight. *The American Journal of Physiology,* **276**: R1038-1045.

Oakes, N.D., Cooney, G.J., Camilleri, S., Chisholm, D.J. and Kraegen, E.W. (1997). Mechanisms of liver and muscle insulin resistance induced by chronic high-fat feeding. *Diabetes,* **46**: 1768-1774.

Ohnuki, K., Haramizu, S., Oki, K., Watanabe, T., Yazawa, S. and Fushiki, T. (2001). Administration of capsiate, a non-pungent capsaicin analog, promotes energy metabolism and suppresses body fat accumulation in mice. *Bioscience, Biotechnology, and Biochemistry,* **65**: 2735-2740.

Oomura, Y. (1985). Feeding control through bioassay of body chemistry. *The Japanese Journal of Physiology,* **35**: 1-19.

Park, J.D. (1996). Recent studies on the chemical constitutents of Korean ginseng (*Panax ginseng* C. A. Meyer). *Korean Journal of Ginseng Science,* **20**: 389-415.

Park, J.K., Nam, K.Y., Hyan, H.C., Jin, S.H., Chepurnov, S.D. and Chepunora, N.E. (1994). Effect of red ginseng trial saponin fraction on the spatial memory function studied with 17-arm radial maze. *Korean Journal of Ginseng Science,* **18**: 32-38.

Paxinos, G. and Watson, C. (1986). The rat brain in stereotaxic coordinates (4th edn). New York Academic Press, San Diego.

Portillo, M.P., Simon, E., Garcia-Calonge, M.A. and Del Barrio, A.S. (1999). Effects of high-fat diet on lypolisis in isolated adipocytes from visceral and subcutaneous WAT. *European Journal of Nutrition,* **38**: 177-182.

Rebuffe-Scrive, M., Surwit, R., Feinglos, M., Kuhn, C. and Rodin, J. (1993). Regional fat distribution and metabolism in a new mouse model (C57BL/6J) of non-insulin-dependent diabetes mellitus. *Metabolism,* **42**: 1405-1409.

Savastano, D.M. and Covasa, M. (2005). Adaptation to a high-fat diet leads to hyperphagia and diminished sensitivity to cholecystokinin in rats. *The Journal of Nutrition,* **135(8)**: 1953-1959.

Schwartz, M.W., Woods, S.C., Orte, D., Seeley, R.J. and Baskin, D.G. (2000). Central nervous system control of food intake. *Nature,* **404**: 661-671.

Shin, H.R., Kim, J.Y., Yun, T.K., Morgan, G. and Vainio, H. (2000). The cancer-preventive potential of *Panax ginseng*: a review of human and experimental evidence. *Cancer Causes & Control,* **11**: 565-576.

Sievenpiper, J.L., Arnason, J.T., Leiter, L.A. and Vuksan, V. (2004). Decreasing, null and increasing effects of eight popular types of ginseng on acute postprandial glycemic indices in healthy humans: the role of ginsenosides. *Journal of the American College of Nutrition,* **23(3)**: 248-258.

Smith, G.P. and Gibbs, J. (1992). The development and proof of the CCK hypothesis of satiety. *In*: Multiple cholecystokinin receptors in the CNS, *Ed.* By Dourish, C.T., Cooper, S.J., Iversen, S.D. and Iversen, L.L. Oxford University Press, Oxford, pp. 166-182.

Sotaniemi, E.A., Haapakoski, E. and Rautia, A. (1995). Ginseng therapy in non-insulin-dependent diabetes patients. *Diabetes Care,* **18**: 1373-1375.

Spiegelman, B.M. and Jeffery, S.F. (2001). Obesity and the regulation of energy balance. *Cell,* **104**: 531-543.

Surwit, R.S., Feinglos, M.N., Rodin, J., Sutherland, A., Petro, A.E., Opara, E.C., Kuhn, C.M. and Rebuffe-Scrive, M. (1995). Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. *Metabolism,* **44**: 645-651.

Weigle, D.S. (2003). Pharmacological therapy of obesity: past, present, and future. *The Journal of Clinical Endocrinology and Metabolism,* **88**: 2462-2469.

West, D.B., Boozer, C.N., Moody, D.L. and Atkinson, R.L. (1992). Dietary obesity in nine inbred mouse strains. *The American Journal of Physiology,* **262**: 1025-1032.

West, D.B. and York, B. (1998). Dietary fat, genetic predisposition, and obesity: lessons from animal models. *The American Journal of Clinical Nutrition,* **67**: 505-512.

Yokosuka, M., Xu, B., Pu, S., Kalra, P.S. and Kalra, S.P. (1998). Neural substrates for leptin and neuropeptide Y. (NPY) interaction: hypothalamic sites associated with inhibition of NPY-induced food intake. *Physiology & Behavior,* **64(3)**: 331-338.

Zhao, H.L., Sim, J.S., Shim, S.H., Ha, Y.W., Kang, S.S. and Kim, Y.S. (2005). Antiobese and hypolipidemic effects of platycodin saponins in diet-induced obsess rats: evidence for lipase inhibition and calorie intake restriction. *International Journal of Obesity,* **29**: 983-990.

7

Carotenoids in Commercially Important Crustaceans from Indian Waters

SACHINDRA N.M.[1,*], BHASKAR N.[1] AND MAHENDRAKAR N.S.[1]

ABSTRACT

Crustacean fishery, particularly shrimps, is one of the major contributors to the Indian economy. Nearly 450,000 MT of crustaceans are produced annually in India, both from natural waters and from culture in marine and brackish water environments. Crustaceans are one of the important groups of marine animals containing carotenoids that are derived from their diets. Carotenoids are known to have several beneficial physiological functions like provitamin A activity, antioxidant activity, antitumor activity and enhancement of immune system. Detailed studies have been carried out at CFTRI on quantitative and qualitative distribution of carotenoids in commercially important crustaceans from Indian waters, and, recovery of carotenoids from shrimp processing waste. The carotenoid content in shrimps was found to vary depending on species and the body components. Shrimps from deep-sea waters had higher carotenoid content compared to those from shallow waters. Highest carotenoid content was observed in the head of deep-sea shrimp Aristeus alcocki. The body carapace of shrimps also had high levels of carotenoids, while the meat portion had low levels of carotenoids. One of the important crabs, Charybdis cruciata, harvested from Indian seas had low levels of carotenoids in both meat and shell. Free astaxanthin, astaxanthin monoester and astaxanthin diester were the major carotenoids in the extracts from different body components of shrimps. β-carotene and zeaxanthin were also observed at low levels in these extracts. In the carotenoid extracts from crab also, astaxanthin and its esters dominated the carotenoid composition.

1. Department of Meat, Fish and Poultry Technology, Central Food Technological Research Institute (CFTRI), Mysore - 570 020, India.
Council of Scientific and Industrial Research Institute, India
* *Corresponding author* : E-mail : sachiprathi@yahoo.com

Saturated fatty acids dominated the fatty acid profile of solvent extracted carotenoids from different body components. The major fatty acids in these extracts were palmitic (C16:0), heptadecanoic (C17:1) and oleic (C18:1) acids. Methods were developed to recover the carotenoids from shrimp waste based on their solubility in organic solvents and vegetable oils. As large quantities of shrimp waste are generated in the Indian shrimp processing industries, there is a high potential for recovering these valuable components from these byproducts. The recovered components would find application as pigment source in aquaculture feeds for shrimps/ornamental fishes and as natural colorant in fish/meat based products, as an alternative to more expensive synthetic carotenoids.

Key words : Crustaceans, shrimp, prawn, crab, carotenoids, astaxanthin

INTRODUCTION

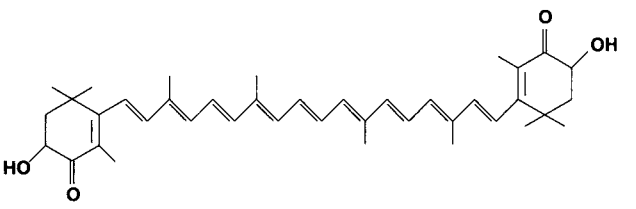
Carotenoids are a group of fat-soluble pigments found principally in plants, algae, photosynthetic bacteria and animals, and are responsible for the various colors found in nature. The carotenoids can be divided into two major classes as, the highly unsaturated carotene hydrocarbons which contain no oxygen (*e.g.* lycopene and β-carotene) and oxygenated derivatives of carotenes called xanthophylls (*e.g.* astaxanthin, zeaxanthin). Generally carotenes have greater distribution in plants than in animals, while xanthophylls are more widely distributed both in plants and animals (Shahidi *et al.*, 1998).

It is generally accepted that animals are unable to synthesize carotenoids *de novo*, but are able to modify dietary plant carotenoids (Buchecker, 1982). Animals incorporate carotenoids from their diets and provide bright coloration, serve as antioxidants and can be a source of vitamin A (Britton *et al.*, 1995).Thus the distribution of carotenoids in animal sources is primarily the result of specific dietary habits, absorption and metabolic transformation (Torrison, 2000). In animals the astaxanthin is the most widely distributed xanthophylls, followed by lutein and zeaxanthin (Haard, 1992).

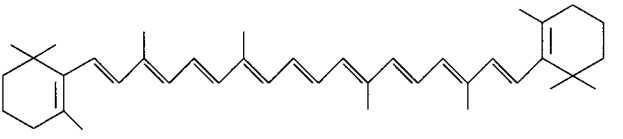
CAROTENOIDS IN MARINE ANIMALS

Carotenoids are responsible for the color of many important fish and shellfish products and color intensity is one of the factors influencing the grading or pricing of several fish and crustaceans (Sacton, 1986). As carotenoids in animal tissues are solely derived from their dietary intake, aquatic animals are no exception. However, they differ in the requirements and assimilation of carotenoids (Shahidi *et al.*, 1998). The primary source of carotenoids for aquatic animals is phytoplankton. The ingested carotenoids may be assimilated as such or may be converted to other form or may be completely

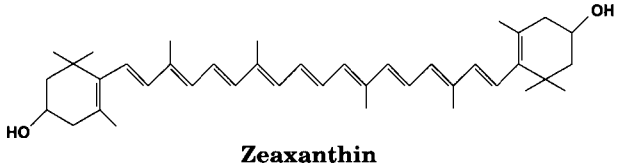
catabolized, or may be passed out via feces (Haard, 1992). In crustaceans, astaxanthin is formed from β-carotene (Fig 1) or zeaxanthin (Fig 1) through oxidative transformation (Katayama *et al.,* 1971). The carotenoids also follow reductive metabolic pathway in some aquatic organisms.



Astaxanthin



β-Carotene



Zeaxanthin

Fig 1. Structure of common carotenoids occurring in crustaceans

Crustaceans such as shrimp, prawn, lobster, krill and crab contain astaxanthin (Fig 1) as their main pigment (Latscha, 1990). Crustaceans absorb the pigments from the diet and deposit them as such or transfer them metabolically to keto or hydroxy derivatives (Castillo *et al.,* 1982). The pigments may be present in free forms, esterified or as bound form to macromolecules such as protein or chitin (Goodwin, 1984). The complex forms of carotenoids in crustaceans were identified to be carotenolipoproteins, chitinocarotenoids and carotenoproteins (Ghidalia, 1985). Carotenolipoproteins are the complex of carotenoids, lipids and proteins found mainly in ovaries and eggs, while chitinocarotenoids are found in exoskeleton, where they are formed by a Schiff base bonds between terminal basic nitrogen bonds of chitin and keto group of carotenoids (Ghidalia, 1985). Carotenoproteins are the carotenoid protein complexes and association of the carotenoids with protein results in display of various colors in crustaceans (Zagalsky, 1985) and cleavage of this complex results in color change due to the liberation of free carotenoid (Nelis *et al.,* 1989).

CAROTENOIDS IN CRUSTACEANS FROM INDIAN WATERS

India, with its vast fishery resources, is one of the major contributors to the world fish production. Major quantity of fish produced in India is consumed fresh (3.9 million tonnes) and 24% of fish harvested is processed, mainly for export. There are 400 seafood freezing plants along the Indian coast, with a built-in-capacity of 7284 tonnes per day. Nearly 190,000 tonnes of crustaceans are processed annually in these export oriented seafood processing industries. The export of seafood from India during 2006-07 was 612,000 tonnes valued at Rs 8363 Crores. Shrimps, valued at Rs. 4506 Crores, contributed 22% of the total quantity of seafood exported. (www.mpeda.com).

Shrimp processing for freezing normally involves removal of head and body carapace. It is estimated that the generation of byproducts in the form of head and body carapace from the Indian seafood industry is around 100, 000 tonnes (Gopakumar, 1993). These byproducts are good sources of protein (35 – 40% DWB), chitin (10 – 15% DWB), minerals and natural carotenoids. At present, these byproducts are being used in small quantities as shrimp meal for use in aquaculture and poultry feed, and for production of chitin/chitosan. A considerable quantity of these byproducts is being wasted, resulting not only in the loss of valuable components but also environment pollution.

Research efforts on effective utilization of shrimp waste were mainly focused on recovery of chitin. Not much attention has been paid towards extraction of other valuable components such as carotenoids. There is a great demand for natural carotenoids for use as colorants in fish products and as pigment source in aquaculture diets. The synthetic pigments like carophyll red (canthaxanthin) and carophyll pink (astaxanthin) presently used in fish culture are very expensive. Thus natural carotenoids recovered from shrimp waste will have profound utility value in fish culture as well as in fish products industries.

The research efforts on characterization of carotenoids in crustaceans were mostly restricted to species from temperate waters. The information on carotenoids in crustaceans from tropical waters, especially from Indian waters is limited. Further, the recovery of carotenoids from byproducts of Indian shrimps of commercial value and their utilization needs attention. Extensive studies have been carried out at Central Food Technological Research Institute (CFTRI), Mysore, India, to characterize the carotenoids in different crustaceans like shrimp and crab from both marine and fresh waters. Further, the recovery of carotenoids from shrimp waste by different methods, factors affecting their recovery has been investigated.

Total Carotenoid Content

Total carotenoid content in different body parts of crustaceans analysed from Indian waters is summarized in Table 1. Total carotenoid content (μg/g) in shrimps from shallow waters (*Penaeus monodon, P. indicus, Parapenaeopsis stylifera* and *Metapenaeus dobosnii)*) and deep sea (*Aristeus alcocki* and *Solonocera indica)* ranged from 10.4 to 21.4 in meat, 35.8 – 185.3 in head and 59.8 – 117.4 in carapace. In case of *Penaeus monodon, P indicus* and *Solonocera indica,* the carotenoid content was higher in carapace than in head, while in *Parapenaeopsis stylifera* and *Aristeus alcocki* the carotenoid content was higher in head than in carapace. Highest carotenoid content was observed in the head of *A. alcocki* (185.3 μg/g) followed by head of *P stylifera* (153.1 μg/g). Comparatively the fresh water prawn *Macrobrachium rosenbergii* had lower carotenoid content. In general the carotenoid content was highest in all the body components of the deep-sea shrimp *A alcocki.* Carotenoid content in the crab was low, highest being 11.0 μg/g in the shell of marine crab.

Table 1. Total carotenoid content (μg/g) in different body parts of crustaceans from Indian waters*

Species	Body part		
	Meat	**Head**	**Carapace**
Penaeus monodon (Shallow water shrimp)	17.4	58.4	86.6
Penaeus indicus (Shallow water shrimp)	10.4	35.8	59.8
Metapenaeus dobsoni (Shallow water shrimp)	11.1	51.3	83.3
Parapenaeopsis stylifera (Shallow water shrimp)	16.0	153.1	104.7
Solonocera indica (Deep-sea shrimp)	15.9	67.7	116.0
Aristeus alcocki (Deep-sea shrimp)	21.4	185.3	117.4
Macrobrachium rosenbergii (Fresh water prawn)	2.7	34.4	40.7
Charybdis cruciata (Marine crab)	3.4	-	11.0
Potamon potamon (Fresh water crab)	4.1	-	6.9

* Source: Sachindra, 2003; Sachindra *et al.*, 2005a, b; 2006a

The total carotenoid content in crustaceans was found to vary depending on species (Lambertson & Brakken, 1971). Okada *et al.* (1994) analysed tiger prawn (*P. monodon*) from waters of Indo-Pacific region and reported that the total carotenoid content varies from 23 – 331 μg/g in the exoskeleton, with a lower level in prawns having a pale blue body color and highest being in prawn having dark gray body color. The carotenoid content in crabs has been reported to be low. Shahidi and Synowiecki (1991) reported that the carotenoid content in the shells of snow crab *Chinocetes opilio,* was 14 μg/g. The carotenoid content in blue crab *Callinectes sapidus* was 4.63 μg/g (Felix-Valenzuela *et al.,* 2001).

The results indicated that the commercially important shrimp species harvested from the Indian waters contain variable level of carotenoids. The waste from the shallow water shrimp *P. stylifera,* and the deep-sea shrimps

contain highest carotenoid level. Fresh water prawn and crabs showed comparatively lower level of carotenoids.

Qualitative Distribution of Carotenoids

Thin layer chromatographic separation of carotenoid extracts from body components of shrimp, prawn and marine crab and comparison with standards indicated the presence of astaxanthin, astaxanthin mono- and di-ester and β-carotene, while the carotenoid extract from fresh water crab contained zeaxanthin in addition to the above four carotenoids.

High performance liquid chromatography (HPLC) of carotenoid extracts indicated that astaxanthin and its esters were the major carotenoids in the extract from shrimp, prawn and marine crab, while zeaxanthin was the major carotenoid fraction in fresh water crab. Content of each carotenoid in the total carotenoid extract as determined by HPLC is summarized in Table 2. In shrimps astaxanthin content (% of total carotenoids) ranged from a low of 14.9 in the carapace of *S. indica* to a high of 42.2 in the meat of *P. stylifera*. Astaxanthin monoester content (% of total carotenoids) ranged from 20.5 in meat of *P. indicus* to 49.8 in the meat of *A. alcocki*. Composition of β-carotene and zeaxanthin was low in carotenoid extracts in shrimp, highest being 10.3% in the meat of *S. indica* and 12.2% in the meat of *P. monodon* respectively. In general, zeaxanthin content was higher in body components of *P. monodon* compared to other species of shrimp. The results indicated that astaxanthin and its esters contribute 63.5 – 92.2% to the total carotenoid content in shrimps analyzed.

Table 2. Composition (% of total carotenoids) of major carotenoids in the carotenoid extract from different species of crustaceans*

Species	Body component	Astaxanthin	Astaxanthin monoester	Astaxanthin diester	β-Carotene	Zeaxanthin	Unidentified
P. monodon	Meat	22.2	43.1	15.2	1.1	12.2	6.2
	Head	24.3	22.6	20.3	4.9	5.7	22.2
	Carapace	28.8	44.0	13.9	1.7	5.5	6.2
P. indicus	Meat	32.9	20.5	17.9	5.5	1.7	21.4
	Head	25.5	27.3	19.3	5.5	1.4	17.7
	Carapace	24.3	26.8	25.1	3.8	1.1	18.8
M. dobsoni	Meat	26.7	21.1	20.8	7.3	0.5	23.6
	Head	24.2	22.4	21.3	6.5	0.9	18.3
	Carapace	33.2	22.4	21.2	4.4	0.6	18.3
P. stylifera	Meat	42.2	26.0	10.3	7.3	1.3	12.7
	Head	22.6	29.1	29.6	4.4	1.7	14.6
	Carapace	18.8	32.1	20.3	1.6	1.0	26.2
S. indica	Meat	23.2	24.3	19.5	10.3	1.8	20.8
	Head	19.4	23.9	20.2	5.5	2.8	24.7
	Carapace	14.9	39.0	19.4	1.1	1.5	25.1

Table 2. *Contd.*

Species	Body component	Astaxanthin	Astaxanthin monoester	Astaxanthin diester	β-Carotene	Zeaxanthin	Unidentified
A. alcocki	Meat	15.1	49.8	24.0	0.8	0.6	9.8
	Head	25.4	46.3	20.5	1.0	1.2	5.8
	Carapace	26.5	40.7	21.0	1.6	4.3	5.8
M. rosenbergii	Meat	29.7	12.3	12.9	21.8	0.3	24.4
	Head	24.6	12.8	14.5	29.6	1.3	16.2
	Carapace	29.8	18.2	16.3	5.5	0.8	29.4
C. cruciata	Meat	17.3	26.4	23.9	3.6	0.5	27.6
	Shell	23.6	15.2	26.7	5.1	5.1	24.4
P. potamon	Meat	9.3	11.2	16.0	7.4	42.0	14.1
	Shell	7.2	3.7	3.8	3.6	74.8	6.9

* Source: Sachindra, 2003; Sachindra *et al.*, 2005a, b, 2006a

In freshwater prawn, *M. rosenbergii,* along with astaxanthin and its esters β-carotene was also found to be a major pigment. β-Carotene content ranged from 5.5% in carapace to 29.6% in head. Astaxanthin content was higher than its esters and the total astaxanthin and esters content ranged from 51.9 to 64.3%. Zeaxanthin content was low in prawns. Astaxanthin and its esters were found to be major pigments in marine crab *Charybdis cruciata*, with a total content of 67.6 in meat and 65.5% in shell. β-Carotene content was 3.6% in meat and 5.1% in shell. In freshwater crab, *Potamon potamon*, zeaxanthin was the major pigment both in meat (42.0%) and shell (74.8%). The total content of astaxanthin and its esters in freshwater crab was 36.5% in meat and 14.8% in shell and β-carotene content was 7.4% in meat and 3.6% in shell.

Astaxanthin and its esters have been found to be the major carotenoids in crustaceans (Shahidi *et al.*, 1998). In the Indian shrimp, *P. stylifera*, Balachandran (1976) reported the presence of astaxanthin as the major pigment. Okada *et al.* (1994) reported that astaxanthin in free, mono and diester forms constitutes 86 – 98% of total pigments in *P. monodon*. They also reported the presence of small amounts of β-carotene (3.6%) and zeaxanthin (1.5%) in the exoskeleton of *P. monodon*. Astaxanthin and its esters have also been isolated as major carotenoid from the shrimp *P. borealis* (Shahidi *et al.*, 1992) and *Penaeus japonicus* (Negre-Sadargues *et al.*, 1993) and in deep-sea shrimp from Atlantic waters (Negre-Sadragues, 2000). Fresh water prawn *M. rosenbergii* can convert dietary β-carotene to astaxanthin. In the present study, β-carotene was also found to be a major pigment along with astaxanthin and its esters. The presence of β-carotene in large quantities may be due to composition of feed for the cultured prawns used in the study.

Fatty Acid Profile of Carotenoid Extracts

The fatty acid profile of carotenoid esters from carotenoid extract of different shrimps and prawn indicated that C16:0, C17:0 and C18:0 are the major saturated fatty acids and C16:1, and C18:1 are the major unsaturated fatty acids, with which carotenoids are esterified in majority of samples analysed (Sachindra, 2003; Sachindra *et al.,* 2005a, 2006a). Short chain fatty acids like C8:0 and C10:0 were present in considerable quantities in the carotenoid esters from carapace of *P. monodon* and C10:0 in meat of deep-sea shrimp *A. alcocki*. Saturated fatty acids predominated in carotenoid esters from all the body components of *P. monodon, P. indicus* and *P. stylifera*, meat and head of *M. dobsoni*, in meat and carapace of *S. indica* and meat of *A.. alcocki* and *M. rosenbergii*. In carotenoid esters from crabs, unsaturated fatty acids were higher than the saturated fatty acids. C16:0 was the major saturated fatty acid in the carotenoid esters from marine crab meat and shell, while C17:0 was the major saturated fatty acid in the shell of fresh water crab (Sachindra *et al.,* 2005b). Among unsaturated fatty acids, C16:1 predominated in carotenoid esters from marine crab shell, C18:1 in marine crab meat, C18:3 in fresh water crab shell and C20:1 in fresh water crab meat.

The results indicated that the major fatty acids associated with the carotenoid extracts in the crustaceans analyzed are palmitic acid (C16:0), heptadecanoic acid (C17:0), stearic acid (C18:0), palmitoleic acid (C16:1) and oleic acid (C18:1). Even though, Snauweart *et al.* (1973) reported the dominance of these fatty acids in carotenoid esters from brown shrimp *Cragnon vulgaris*, Renstrom and Liaaen-Jensen (1981) observed no such preferential selection of fatty acids in the carotenoid esters of shrimp *P. borealis*. No such observations were made in the present study. They also reported the high composition of unsaturated fatty acids in carotenoid esters and concluded that the marine animals living in cold waters contain more of unsaturated fatty acids than those living in warm waters.

Gopakumar and Nair (1975) reported that the composition of fatty acids in the lipid extract of the meat from three species of Indian shrimps is almost equally distributed between saturated and unsaturated fatty acids, with predominance of C16:0, C18:0 and C18:1 fatty acids. Palmitic acid, palmitoleic acid, stearic acid and oleic acid were also found to be the major fatty acids in the lipids of fresh water prawn *M. rosenbergii* (Nair & Gopakumar, 1984). The reported fatty acid profiles of meat from the Indian shrimps and prawn and the fatty acid profile of carotenoid esters indicate that the carotenoids are esterified with the predominant fatty acid present in the body of crustaceans.

RECOVERY OF CAROTENOIDS FROM CRUSTACEAN WASTE

Several studies have been carried out to recover the pigments from crustacean processing discards. Methods such as extraction of carotenoids using organic solvents and edible oils and recovery of carotenoids as carotenoprotein have been attempted. Solvent extraction process for recovery of carotenoids from crustacean waste has been limited to analytical purposes. Meyers and Bligh (1981) extracted pigments from heat processed crawfish waste using a ternary system of ether, acetone and water. Britton (1985) outlined the protocols for solvent extraction of carotenoids as analytical tool. Carotenoids in shrimp waste can be extracted using cold acetone and subsequently partitioned using petroleum ether (Mandeville *et al.,* 1991). Masatoshi and Junji (1999) used acetone for extraction of carotenoids from acidified shrimp waste. Supercritical CO_2 method with ethanol as co-solvent has also been attempted for astaxanthin extraction from crawfish shells (Charest *et al.,* 2001).

Studies have been carried in our laboratory to develop methods for extraction of carotenoids using organic solvents (Sachindra *et al.,* 2006b). The solvent extracted carotenoid was in the paste form with an orange red color (Fig 2). Highest carotenoid yield (43.9 µg/g waste) from waste of *P indicus* was obtained when the carotenoids were extracted with a mixture of isopropyl alcohol (IPA) and hexane, followed by IPA (40.8 µg/g) and acetone alone (40.6 µg/g). The lowest carotenoid yield was obtained with two non-polar solvents, petroleum ether (12.1 µg/g) and hexane (13.1 µg/g). Maximum quantity (77.8% of total carotenoids) of carotenoids was extracted in the first extraction itself, when extracted with a 50:50 mixture of IPA and hexane The 2nd extraction yielded 15.6% of total carotenoid. The optimized conditions for the solvent extraction of carotenoid from shrimp waste was found to be

Fig 2. Solvent extracted carotenoid

60% hexane in the solvent mixture of IPA and hexane, solvent to waste level of 5 in each extraction and 3 number of extractions. The flow diagram for extraction of carotenoids using organic solvents is given in Fig 3.

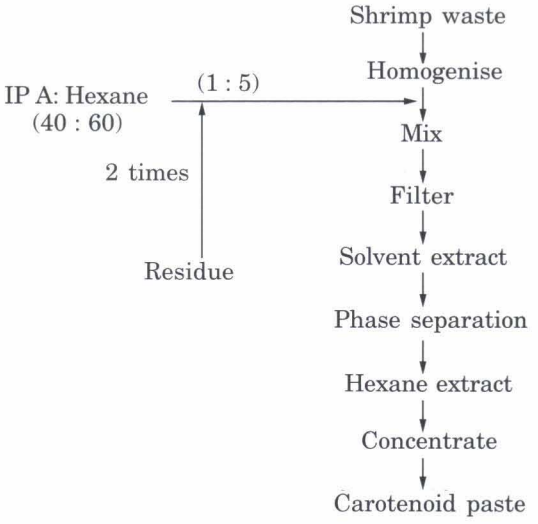


Fig 3. Flow diagram for recovery of carotenoids from shrimp waste using organic solvents (Sachindra *et al.*, 2006b)

The use of IPA and hexane instead of normally used acetone is beneficial in the large-scale extraction of carotenoids from shrimp waste, as cost of IPA and hexane is lower than that of acetone and the yield of carotenoid is higher. The present experiment was conducted using the waste from the shrimp *P. indicus,* which has shown lowest level of carotenoids among the marine shrimps analyzed. The results obtained would be applicable to waste from other species of shrimps and prawns as well.

Britton (1985) recommended the use of water miscible polar organic solvents, usually acetone, methanol or ethanol for extraction of carotenoids from tissues containing water. Delgado-Vargus *et al.* (2000) discussed the advantages and disadvantages of various organic solvents for extraction of carotenoids and suggested that polar solvents are generally good extraction media for xanthophylls but not for carotenes. For wet tissues, use of non-polar solvents is not recommended as their penetration through the hydrophobic mass that surrounds the pigment is limited (Delgado-Vargus *et al.,* 2000). The increased extraction yield of carotenoids by the mixture of IPA and hexane may be due to the reason that along with xanthophylls, increased amount of carotenes are also extracted due to the inclusion of a non-polar solvent in the extraction medium. Further it is stated that, when IPA or mixture of IPA and hexane was used for oil extraction, more antioxidants were extracted and oils with extended stability were obtained

(Procter & Bowen, 1996). Shrimp waste is known to contain antioxidants (Li *et al.,* 1998), thus use of IPA and hexane for extraction of carotenoids may improve their stability during storage.

As carotenoids in crustacean wastes are fat soluble, vegetable oils have been used to extract pigments from waste. Chen and Meyers (1982) used enzymatic hydrolysis of homogenised crawfish waste with a protease and subsequent extraction with soy oil for recovery of carotenoids. The extraction of carotenoids using different oils such as soybean, cottonseed, herring, menhaden and salmon oil was attempted by Chen and Meyers (1984). No and Meyers (1992) demonstrated that the process of oil extraction of carotenoids from crawfish waste can be integrated with production of chitin and chitosan. Cod liver oil also has been used to extract pigments from processing discards of snow crab and shrimp waste (Shahidi & Synowiecki, 1991).

Our efforts on developing a suitable oil extraction method (Fig 4) for carotenoids from shrimp waste resulted in optimization of condition for maximum carotenoid yield (Sachindra & Mahendrakar, 2005). The pigmented oil recovered was orange red in color (Fig 5). Comparison between different vegetable oil for carotenoid extraction indicated that sunflower oil gives highest carotenoid yield while mustard oil gives lowest yield. The extraction yield of carotenoid in coconut oil and rice bran oil was comparable with that of soy oil. The optimized conditions for the extraction of carotenoids from shrimp waste using soy oil were found to be adding oil to the waste in a ratio of 2 : 1 and heating the mixture at 70°C for 150 min. The pigmented oil can be recovered by centrifuging the treated waste and phase separation.

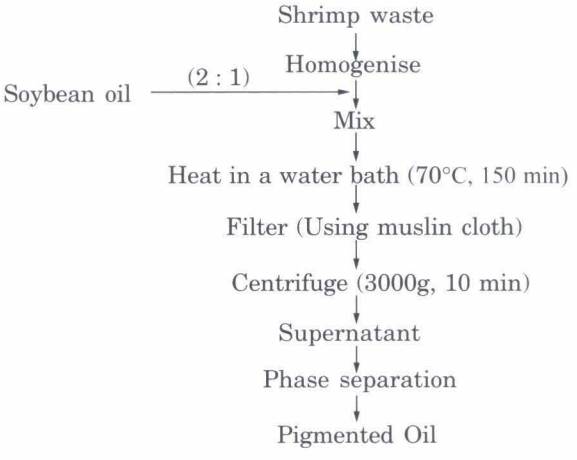


Fig 4. Flow diagram for recovery of carotenoids from shrimp waste using vegetable oil (Sachindra & Mahendrakar, 2005)

Fig 5. Oil extracted carotenoid

The carotenoid yield by oil extraction was found to be lower than that obtained by solvent extraction. However the advantage of oil extraction process is that the pigmented oil finds use as carotenoid source in aquaculture feeds, as oil serves as pigment carrier as well a source of energy (Spinelli & Mahnken, 1978). The use of oils as an ingredient in feed preparation is mainly as source of energy. Thus concentration of carotenoids in the oil would be advantageous, as required carotenoid concentration in the feed and can be achieved by minimum addition of pigmented oil without affecting the energy balance.

Torrison *et al.* (1981) attempted acid ensilaging as a method for stabilization of astaxanthin in shrimp waste during storage prior to oil extraction. Acid ensilaging of crawfish waste was found to stabilize the astaxanthin in the waste and also increase the recovery of astaxanthin in soy oil (Chen & Meyers, 1983). Guillou *et al.* (1995) observed that silaging treatment of shrimp waste was effective in stabilizing astaxanthin in the waste and also increasing the carotenoid recovery by solvent extraction. Our studies on effect of ensilaging on stability and recovery of carotenoids from shrimp waste (Sachindra *et al.,* 2007a) indicated that acid ensilaging results in reduction of solvent extraction yield of carotenoids but oil extraction yield was not affected. Further fermentation ensilaging was found to be better option for stabilizing the carotenoids in shrimp waste without affecting its recovery (Bhaskar *et al.,* 2007).

As carotenoids are more stable as complex with proteins, studies have been carried out on recovery of carotenoids as carotenoproteins. Simpson and Haard (1985) developed an enzymatic technique for extraction of carotenoprotein from shrimp waste using chelating agents like EDTA and the proteolytic enzyme trypsin. Cano-Lopez *et al.* (1987) used trypsin from Atlantic cod instead of bovine trypsin for increased recovery of carotenoprotein from shrimp waste. Trypsin hydrolysis of snow crab waste

followed by ammonium sulphate precipitation yielded carotenoprotein with increased carotenoid content (Manu-Tawiah & Haard, 1987). Carotenoprotein from crawfish waste has also been extracted by a fermentation process (Cremades *et al.,* 2001). Lyophilised fermentation liquor from Indian shrimp waste was found to be rich in carotenoids and exhibited strong antioxidant activity (Sachindra & Bhaskar, 2008).

CONCLUSIONS

As shrimp processing is one of the major export oriented food processing industries in India, and large quantities of byproducts are generated during processing, R & D efforts should be more focused on recovery of valuable biomolecules from these wastes. Our efforts have indicated the potential of shrimp waste as an important source of carotenoids. Carotenoids from marine source are known to possess various biofunctions such as antioxidant activity, anticancer activity and antiobesity effects (Sachindra *et al.,* 2007b). Thus, further studies should be carried out on the biological functions of these recovered carotenoids and the use of these carotenoids in food and feed needs to be explored.

REFERENCES

Balachandran, K.K. (1976). On the carotenoid pigments in Indian prawns. *Fisheries Technology*, **13**: 121-125.

Bhaskar, N., Suresh, P.V., Sakhare, P.Z. and Sachindra, N.M. (2007). Shrimp biowaste fermentation with *Pediococcus acidolactici* CFR2182: Optimization of fermentation conditions by response surface methodology and effect of optimized conditions on deprotenization/demineralization and carotenoid recovery. *Enzyme and Microbial Biotechnology,* **40**: 1427-1434.

Britton, G. (1985). General carotenoid methods. *Methods in Enzymology,* **111**: 113-149.

Britton, G., Liaaen-Jensen, S. and Pfander, H. (1995). Carotenoids today and challenges for the future. *In*: Carotenoids Vol. 1 A Isolation and analysis. *Ed.* By Britton, G., Liaaen-Jensen, S. and Pfander, H., Birkhauser, Basel.

Buchecker, R. (1982). A chemist's view of animal carotenoids. *In*: Carotenoid chemistry and biochemistry. *Ed.* By Britton, G. and Goodwin, T.W, Pergamon Press, Oxford.

Cano-Lopez, A., Simpson, B.K. and Haard, N.F. (1987). Extraction of carotenoprotein from shrimp process waste with the aid of trypsin from Atlantic cod. *Journal of Food Science*, **52**: 503-506.

Castillo, D.F., Lee, W.L. and Zagalsky, P.F. (1982). General survey of the carotenoids in crustacea. *In*: Carotenoid chemistry and biochemistry, *Ed.* By Britton, G. and Goodwin, T.W, Pergamon Press, Oxford.

Charest, D.J., Bulaban, M.O., Marshall, M.R. and Cornell, J.A. (2001). Astaxanthin extraction from crawfish shells by supercritical CO_2 with ethanol as cosolvent. *Journal of Aquatic Food Product Technology*, **10**: 79-93.

Chen, H.M. and Meyers, S.P. (1982). Extraction of astaxanthin pigment from crawfish waste using a soy oil process. *Journal of Food Science,* **47**: 892-896.

Chen, H.M. and Meyers, S.P. (1983). Ensilage treatment of crawfish waste for improvement of astaxanthin pigment extraction. *Journal of Food Science,* **48**: 1516-1520.

Chen, H.M. and Meyers, S.P. (1984). A rapid quantitative determination of astaxanthin pigment concentrate in oil extraction. *Journal of American Oil Chemists Society*, **61**: 1045-1047.

Cremades, O., Ponce, E., Corpas, R., Gutierrez, J.F., Jover, M., Alvarez-Ossorio, M.C., Parrado, J. and Bautista, J. (2001). Processing of crawfish (*Procambarus clarkii*) for

the preparation of carotenoproteins and chitin. *Journal of Agriculture and Food Chemistry,* **49**: 5468-5472.

Delgado-Vargus, F., Jimenez, A.R. and Peredes-Lopez, O. (2000). Natural pigments: carotenoids, anthocyanins and betalains: characteristics, biosynthesis, preparation and stability. *CRC Critical Reviews in Food Science and Nutrition,* **40**: 173-289.

Felix-Valenzuela, L., Higuera-Ciapara, I., Goycoolea-Valencia, F. and Arguelles-Monal, W. (2001). Supercritical CO_2/ethanol extraction of astaxanthin from blue crab (*Callinectes sapidus*) shell waste. *Journal of Food Processing Engineering,* **24**: 101-112.

Ghidalia, W. (1985). Structural and biological aspects of pigments. *In*: The biology of crustacea. Vol. 9, *Ed.* By Bliss, D.E. and Mantel, L.H, Academic Press, New York.

Goodwin, T.W. (1984). The biochemistry of carotenoids Vol. 2. Animals. Chapman and Hall, London.

Gopakumar, K. (1993). Shrimps and crab shell utilization. *Fishing Chimes,* **13**: 67-69.

Gopakumar, K. and Nair, M.R. (1975). Composition of five species of Indian prawns. *Journal of Science of Food and Agriculture,* **26**: 319-325.

Guillou, A., Khalil, M. and Adambounou, L. (1995). Effects of silage preservation on astaxanthin forms and fatty acid profiles of processed shrimp (*Pandalus borealis*) waste. *Aquaculture,* **130**: 351-360.

Haard, N.F. (1992). Biochemistry and chemistry of color and color changes in seafood. *In*: *Advances in seafood biochemistry, composition and quality. Ed.* By Flick, G.J. and Martin, R.E., Technomic Publishing Co. Inc, Lancaster.

Katayama, T., Hirata, K. and Chichester, C.O. (1971). The biosynthesis of astaxanthin IV. The carotenoids in the prawn *Penaeus japonicus. Bulletin Japanese Society of Scientfic Fisheries,* **37**: 614-620.

Lambertsen, G. and Braekkan, O.R. (1971). Methods of analysis of astaxanthin and its occurrence in some marine products. *Journal of Science of Food and Agriculture,* **22**: 99-101.

Latscha, T. (1990). Carotenoids: Their nature and significance in animal feeds. Haffman – La Roch Ltd, Basel, Switzerland.

Li, S.J., Seymour, T.A., King, A.J. and Morrissey, M.T. (1998). Color stability and lipid oxidation of rockfish as affected by antioxidant from shrimp shell waste. *Journal of Food Science,* **63**: 438-441.

Mandeville, S., Yaylayan, V., Simpson, B.K. and Ramaswamy, H. (1991). Isolation and purification of carotenoid pigments, lipids and flavor active components from raw commercial shrimp waste. *Food Biotechnology,* **5**: 185-195.

Manu-Tawaiah, W. and Haard, N.F. (1987). Recovery of carotenoprotein from the exoskeleton of snow crab *Chinocetes opilio. Canadian Institute of Food Science Technology Journal,* **20**: 31-33.

Masatoshi, M. and Junji, S. (1999). Method for simultaneously producing astaxanthin and chitosan from shell waste. *Japanese Patent* No. JP 11049972A2, February 1999

Meyers, S.P. and Bligh, D. (1981). Characterization of astaxanthin pigment from heat processed crawfish waste. *Journal of Agriculture and Food Chemistry,* **29**: 505-508.

Nair, P.G.V. and Gopakumar, K. (1984). Lipid and Fatty acid composition of fish and shellfish. *Journal of Food Science and Technology,* **21**: 389-392.

Negre-Sadargues, G., Castillo, R. and Seginzac, M. (2000). Carotenoid pigments and tropic behavior of deep-sea shrimps from a hydrothermal area of the mid-Atlantic ridge. *Comparative Biochemistry and Physiology,* **127A**: 293-300.

Negre-Sadargues, G., Castillo, R., Petit, H., Sance, S., Gomez, R., Milicua, J.C.G. and Trilles, J.P. (1993). Utilization of synthetic carotenoids by prawn, *Penaeus japonicus* reared under laboratory condition. *Aquaculture,* **110**: 151-159.

Nelis, H.J., Lavens, P., Moens, L., Sorgeloss, P. and De Leenheer, A.P. (1989). Carotenoids in relation to *Artemia* development. *In*: Biochemistry and cell biology of Artemia. *Ed.* By MacRae, T.H., Bagslaw, J.C. and Warner, A.H. CRC Press, Boca Raton.

No, H.K. and Meyers, S.P. (1992). Utilization of crawfish processing wastes as carotenoid, chitin and chitosan source. *Journal of Korean Society of Food and Nutrition,* **21**: 319-326.

Okada, S., Nur-E-Borhan, S.A. and Yamaguchi, K. (1994). Carotenoid composition in the exoskeleton of commercial black tiger prawns. *Fisheries Science,* **60**: 213-215.

Procter, A. and Bowen, D.J. (1996). Ambient temperature extraction of rice bran oil with hexane and isopropanol. *Journal of American Oil Chemists Society*, **73**: 811-813.

Renstrom, B. and Liaaen-Jensen, S. (1981). Fatty acid composition of some esterified carotenols. *Comparative Biochemistry and Physiology,* **69B**: 625-627.

Sachindra, N.M. (2003). Studies on some crustaceans of tropical waters with special reference to pigments. Ph.D. Thesis, University of Mysore, India, 2003.

Sachindra, N.M. and Bhaskar, N. (2008). *In vitro* antioxidant activity of liquor from fermented shrimp biowaste. *Bioresource Technology,* **99**: 9013-9016.

Sachindra, N.M. and Mahendrakar, N.S. (2005). Extractability of carotenoids from shrimp waste in vegetable oils and process optimization. *Bioresource Technology*, **96**: 1195-1200.

Sachindra, N.M., Bhaskar, N. and Mahendrakar, N.S. (2005a). Carotenoids in different body components of Indian shrimps. *Journal of Science of Food and Agriculture,* **85**: 167-172.

Sachindra, N.M., Bhaskar, N. and Mahendrakar, N.S. (2005b). Carotenoids in crabs from marine and fresh waters of India. *LWT Food Science and Technology*, **38**: 221-225.

Sachindra, N.M., Bhaskar, N. and Mahendrakar, N.S. (2006a). Carotenoids in *Solonocera indica* and *Aristeus alcocki*, Deep-Sea Shrimps from Indian Waters. *Journal of Aquatic Food Products Technology*. **15**: 5-16.

Sachindra, N.M., Bhaskar, N. and Mahendrakar, N.S. (2006b). Recovery of carotenoids from shrimp waste in organic solvents. *Waste Management,* **26**: 1092-1098.

Sachindra, N.M., Bhaskar, N., Siddegowda, G.S., Sathisha, A.D. and Suresh, P.V. (2007a). Recovery of carotenoids from ensiled shrimp waste. *Bioresource Technology,* **98**: 1642-1646.

Sachindra, N.M., Hosokawa, M. and Miyashita, K. (2007b). Biofunctions of marine carotenoids. *In*: Biocatalysis and biotechnology for functional foods and industrial products. *Ed.* By Hou, C.T. and Shaw, J., CRC Press, NY.

Sacton, J. (1986). The seafood handbook: Seafood business. Seattle, WA, 70.

Shahidi, F. and Synowiecki, J. (1991). Isolation and characterization of nutrients and value added products from snow crab (*Chinoecetes opilio*) and shrimp (*Pandalus borealis*) processing discards. *Journal of Agriculture and Food Chemistry,* **39**: 1527-1532.

Shahidi, F., Synowiecki, J. and Naczk, M. (1992). Utilization of shellfish processing discards. *In*: Seafood Science and Technology. *Ed.* By Bligh, E.G., Fishing News Books, Oxford.

Shahidi, F., Metusalach. and Brown, J.A. (1998). Carotenoid pigments in seafoods and aquaculture. *CRC Critical Reviews in Food Science*, **38**: 1-67.

Simpson, B.K. and Haard, N.F. (1985). The use of enzymes to extract carotenoprotein from shrimp waste. *Journal of Applied Biochemistry*, **7**: 212-222.

Snauwaert, F., Tobback, P.P. and Maes, E. (1973). Carotenoid pigments in brown shrimp (*Crangon vulgaris*). *Lebens Wissen Technologie,* **6**: 43-47.

Spinelli, J. and Mahnken, C. (1978). Carotenoid deposition in pen reared salmonids fed diets containing oil extracts of red crab (*Pleuronnocodes planipes*). *Aquaculture*, **13**: 213-216.

Torrison, O.J. (2000). Dietary delivery of carotenoids. *In*: Antioxidants in muscle foods. *Ed.* By Decker, E., Faustman, C. and Lopez-Bote, C.L., John Wiley & Son.

Torrison, O.J., Tideman, E., Hansen, F. and Raa, J. (1981). Ensilaging in acid – a method to stabilize astaxanthin in shrimp processing by-products and improve uptake of this pigment by rainbow trout (*Salmo gairdneri*). *Aquaculture,* **26**: 77-83.

Zagalsky, P.F. (1985). Invertebrate carotenoproteins. *Methods in Enzymology,* **111**: 216-247.

"This page is Intentionally Left Blank"

8

Therapeutic Uses of Venoms

RUMI GHOSH[1]* AND VAIDEHI THANAWALA[1]

ABSTRACT

Venoms comprise a highly complex mixture of peptides which are currently being explored for their therapeutic options. The medicinal applications of venoms have been described in folk medicine, alternative medicine and the Ayurveda for centuries but it was only till about forty years ago that venoms were considered as viable pharmacological tools to unravel targets for the treatment of a number of disease states. Recently, a great deal of attention is being paid to the therapeutic applications of venom peptides. Many of these peptides have undergone clinical trials for a wide range of conditions ranging from diabetes mellitus to cancer. This review attempts to elucidate the pharmacology of venoms, their applications and recent developments in research on venoms as novel ligands with therapeutic potential.

Key words : Applications, clinical trials, potential, therapeutic, venoms

INTRODUCTION

Animals have evolved numerous ways to cope with feeding and defense, and one of these ways is by using poisons and venoms. Venomous animals use a toxin to kill prey or to defend themselves. Venoms are produced in specialized tissues or glands which often are connected with application structures. These animals are referred to as venomous (Meier & White, 1995). They deliver the toxin with specialized organs, like stingers, fangs, hollow fangs, a proboscis, or tentacles.The animals most widely known to use venom are snakes, some spiders and centipedes, scorpions and stinging insects, such as bees and wasps. Insects like ants also contain venom. Many

1. Bharati Vidyapeeth's College of Pharmacy, Sector 8, C.B.D. Belapur, Navi Mumbai - 400 614, India.

* *Corresponding author* : E-mail : rumi1968@hotmail.com

caterpillars have defensive venom glands associated with specialized bristles on the body, known as urticating hairs, and can be lethal to humans (*e.g.* that of the *Lonomia* moth). Besides snakes, venom is also present in other reptiles, such as the gila monster and Mexican beaded lizard. Apart from these some fishes, such as the cartilaginous fishes – stingrays, sharks, and chimaeras – and the teleost fishes including monognathus eels, catfishes, stonefishes and waspfishes, scorpionfishes and lionfishes, gurnard perches, rabbitfishes, surgeonfishes, scats, stargazers, weevers, carangids, saber-toothed blenny, and toadfish possess venom. In fact, recent studies have shown that there are more venomous ray-finned fishes than all other venomous vertebrates combined. There are many other venomous invertebrates, including jellyfish and cone snails. The box jellyfish is widely considered the most venomous creature in the world. Some mammals that are found to be venomous include solenodons, shrews, the slow loris, and the male platypus.

Venoms are complex mixtures of bioactive molecules that have evolved for prey capture and defense, many of these molecules have a high selectivity for physiological processes, including modulation of ion channel function, which has not been matched by man made molecules. The venom of animals have been used for healing various diseases since ancient times.

The biochemical effects of poisons and the toxins found in venomous and poisonous organisms have been the subject of much research for many decades. This research, resulted in elucidation of evenomation processes and the development of anti-venoms for the clinical treatment of snakebites. However, it has only been in the past decade that there has been an increased interest in biologically active toxins and poisons for use in therapeutically active drugs or pharmacological targets. The venom from snakes, bees, snails, toads and spiders are currently being studied for their therapeutic potential.

SNAKE VENOMS

Snake venoms are the natural sources of many compounds that fascinated people through the history of pharmacology. Current drug design research considers venoms of many snakes as a valuable source for identification of new compounds with potential application in the pharmacology of many diseases. Moreover, toxic and non toxic components of snake venoms are very useful reagents for conducting medical research and understanding the physiology and pathology of many processes occurring in the body. Good examples are the snake venom disintegrins which have facilitated the discovery of new anti-platelet peptides and peptidomimetics that are currently used as therapy for certain cardiovascular diseases.

Snake bites can be deadly, but the venoms also contain components of medical and biotechnological value. The proteomic characterization of snake venom proteomes, snake venomics, has thus a number of potential benefits for basic research, clinical diagnosis, and development of new research tools and drugs of potential clinical use (Calvette *et al.*, 2004; Hogg, 2006). Snake venoms are complex mixtures of pharmacologically active proteins and peptides. There is a wide variation in the composition of venoms within a species. The venom, the most complex of all poisons is a mixture of enzymatic and non-enzymatic compounds as well as other non-toxic proteins including carbohydrates and metals. There are over 20 different enzymes including phospholipases A2, B, C, D hydrolases, phosphatases (acid as well as alkaline), proteases, esterases, acetylcholinesterase, transaminase, hyaluronidase, phosphodiesterase, nucleotidase and ATPase and nucleosidases (Matsui *et al.*, 2000). The non-enzymatic components are loosely categorized as neurotoxins and hemorrhagens (Bhattacharya & Chakraborty, 2007). Different species have differing proportions of most if not all of the above mixtures - this is why poisonous species were formerly classified exclusively as neurotoxic, haemotoxic or myotoxic. The pathophysiological basis for morbidity and mortality is the disruption of normal cellular functions by these enzymes and toxins. Some enzymes such as hyaluronidase disseminate venom by breaking down tissue barriers.

The broad medical classification of snake venom activities can be given as shown in Fig 1 and their clinical effects are elaborated in Table 1 and some of them are discussed in detail below:

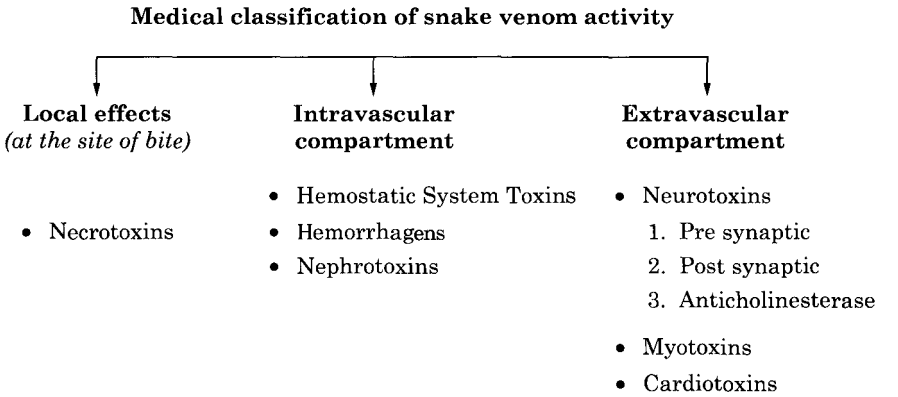


Fig 1. Broad medical classification of snake venom activity

Neurotoxins

The venoms of different snakes contain small proteins that have a similar size and homologous dispositions of disulfide bridges are called α-neurotoxins.

Table 1. Clinical effects of snake venom

	Activity	Clinical effects
Local effects	Necrotoxins	Direct tissue injury at the bite site/bitten limb
Intravascular compartment	Haemostatic System Toxins	Interfere with normal hemostasis, causing either bleeding or thrombosis
	Hemorrhagens	Damage vascular wall, causing bleeding
	Nephrotoxins	Direct renal damage
Extravascular compartment	Neurotoxins	Flaccid paralysis
	• Presynaptic	Resistant to late antivenom therapy
	• Post synaptic	Often reversal with antivenom therapy
	• Anticholinesterase	Fasciculation

Neurotoxins

The venoms of different snakes contain small proteins that have a similar size and homologous dispositions of disulfide bridges are called α-neurotoxins. Most neurotoxins competitively bind to the nicotinic acetylcholine receptor and contain 60–75 amino acid residues and are fixed by 4–5 disulfide bridges. These α-neurotoxins are comprised of two main structural forms, Type I (formerly short-chain) and Type II (formerly long-chain). The two main groups of toxins are similar in action but differ in size, from an average of slightly above sixty amino acids to an average of seventy-three amino acids having either four or five disulfide bridges respectively. These structural differences are due to the Type I neurotoxins having a primary structure similar to the Type II but with the latter having a C-terminal extension. The toxins bind with high affinity to 2α forms of skeletal nicotinic acetylcholine receptors but only the Type II neurotoxins bind to 7α neuronal acetylcholine receptors. In contrast kappa-neurotoxins, such as kappa-bungarotoxin, target the 3α-2β neuronal AChRs. The muscarinic toxins were first isolated from the venom of the Eastern green mamba (*Dendroaspis angusticeps*) with other toxins following from other members of this genus. Muscarinic toxins can be broken into two groups, A and B. Group A toxins have 65 or 66 amino acids, four disulfide bonds and a number of invariant residues. All of the group A toxins have a Lys or Arg at residue 34, differing from loop two of the α-neurotoxins and Trp 28, Tyr 30 and Tyr 36 are other essential and invariant residues (Tsetlin,1999). Cardiotoxins are basic membrane-active polypeptides that disorganize the structure of membranes. Cardiotoxins (or cytotoxins) do not have a specific receptor or ion channel target, rather they appear form channels in membranes (Bilwes *et al.*, 1994). Venoms from mamba snakes (*Dendroaspis* species) contain many neurotoxins which are pharmacologically different from toxins found in other related snake venoms (McDowell *et al.*, 1992). The binding of toxins to the outer parts of receptor molecules probably accounts for their much higher specificity for individual receptor subtypes than is seen with smaller ligands. Toxins are useful for identifying and blocking m1 and m4 receptors with

high specificity. Muscarinic toxins have shown to be useful in the characterization of receptor sub-types found in particular tissues. MT-3 was shown to be a reversible competitive antagonist at the m4 subtype muscarinic receptor. This toxin was used to identify the presence of m4 receptors in the rat brain through the selective inhibition of adenylate cyclase activity. M1 toxins were used to bind the m1 receptor on rat striated muscle tissue and m4-specific toxins were used to show that m4 subtype muscarinic receptors make up the vast majority of the remaining receptors. These receptors are being currently investigated the treatment of Parkinson's disease and Huntington's disease.

Other toxins have been shown to inhibit the binding of muscarinic ligands, specifically a 13,600 MW component from *Naja sputatrix* and a 13,800 MW component from *Crotalus atrox*. However, rather than being peptides such as in the mamba muscarinic toxins, these components are larger phospholipase A2 (PLA2) enzymes. These toxins were significantly less potent than those isolated from the mambas and displayed none of the useful specificity.

Myotoxins

Myotoxins are also called myonecrotic toxins and are found in venoms of rattlesnakes and other pit vipers. One of the best-known myotoxins is myotoxin-a, isolated from the venom of the Prairie rattlesnake *C. viridis viridis* (Ownby & Colberg, 1996). It is a small (4600 Da), basic protein devoid of enzymatic activity. Mytotoxin-a, binds specifically to the sarcoplasmic reticulum of muscles, causing a change in ion permeability of the sarcoplasmic reticulum leading to swelling and disintegration of both the sarcoplasmic reticulum and muscle fibrils. Most snake venom myotoxins are based on phospholipase A2 and cause systemic myolysis of skeletal muscle, rarely affecting cardiac or smooth muscle (Teixeira *et al.*, 1993). The damage occurs to individual muscle cells without affecting the basement membrane, thus regeneration of muscle usually occurs, commencing about 3 days after the bite and is complete after about 28 days. Interestingly, there are a number of Viperid species whose venom is neurotoxic and causes paralysis and but produces no or minimal effect on either local tissues at the bite site or on the haemostatic system.

Hemotoxins

Hemotoxins are toxins that cause hemolysis, disrupt blood clotting, and/or cause organ degeneration and generalized tissue damage. Snake venom components have been classified into various families, such as serine proteases, metalloproteinases, C-type lectins, disintegrins and phospholipases. The various members of a particular family act selectively on different blood coagulation factors, blood cells or tissues. The human hemostatic system is a target of snake venoms. The venom of the snake families Colubridae, Elapidae, Viperidae are known to contain hemotoxins.

The toxin groups include procoagulants, anticoagulants, platelet aggregation inhibitors and promoters. The effect of most of these toxins is to increase bleeding and in some cases clinical thrombosis. The mechanisms of increased bleeding vary, but most components acting as procoagulants exert their effect by consumption of fibrinogen, resulting in defibrination. Some venoms cause defibrination by direct action on fibrinogen, splitting fibrinopeptides. The biochemical nature and structure of these diverse toxins varies from comparatively small molecules to large, complex multi-component toxins that mimic normal clotting complexes such as the prothrombinase complex.

For almost every factor involved in coagulation or fibrinolysis there is a venom protein that can activate or inactivate it. Venom proteins affect platelet function by binding or degrading vWF or platelet receptors, activating protease-activated receptors or modulating ADP release and thromboxane A2 formation. Some venom enzymes cleave key basement membrane components and directly affect capillary blood vessels to cause hemorrhaging. L-amino acid oxidases activate platelets via H_2O_2 production (Lu *et al.*, 2005).

Uses

Fibrinolytic

Envenomations of mammals, including humans, by snakes, especially by those belonging to the Crotalidae and Viperidae families, are characterized by an inhibition of blood coagulation due to the action of fibrino (geno) lytic enzymes. Recently many enzymes have been isolated which demonstrate fibrinolytic effects (Marsh & Williams, 2005; Siigur *et al.*, 2001).

Beta-blocker

Rajagopalan *et al.* (2007) isolated and characterized a protein from *Ophiophagus hannah* (king cobra) venom gland tissue. Radioligand displacement studies showed that this protein targets β-adrenergic receptors with a binding affinity (K_i) of 5.3 and 2.3 μM toward β_1 and β_2 subtypes, respectively, to bring about its effect, and hence, it was named as β-cardiotoxin. This is the first report of a natural exogenous beta-blocker.

Antinociception

The high selectivity and specificity of animal toxins have enabled their use as potential therapeutics in the treatment of pain and candidates for the development of new analgesic drugs (Cury & Picolo, 2006). Recently, a compound analgesic formulation combining cobrotoxin, tramadol hydrochloride and ibuprofen (Compound Keluoqu, CKLQ) has become available in China. It was found be of use in the alleviation of pain in cancer patients (Xu *et al.*, 2006). A long form α–neurotoxin was studied for its ant-inociceptive activity and its effects were mediated by the central

cholinergic system and not the opioid system (Chen *et al.*, 2006). Cobra venom has been investigated to treat pain associated with rheumatoid arthritis (Gaede *et al.*, 1995).

HIV

A low CD4 count and multi–drug resistance is a major problem faced by physicians while treating HIV + patients. Snake venoms have been found to reduce viral load and an increase in CD4 counts (Alrajhi & Almohaizeie, 2008) A novel L-amino acid oxidase, named TSV-LAO, has been purified and cloned from the snake *Trimeresurus stejnegeri* and may prove to play a role in treatment of HIV (Zhang *et al.*, 2003).

Tuberculosis

A small peptide from *Naja atra* (isolated from Yunnan province of China) venom had *in vitro* activity against clinically isolated multidrug-resistant strains of *M. tuberculosis* (Xie *et al.*, 2003).

Angiotensin converting enzyme (ACE) Inhibitors

The toxic effects of venom from a Brazilian viper (*Bothrops jararaca*) were found to be due to a sudden, massive drop in blood pressure. When the snake venom peptide was injected before the angiotensin, the blood pressure increased after angiotensin II but not after angiotensin I, as should be the case if ACE is inhibited in the body. This led to the discovery of captopril which revolutionized the treatment of hypertension (Smith & Vane, 2004.) ACE inhibitors have been identified from snake venom. Bradykinin-potentiating peptides (BPPs) are inhibitors of the angiotensin-converting enzyme. The venom of the *Bothrops moojeni* snake was shown to have such activity and its sequence is being elucidated (Menin *et al.*, 2008).

Anti-angiogenesis

Integrins, a family of cell surface molecules, are known to play a vital role in pathological angiogenesis. Peptides rich in disulphides known as disintegrins, isolated from the venoms of various snake species have been found to bind integrins with extremely high affinity and block their function. Disintegrins are a class of molecules that can potentially be used to inhibit angiogenesis and augment currently available chemotherapeutic agents in the treatment of cancer (Swenson *et al.*, 2007). Contortrostatin (CN), the disintegrin from southern copperhead venom, is a homodimer with a molecular weight of 13,500. *In vivo* studies using the human metastatic breast cancer cell line MDA-MB-435, in an orthotopic xenograft model in nude mice, revealed that CN has potent anti-tumor and anti-angiogenic activity (Swenson *et al.*, 2005).

Anti-inflammatory

Snake venoms possess both pro-inflammatory and anti-inflammatory components. The possible anti-inflammatory activity is currently being investigated for any therapeutic implications (Farsky *et al.*, 2005).

Multiple sclerosis

The use of snake venom in the treatment of multiple sclerosis has been controversial. Cobratoxin, a neurotoxin obtained from the venom of the Thailand cobra, has demonstrated several pharmacological activities that strongly support its use in this application. By employing a chemical detoxification step, the neurotoxin can be rendered safe for administration to humans with minimal side effects. This modified neurotoxin has demonstrated neuromodulatory, antiviral, and analgesic activity, elements associated with the multiple sclerosis condition. Modified cobratoxin has demonstrated potent immunosuppressive activity in acute and chronic animal models of the disease. The drug is under investigation for use in adrenomyeloneuropathy and clinical trials in multiple sclerosis are planned (Reid, 2007).

BEE VENOM

The therapeutic application of bee venom (BV) is known as Apitherapy or Bee venom therapy (BVT). It has been used in traditional medicine to treat diseases, such as arthritis, rheumatism, pain, cancerous tumors, and skin diseases. Honey bee venom contains at least 18 active substances. It includes a variety of components like melittin, apamin, adolapin, mast cell degranulating (MCD) peptide, phospholipase [PL] A2. They have been elaborated in Table 2.

Table 2. Components of bee venom and their characteristics

Class	Component	Molecular weight	Characteristics	
			Number of amino acids	Activity
Peptides	Melittin	2840	26	• Enhance of PLA_2 activity • Cytotoxic effects against cancer cells • Anti-inflammatory and anti-arthritic effects
	Apamin	2036	10	• Inhibition of Ca^{2+}-activated K^+ channel • Cytotoxic effect against cancer • Nociceptive effect • Anti-inflammatory properties
	MCD peptide	2588	22	• Anti-inflammatory and analgenic effect

Table 2. *Contd.*

Class	Component	Molecular weight	Characteristics	
			Number of amino acids	Activity
	Adolapin	11,500	-	• Histamine release (low dose) • Histamine release inhibition (high dose) • Anti-allergic effect • Inhibition of PLA_2 and COX activity • Anti-inflammatory activity • Analgesic effect
	Protease inhibitor	9,000	-	-
	Minimine	6,000	-	-
	Procamine	-	-	-
	Secarpin	-	-	-
	Tertiapin	-	-	-
Enzymes	PLA_2	19,000		• Cytotoxic effects against cancer cells • Inflammatory effects • Anti-tumor effects
	Hyaluronidase	38,000		• Selectively attacks tissue hyaluronic acid polymers • Increase the capillary permeability • Immune response and tissue spread properties • Antigenic
	Glucosidase	170,000	-	
	Acid phosphomo-noesterase	55,000	-	
Amine	Histamines	307.14	-	
	Dopamine	189.64	-	
	Norepinephrine	169.18	-	
Others	Carbohydrates	307.14	-	
	r-Aminobutyric acid	189.64	-	
	B-Amino iso butyric acid	169.18	-	

Melittin, a major peptide component of BV, has anti-inflammatory and anti-arthritis properties, and its inhibitory activity on nuclear factor kappa B (NF-κB) may be essential for the effects of BV. The antinociceptive effects of BV have also been demonstrated in thermal, visceral, and inflammatory pain models. Apcupoint stimulation (apipuncture) therapy into subcutaneous region may be important in the BV-induced antinociceptive effects. Multiple mechanisms, such as activation of the central and spinal opioid receptor, and α2-adrenergic activity, as well as activation of the descending serotonergic pathway have been suggested. The inhibition of c-Fos expression in the spinal cord by BV apipuncture in several nociceptive

models is also reported to be a possible mechanism. BV also has anti-cancer activity. The cell cytotoxic effects through the activation of PLA2 by melittin have been suggested to be the critical mechanism for the anti-cancer activity of BV. The conjugation of cell lytic peptide (melittin) with hormone receptors and gene therapy carrying melittin can be useful as a novel targeted therapy for some types of cancer, such as prostate and breast cancer (Son *et al.*, 2007).

Uses

Arthritis and other systemic inflammations

Bee venom therapy can be useful in both rheumatoid and osteoarthritis, helping with both pain and swelling. In the case of rheumatoid arthritis, rheumatoid nodules can lessen in size. Other connective tissue diseases such as scleroderma have been ameliorated by BVT. Even systemic inflammations not related to joints, such as ulcerative colitis or even asthma, may warrant a trial of bee venom. This is presumably due to stimulation of endogenous cortisol through the hypothalamus-pituitary-adrenal axis. Bursitis, tendonitis and other areas of injury respond well to bee venom therapy. In this case, the effect is probably a local anti- inflammatory effect, involving the humoral and cellular immune responses to a foreign protein. Chronic back and neck pain may respond, as can other aches and pains (Behrens *et al.*, 1994).

Anti-cancer effect of Bee venom

It was found that melittin has cytotoxic properties. Melittin could enter the phospholipid bilayers and exhibit surfactant activity. The association between melittin and the cellular membranes results in

A) A disturbance of the acyl groups of phospholipids

B) Increased phospholipid susceptibility to hydrolysis by PL

C) Increased synthesis of PG from the arachidonic acid released from the phospholipids.

The above mechanisms are expected to contribute to its anti cancer effect (Son *et al.*, 2007).

Scar tissue

Keloids and other scar tissue are broken down and softened by the substances in the venom, and can flatten out and fade in color. Internal scar tissue, such as adhesions from previous surgery, may respond to treatment over the area.

Multiple sclerosis

This use of bee venom is poorly understood, and needs to be studied further. The treatment is prolonged but the common responses are increased stability, less fatigue, and less spasm (Behrens *et al.*, 1994).

SNAIL VENOM

Marine snails of the genus *Conus* provide some of the most intriguing marine natural products. Cone snails are found in coral reefs in tropical waters throughout the world. Each *Conus* species contains 100–200 small, highly structured venom peptides (colloquially known as conotoxins), which as part of a defensive and feeding strategy, are synthesized and secreted in a venom duct.use complex-venom delivered through a specialized radular tooth. The venoms of the 700 species of predatory cone snails (genus *Conus*) have been systematically characterized. The biomedical potential of these small venom peptides is now well established (Olivera, 2002; Olivera & Terlau, 2004; Olivera *et al.*, 1990; Shen *et al.*, 2000). The various conopeptides can be classified as shown in Table 3.

Table 3. Classification of conopeptides (Shen *et al.*, 2001)

Class	Example	Pharmacological target
Conantokins	Conantokin-G	NMDA-receptor antagonists
Contryphans	Contryphan	Unknown
α-Conopeptides	MII	Nicotinic acetylcholine receptor
ω-Conopeptides	MVIIA	Calcium channels
μ-Conopeptides	GIIIA	Sodium channels

The Food and Administration in December 2004 approved a *Conus* peptide as a commercial drug for intractable pain. The product, Prialt (generically called ziconitide) is identical to the natural peptide produced by the magician's cone snail, *Conus magus*, originally designated ω-conotoxin MVIIA (Olivera, 2006). Other *Conus* peptides alongwith the stages of trial and their uses are listed in Table 4.

Table 4. Conopeptides and their stages of development (Olivera, 2006)

Peptide	Commercial name	Company	Target	Use	Development stage
ω-MVIIA	Prialt, zicontide	Elan (Neurex)	N-type calcium channel	Anti-nociception	Approved by FDA
ω-CVID	AM336	Amrad	N-type calcium channel	Anti-nociception	Phase 1
Contulakin-G	CGX-1160	Cognetix	Neurotensin receptor	Anti-nociception	Phase 1
MrIA (derivative)	Xen-2174	Xenome	Norepinephrine transporter	Anti-nociception	Phase 1
α-Vc1.1	ACV-1	Metabolic	Nicotinic receptors	Anti-nociception	Phase 1
Conantokin-G	CGX-1007	Cognetix	NMDA receptors	Epilepsy, Anti-nociception	Phase 1
κ-PVIIA	CGX-1051	Cognetix	K^+ channels	Myocardial infarction	Pre-clinical
μO-MrVIB	CGX- 1002	Cognetix	Na^+ channels	Anti-nociception	Pre-clinical

Individually, the *Conus* venom components have diverse molecular targets, including G protein-coupled receptors and neurotransmitter transporters; some *Conus* venom components even have enzymatic activity. However, the majority of biologically active *Conus* venom constituents that are characterized at present are small, structured peptides that target ion channels, either of the ligand-gated or voltage-gated class. Most *Conus* peptides have a specific ion channel as the physiologically relevant target (Olivera & Terlau, 2004).

TOAD VENOM

There are many different kinds of toads on earth, poisonous or not, in the earth's ecosystem. One genus of toads that is poisonous is the genus *Bufo*. There are more than 200 species that are known in this genus of toads. *Bufo marinus* and *Bufo alvarius* are the two main species under this genus that are under discussion.

Both *Bufo marinus* and *Bufo alvarius* also have large parotoid glands on their backs that secrete their venom. These parotoid glands are kidney-shaped and are also located on each side of their neck (Davis & Weil, 1992; Lyttle *et al.*, 1996). Toad venom contains a huge mixture of different classes of chemicals. There are over 26 different biologically active compounds in toad venom. The concentration of these varies from species to species. The components are:

1) *Cardiac glycosides*

There are two classes of cardiac glycosides present in toad venom, bufogenins and its derivative bufotoxin (Davis & Weil, 1992). The bufogenins and bufotoxins together are known as bufodienolides. These classes of chemicals in the venom are known for their effects on the cardiovascular system, including the heart and blood vessels (Chi *et al.*, 1998; Davis & Weil, 1992; Lim *et al.*, 1997; Lyttle *et al.*, 1996).

2) *Phenethylamines and their derivative*

These include catecholamines such as dopamine, norepinephrine and epinephrine. These classes of chemicals in the toad venom generally do not have as prominent an effect as the bufodienolides in exerting a quick and obvious physiological effect in animals, but it is thought that they probably contribute to vasoconstriction in human placental fetal blood vessels (Lim *et al.*, 1997).

3) *Tryptamines and its derivatives*

These include:

- 5-hydroxytryptamine (5-HT, serotonin). Serotonin is found endogenously in both humans and Bufo toads.

- 5-hydroxy-dimethyltryptamine (5-hydroxy-DMT, bufotenine), which is found in all species of Bufo toads, but in different concentrations. Bufotenine is toxic physiologically because it is thought to have pressor effects, meaning that it can affect the cardiovascular system, such as the heart rate and/or blood pressure (Lyttle *et al.*, 1996). Inhaling the vapors of toad venom from *Bufo marinus*, it is found to have hallucinogenic and/or psychoactive effects due to Bufotenine. But it has been postulated that it is very unlikely that the venom of *Bufo marinus*, which contains bufotenine, will have any psychoactive agents because of the lack of any hallucinogenic contents found in the venom after its analysis (Davis & Weil, 1992).
- 5-methoxy-N, N-dimethyltryptamine (5-MeO-DMT) is also a tryptamine, and it is found only in the venom of one species of the *Bufo* genus, *Bufo alvarius*. There is evidence that it is the only substance found endogenously in *Bufo alvarius* that have psychoactive and/or psychedelic effects when their venom are inhaled (Davis & Weil, 1992; Lyttle *et al.*, 1996). However, when toad venom is administered orally or topically, the venom can have toxic effects on animals and humans. This is most likely due to the presence of the bufodienolides in the venom.

4) *Non-cardiac sterols*

These include cholesterol, provitamin D, ergosterol, and gamma sitosterol. They do not have any toxic effects in toad venom.

SPIDER VENOM

Two types of spiders are implicated in most of the medically significant spideren venomations: the black widow (genus *Latrodectus*) and the brown recluse (genus *Loxosceles*). Spider venoms are composed of complex proteins and proteolytic enzymes that are either designed to initiate digestion of prey entrapped by web-spinners or to incapacitate prey ambushed by hunting spiders.

Most spider species are harmless to humans, so peptides or drug molecules from these spiders are likely to be safe. By modifying the molecular surfaces and active sites of peptides and enzymes from spiders, whilst keeping the spider scaffold, it is possible to gain specificity and/or affinity for a given receptor. Therefore, acylpolyamines, peptides and enzymes from spider venoms represent an interesting source of molecules for the design of novel pharmaceutical drugs.

Uses

The potential medical uses of spider venoms are largely due to their selectivity and affinity for ion channels and other receptors. This makes them suitable for studying cell function and for designing therapeutic drugs.

Cardioprotective

The venom of the theraphosid *Grammostola spatulata* from South America contains a peptide, GsMtx-4, that blocks stretch-activated ion channels. These channels are sensitive to muscle contraction and blood pressure and play an important role in coordinating a heartbeat. Potentially, GsMtx-4 can be used to prevent atrial fibrillation after a heart attack and to treat cardiac patients (Itabashi *et al.*, 2005). The venom of the spider *Heteropoda venatoria* contain toxins that were shown to block I_{to1} in isolated cardiac myocytes and Kv4.2 channels expressed in *X. laevis* oocytes. These toxins, known as heteropodatoxins, represent a new class of modulators that will be useful in defining the physiologic role of Kv4.2 channels in a variety of cell types and will aid in structure function studies of these channels (Sanguinetti *et al.*, 1997).

Analgesic

Peptides make up a substantial part of spider venom, and modulate ionic currents across Ca^{2+}, Na^{+}, or K^{+} ion channels. Some spider peptides can discriminate between ion channel subtypes and several will inhibit peripheral neurons, the nerve cells that are associated with supplying sensation to the skin and skeletal muscles. Spider toxins that block the neuronal Ca^{2+} ion channel could prove important for the treatment of chronic pain. Acylpolyamines can also act as pain-killers, by blocking capsaicin receptor channels, non-selective cation channels in sensory neurons that respond to pain-causing stimuli. The venom of the Chinese bird spider *Ornithoctonus huwena* (Wang) [= *Selenocosmia huwena* wang] contains huwentoxin-I (HWTX-I), a N-type calcium channel blocker. The intrathecal administration of HWTX-I was found to be effective in antinociception in the rat model of the formalin test (Chen *et al.*, 2004).

Antibacterial

A group of the spider peptides are amphipathic. These form α-helical structures that insert into cell membranes to form pores, resulting in loss of cell function. Most of these peptides will destroy red blood cells and hence they could potentially be used in topical applications, such as antibacterial coatings for medical implants, in inhibiting the growth of oral bacteria associated with tooth decay and early plaque formation and in treating skin infections (Yan & Adams, 1998).

Antiepileptic

Of all the venom components, the acylpolyamines represent the vast majority of the molecules in the mixture. These have been shown to suppress epileptic activity in brain tissue. Moreover, brain damage caused by restricted blood flow, for example during a stroke, can be prevented with acylpolyamines. The compounds work by blocking Ca^{2+} voltage-gated ion channels or preventing glutamate release, both of which are implicated in neuronal death (Salamoni *et al.*, 2005).

Anti-tumor

A component of brown recluse spider venom, hyaluronidase, is a compound that has shown medical potential for tumor treatments. Recently, it was demonstrated that the production and action of lysophosphatidic acid (LPA) is required for the toxic effects of envenomation by the *Loxosceles reclusa* or brown recluse spider. There are distinct similarities between LPA signaling and the noxious effects of envenomation: the production of interleukin-8 and growth-regulated oncogene α, platelet aggregation, and endothelial permeability. Thus, cancer and the toxic effects of spider envenomation may represent a striking convergence (Murph *et al.*, 2006).

CURRENT ADVANCES

A number of venom products and derivatives are under clinical trials. Venoms derived from cone snails that are being studied currently have been included in Table 4. Apart from these, venoms from other sources under study have also been discussed below:

- SYN®-AKE, is a synthetic dipeptide, which is identified by the chemical name dipeptide diaminobutyroyl benzalamide diacetate, which mimics the action of a neuromuscular blocking compound of the venom of the Temple Viper (*Tropidolaemus wagleri*). It is being manufactured by Pentapharm Ltd., Switzerland. The actual venom paralyzes the muscles; this peptide only mimics Waglerin 1®, the component which relaxes the muscles. It has been postulated that it acts similarly to Argireline® by interfering with the transmission of nerve signals to the muscles directing them to contract. However, research indicates that SYN®-AKE is significantly more powerful than Argireline. A clinical trial was conducted with 45 subjects for 28 days with twice daily application (Web ref. 1).
- TM-601 is a novel, synthetic version of a peptide, or protein particle. It naturally occurs in the venom of the Giant Yellow Israeli scorpion. TM-601 binds to glioma cells and has an unusual ability to pass through the blood-brain barrier that blocks most substances from reaching brain tissue from the bloodstream. It is highly specific and selective in targeting both primary tumors and metastases. TM-601 targets and binds to receptors expressed on tumor cells, but not on normal, healthy cells. When ^{131}Iodine radiolabeled TM-601 is administered, it is actively taken up into these tumor cells, delivering a highly concentrated dose of radiation to kill the tumor cells without affecting nearby healthy cells. TM-601 is primarily being used as a carrier to transport radioactive iodine to glioma cells, although there are data to suggest that it may also slow down the growth of tumor cells. If studies continue

to confirm this, it may find use in conjunction therapy with other treatments, such as chemotherapy, because there may be a synergistic effect. In other words, TM-601's ability to impede cancer growth could provide for reduction of the dose of chemotherapy required to achieve a therapeutic effect. After a Phase I clinical trial conducted in 18 patients showed the approach to be safe, a larger Phase II trial is underway to assess the effectiveness of multiple doses. The data obtained from preclinical and clinical studies also suggest that native TM-601 may affect a tumor's ability to grow and spread without added radiation through an anti-angiogenic mode-of-action. The FDA has granted the radiolabeled drug, ^{131}I-TM-601, orphan drug status for patients with high-grade and malignant glioma, as well as a Fast Track designation. Unlabeled TM-601 has orphan status in the US for malignant glioma (Mamelak *et al.*, 2006).

- Xen2174 is a synthetic drug modelled on a peptide isolated from the venom of a cone shell found on Australia's Great Barrier Reef. Xen2174 selectively targets the norepinephrine transporter (NET), a well-established pharmaceutical target for a number of conditions. Inhibition of this transporter elevates the levels of norepinephrine in the spinal cord, preventing pain signals from reaching the brain. Xen2174 successfully completed a Phase 1 human safety trial in healthy volunteers in 2005, where it was tested in systemic circulation, via intravenous administration. Xen2174 is currently being tested in a Phase 2 clinical trial targeting chronic intractable pain in cancer patients *via* single dose intrathecal injection. This study again focuses on safety under this type of administration as well as pharmacokinetics and signs of efficacy at various doses (Nielsen *et al.*, 2005).
- Exendin-4 is a drug derived from the saliva of the Gila monster (*Heloderma suspectum*). The lizard venom contains many bioactive peptides. Two of these peptides, exendin-4 and exendin-3 have been isolated from the exocrine glands (oral secretions) of the lizard, although they were shown to have endocrine action. Both these peptides differ in amino acid sequence at position 2 and 3. Exenatide is synthetic Exendin-4, it contains 39 amino acids in its peptide chain. It is a potent agonist of mammalian glucacon like peptide-I receptor (GLP-I R). Exenatide injection is commercially marketed as Byetta. On the 30th of April 2005, the United States FDA (USFDA) approved the use of Byetta for the treatment of type 2 diabetes mellitus. It has been approved for use as adjunctive therapy to improve blood sugar control in patients. Exenatide is the first in a new class of drugs for the treatment of type 2 diabetes called incretin mimetics (Ghosh *et al.*, 2007).

CONCLUSIONS

Even though venoms have been postulated to have medicinal properties since ancient times, the utilization of venoms as pharmacological tools is a relatively recent advancement in venom therapeutics. Venoms have been shown to possess pharmacologically beneficial properties that can be used to prepare derivatives of therapeutic importance. Various animal sources producing venoms have been studied and some of them have been successfully isolated and prepared as agents that are under study, in clinical trials or are in use and have already been approved by the FDA.

ACKNOWLEDGEMENTS

The authors would like to thank Ms. Usha Satypal and Mr. Sunil Nighot for assisting in data compilation and Dr. Vilasrao J. Kadam for providing the necessary facilities that aided the processing and completion of the work.

REFERENCES

Alrajhi, A.A. and Almohaizeie, A. (2008). Snake venom preparation for drug-resistant human immunodeficiency virus. Jun 10. [Epub ahead of print].

Behrens, M.I., Jalil, P., Serani, A., Vergara, F. and Alvarez, O. (1994). Possible role of apamin-sensitive K^+ channels in myotonic dystrophy. *Muscle and Nerve*, **17**: 1264-1230.

Bhattacharya, P. and Chakraborty, A. (2007). Neurotoxic snake bite with respiratory failure. *Indian Journal of Critical Care Medicine*, **11**: 161-164.

Bilwes, A., Rees, B., Moras, D., Menez, R. and Menez, A. (1994). X-ray structure at 1.55Å of toxin γ, a cardiotoxin from *Naja nigricollis* venom: crystal packing reveals a model for insertion into membranes. *Journal of Molecular Biology*, **239**: 122-136.

Calvette, J.J., Juarez, P. and Sanz, L. (2004). Snake venomics. Strategies and applications. *Journal of Mass Spectrometry*, **42**: 1405-1414.

Chen, J.Q., Zhang, Y.Q., Dai, J., Luo, Z.M. and Liang, S.P. (2004). Antinociceptive effects of intrathecally administered huwentoxin-I, a selective N-type calcium channel blocker, in the formalin test in conscious rats. *Toxicon*, **45**: 15-20.

Chen, Z.X., Zhang, H.L., Gu, Z.L., Chen, B.W., Han, R., Reid, P.F., Raymond, L.N. and Qin, Z.H. (2006). A long-form alpha-neurotoxin from cobra venom produces potent opioid-independent analgesia. *Acta Pharmacologica Sinica*, **27**: 402-408.

Chi, H.T., Hung, D.Z., Hu, W.H. and Yang, D.Y. (1998). Prognostic implications of hyperkalemia in toad toxin intoxication. *Human and Experimental Toxicology*, **17**: 343-346.

Cury, Y. and Picolo, G. (2006). Animal toxins as analgesics — an overview. *Drug News Perspective*, **19**: 381-392.

Davis, W. and Weil, A.T. (1992). Identity of a new world psychoactive toad. *Ancient Mesoamerica*, **3**: 51-59.

Farsky, S.H., Antunes, E. and Mello, S.B. (2005). Pro- and anti-inflammatory properties of toxins from animal venoms. *Current Drug Targets of Inflammation and Allergy*, **4**: 401-411.

Gaede, K.I., Baumeister, E. and Heesemann, J. (1995). Decomplementation by cobra venom factor suppresses Yersinia-induced arthritis in rats. *Infection and Immunity*, **63**: 3697-701.

Ghosh, R., Thanawala, V. and Kadam, V.J. (2007). Novel Peptides: An alternative approach for the treatment of diabetes mellitus. *Current Drug Therapy*, **2**: 196-204.

Hogg, R.C. (2006). Novel approaches to pain relief using venom-derived peptides. *Current Medicinal Chemistry,* **13(11)**: 3191-3201.

http://www.wipo.int/pctdb/en/wo.jsp. (WO/2008/020954) Cosmetic Composition to accelerate repair of functional wrinkles.

Itabashi, Y., Shunichiro, M., Yuasa, S., Fujita, J., Shimizu, T., Okano, T., Fukuda, K. and Ogawa, S. (2005). Analysis of the electrophysiological properties and arrhythmias in directly contacted skeletal and cardiac muscle cell sheets. *Cardiovascular Research,* **67**: 561-570.

Lim, T.H., Leitch, I.M., Boura, A.L.A., Read, M.A. and Walters, W.A.W. (1997). Effects of *Bufo marinus* skin toxins on human fetal extracorporeal blood vessels. *Toxicon,* **35**: 293-304.

Lu, Q., Clemetson, J.M. and Clemetson, K.J. (2005). Snake venoms and hemostasis. *Journal of Thrombosis and Haemostasis,* **3**: 1791-9.

Lyttle, T., Goldstein, D. and Gartz, J. (1996). Bufo toads and bufotenine: Fact and fiction surrounding an alleged psychedelic. *Journal of Psychoactive Drugs,* **28**: 267-290.

Mamelak, A.N., Rosenfeld, S., Bucholz, R., Raubitschek, A., Nabors, L.B., Fiveash, J.B., Shen, S., Khazaeli, M.B., Colcher, D., Liu, A., Osman, M., Guthrie, B., Schade-Bijur, S., Hablitz, D.M., Alvarez, V.L. and Gonda, M.A. (2006). Phase I single-dose study of intracavitary-administered iodine-131-TM-601 in adults with recurrent high-grade glioma. *Journal of Clinical Oncology,* **24**: 3644-3650.

Marsh, N. and Williams, V. (2005). Practical applications of snake venom toxins in haemostasis. *Toxicon,* **45**: 1171-1181.

Matsui, T., Fujimura, Y. and Titani, K. (2000). Snake venom proteases affecting hemostasis and thrombosis. *Biochimica et Biophysica Acta,* **1477**: 146-156.

McDowell, R.S., Dennis, M.S., Louie, A., Shuster, M., Mulkerrin, M.G. and Lazarus, R.A. (1992). Mambin, a potent glycoprotein IIb-IIIa antagonist and platelet aggregation inhibitor structurally related to the short neurotoxins. *Biochemistry,* **31**: 4766-4772.

Meier, J. and White, J. (1995). Handbook of Clinical Toxicology of Animal Venoms and Poisons. CRC Press, USA.

Menin, L., Perchuć, A., Favreau, P., Perret, F., Michalet, S., Schöni, R., Wilmer, M. and Stöcklin, R. (2008). High throughput screening of bradykinin-potentiating peptides in *Bothrops mooJeni* snake venom using precursor ion mass spectrometry. *Toxicon,* **51**: 288-302.

Murph, M., Tanaka, T., Liu, S. and Mills, G.B. (2006). Of Spiders and Crabs: The emergence of lysophospholipids and their metabolic pathways as targets for therapy in cancer. *Clinical Cancer Research,* **12**: 6598-6602.

Nielsen, C.K., Lewis, R.J., Alewood, D., Drinkwater, R., Palant, E., Patterson, M., Yaksh, T.L., McCumber, D. and Smith, M.T. (2005). Anti-allodynic efficacy of the X-conopeptide, Xen2174, in rats with neuropathic pain. *Pain,* **118**: 112-124.

Olivera, B.M. (2002). Conus venom peptides: Reflections from the Biology of Clades and Species. *Annual Review of Ecology and Systematics,* **33**: 25-47.

Olivera, B.M. (2006). Conus peptides: Biodiversity-based discovery and exogenomics. *Journal of Biological Chemistry,* **281**: 31173-31177.

Olivera, B.M. and Terlau, H. (2004). Conus venoms: A rich source of novel ion channel-targeted peptides. *Physiological Reviews,* **84**: 41-68.

Olivera, B.M., Rivier, J., Clark, C., Ramilo, C.A., Corpuz, G.P., Abogadie, F.C., Mena, E.E., Woodward, S.R., Hillyard, D.R. and Cruz, L.J. (1990). Diversity of conus neuropeptides. *Science,* **249**: 257-263.

Ownby, C.L. and Colberg, T.R. (1996). Isolation and characterization of a PLA_2 myotoxin from prairie rattlesnake (*Crotalus viridis viridis*) venom. *Toxicon,* **34**: 317-317.

Rajagopalan, N., Pung, Y.F., Zhu, Y.Z., Wong, P.T.H., Kumar, P.P. and Kini, R.M. (2007). β-Cardiotoxin: A new three-finger toxin from *Ophiophagus hannah* (king cobra) venom with beta-blocker activity. *The FASEB Journal,* **21**: 3685-3695.

Reid, P.F. (2007). Alpha-cobratoxin as a possible therapy for multiple sclerosis: A review of the literature leading to its development for this application. *Critical Review of Immunology,* **27**: 291-302.

Salamoni, S.D., da Costa, J.C., Palma, M.S., Konno, K., Nihei, K., Tavares, A.A., Souza de Abreu, D., Venturin, G.T., de Borba Cunha, F., de Oliveira, R.M. and Vaz Breda, R. (2005). Antiepileptic effect of acylpolyaminetoxin JSTX-3 on rat hippocampal CA1 neurons *in vitro*. *Brain Research,* **1048**: 170-176.

Sanguinetti, M.C., Johnson, J.H., Hammerland, L.G., Kelbaugh, P.R., Volkmann, R.A., Saccomano, N.A. and Mueller, A.L. (1997). Heteropodatoxins: Peptides isolated from spider venom that block Kv4.2 potassium channels. *Molecular Pharmacology,* **51**: 491-498.

Shen, G.S., Layer, R.T. and McCabe, R.T. (2000). Conopeptides: From deadly venoms to novel therapeutics. *Drug Discovery Today,* **5**: 98-106.

Siigur, J., Aaspõllu, A., Tõnismägi, K., Trummal, K., Samel, M., Vija, H., Subbi, J. and Siigur, E. (2001). Proteases from *Vipera lebetina* venom affecting coagulation and fibrinolysis. *Haemostasis,* **31**: 123-132.

Smith, C.G. and Vane, J.R. (2004). The discovery of captopril. *The FASEB Journal*, **18**: 225.

Son, D.J., Lee, J.W., Lee, Y.H., Song, H.S., Lee, C.K. and Hong, J.T. (2007). Therapeutic application of anti-arthritis, pain-releasing, and anti-cancer effects of bee venom and its constituent compounds. *Pharmacology and Therapeutics,* **115**: 246-270.

Swenson, S., Costa, F., Ernst, W., Fujii, G. and Markland, F.S. (2005). Contortrostatin, a snake venom disintegrin with anti-angiogenic and anti-tumor activity. *Pathophysiology of Haemostasis and Thrombosis,* **34**: 169-176.

Swenson, S., Ramu, S. and Markland, F.S. (2007). Anti-angiogenesis and RGD-containing snake venom disintegrins. *Current Pharmaceutical Design,* **13**: 2860-71.

Teixeira, C.F.P., Landucci, E.C.T., Antunes, E., Chacur, M. and Cury, Y. (1993). Inflammatory effects of snake venom myotoxic phospholipases A_2. *Toxicon*, **42**: 947-962.

Tsetlin, V. (1999). Snake venom α-neurotoxins and other 'three finger' proteins. *European Journal of Biochemistry,* **264**: 281-286.

Xie, J.P., Yue, J., Xiong, Y.L., Wang, W.Y., Yu, S.Q. and Wang, H.H. (2003). *In vitro* activities of small peptides from snake venom against clinical isolates of drug-resistant *Mycobacterium tuberculosis*. *International Journal of Antimicrobial Agents,* **22**: 172-174.

Xu, J.M., Song, S.T., Feng, F.Y., Huang, F.L., Yang, Y., Xie, G.R., Xu, L.G., Zhang, C.Z., Bruno, M. and Paradiso, A. (2006). Cobrotoxin-containing analgesic compound to treat chronic moderate to severe cancer pain: Results from a randomized, double-blind, cross-over study and from an open-label study. *Oncology Reports*, **16**: 1077-84.

Yan, L. and Adams, M.E. (1998). Lycotoxins, antimicrobial peptides from venom of the wolf spider *Lycosa carolinensis*. *Journal of Biochemical Chemistry,* **273**: 2059-2066.

Zhang, Y.J, Wang, J.H., Lee, W.H., Wang, Q., Liu, H., Zheng, Y.T. and Zhang, Y. (2003). Molecular characterization of *Trimeresurus stejnegeri* venom L-amino acid oxidase with potential anti-HIV activity. *Biochemical and Biophysical Research Communications.* **309**: 598-604.

"This page is Intentionally Left Blank"

9

Potentials of Bryophytes for Biotechnological Use

MARKO SABOVLJEVIC[1]* AND ANETA SABOVLJEVIC[1]

ABSTRACT

Bryophytes are the second biggest group of terrestrial plants. They have not received much attention in biotechnology due to their small size and biomass. However, lately many new features have been highlighted and the interest for the potentials use of these plants increased. Scientifically new chemical compunds are described from bryophytes. Bryophyte extracts are shown to be biologically active with particular reference for their use in pharmacology, medicine and agriculture for all round benefit of living beings. There are some reports that some bryophyte extract are able to cure or prevent diseases such as AIDS and cancer. With axenic culturing of some species, the problems of enough mass and clean bryophyte culture are partly solved. Due to their properties and life span where the dominant life phase is haploid, bryophytes represent easy tools for molecular and genetic investigation. The model bryophyte plant whose genome has been sequenced is Physcomitrella patens. Bryophytes also have extraordinary values in environmental bioindication, or microdust attachment. They received attention as ornamental plants and covering plants in restoration and landscape ecology. Still, many things remain to be proven in the biology of these organisms. This chapter gives the overview of the biotechnological use of bryophytes today and their further use potentials.

Key words : Biotechnology, bryophytes, bryoreactor, ethno-medicine, *in vitro* culture, secondary metabolites

1. Institute of Botany and Botanical Garden, Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia.

* *Corresponding author* : E-mail : marko@bio.bg.ac.rs

INTRODUCTION

Bryophytes are an evolutionary old polyphyletic group of land plants and with some 25000 species world wide the second largest group of terrestrial plants after flowering plants. They comprise very diverse plants like peat-mosses (Sphagnopsida), genuine mosses (Bryopsida), leafy liverworts (Jungermanniopsida), tallous liverworts (Marchantiopsida), latern mosses (Andreaeopsida), hornworts (Anthocerotopsida) and some other smaller groups. Bryophytes live in all ecosystems, from desertic to alpine in all climate types and in some of them highly contribute to the total biomass production of ecosystems like in tundras or cloud forests. Already, this morpho-anatomical, eco-physiological diversity and wide geographical range give advantages to bryophytes to be good object in biotechnological applications.

However, the small size of these plants, as well as problems in achieving enough content of single species from nature, made these plants neglected and avoided for wide use. Eventhough, in the regions where they could be collected in significant amount they are used in ethno-medicine, phytotherapy and human environment improvement.

Comparing to flowering plants, bryophytes have no high economic value. However, they are used in the market of packing, plugging and decoration. *Sphagna* or peat-mosses are used as an energent in the northern countries. Bryophytes are respected horticultural material in Far East (Thieret, 1956; Frahm, 2004; Saxena & Harinder, 2004) .

One of the ways to overcome the problems of bryophyte material purity is to establish axenic culture whether in *in vitro* or *ex vitro* conditions. The idea of monoculturing bryophytes like crops has not been successful up to date. The *in vitro* cultivation is solution for achieving enough quantity of bryophyte material for further studies and analyses, as well as for propagation and conservation of rare and vulnerable material. However, even the first axenic culture was established with moss (Serrvetaz, 1913), the technique is not yet advanced one.

The major advances deriving almost exclusively from axenically cultured plants, highlight a widely held belief that bryophytes have great advantages as experimental organisms, is that they are easy to grow and exhibit rapid, simple, sequential, highly ordered development. Closer scrutiny of the literature, however, soon reveals that the vast majority of publications embrace just three mosses, *Ceratodon purpureus, Funaria hygrometrica* and *Physcomitrella* (*Aphanorhegma*) *patens*, with just a smattering of contributions on other taxa (Chopra, 1994; Sabovljevic *et al.*, 2002, 2003, 2005, 2006a; Bijelovic & Sabovljevic, 2003; Bijelovic *et al.*, 2004; Cvetic *et al.*, 2005, 2007). The simple fact remains that well in excess of 95% of all bryophytes have never been cultured (Duckett *et al.*, 2004).

Many problems remains, and due to the high diversity of bryophyte group, for many taxa it is *species* specific procedure for establishing and maintaining axenic culture, and some of the developing stages is hard or impossible to get in certain taxa simply because of lack of species biology knowledge. Experimental work on *in vitro* bryophytes turn to be starting point for many applied bryology subjects and still in need to be developed. Bryophytes *in vitro* cultivation is not always as simple as those living in a "physcomitrellocentric" world might have believe (Duckett *et al.*, 2004).

Considering that recent investigations on bryophytes in *in vitro* conditions are focused on model moss *Physcomitrella patens*, there are just a few approaches which bring new details for different bryophyte species (Fig 1) (Sabovljevic *et al.*, 2002, 2003; Bijelovic & Sabovljevic, 2003; Bijelovic *et al.*, 2004; Vukojevic *et al.*, 2004; Cvetic *et al.*, 2005; Sabovljevic *et al.*, 2005; Rowntree, 2006). Sabovljevic *et al.* (2006a) compared chemical compounds and ethanol extracts of moss plants *Bryum argenteum* grown in *in vitro* culture and collected in nature, and found minor diferences on their anti-microbial properties.

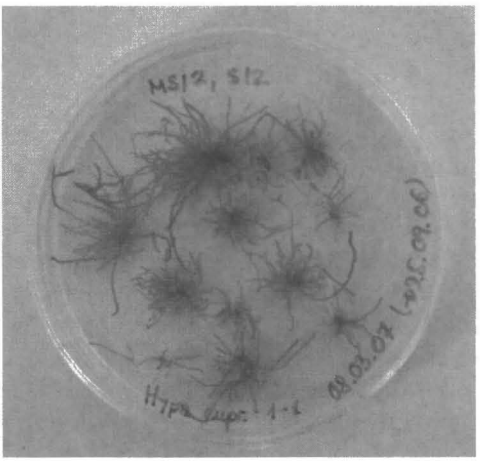


Fig 1. An *in vitro* culture of the moss *Hypnum cupressiforme*

In general, bryophytes are not easy to establish and grow in *in vitro* culture. Another huge advantage of bryophytes is that thay have haploid phase as a dominant in their life cycle and a high rate of homologous recombination (Haltorf *et al.*, 2004). The dominant haploid state is advantageous when performing forward genetic screens because of loss of a certain gene function is not counterbalanced by a second allele. Precise gene disruption in the moss-model *Physcomitrella patens* that occurs in a haploid plant overcome these difficulties with all other higher plants, because single gene knockout mutants are expected to show a more

comprehensive phenotypes. Apart from haploidy advantage, the moss *Physcomitrella* is the only plant model system studied so far able to integrate transforming DNA at a high frequency by the way of gene targeting (Schaefer & Zryd, 1997, 2004) with the homologous recombination rate being several orders of magnitude higher than in seed plants (Reski, 1998, 1999). Contemporary plant scientists are considering bryophytes as sources of genes for modifying crop plants to withstand the physiological stresses of the modern world.

Bryophytes turn to be easily applicable for molecular farming and metabolic engineering. Unique features of the moss system are the opportunities for targeted gene deletion and the purification of secreted proteins from the culture medium. Targeted gene deletion has been employed to knock out nuclear genes for glycosylation, the first step towards the long-term goal of re-engineering the protein modification machinery in plants for 'humanization' of plant-produced pharmaceuticals, setting a new standard for all plant expression systems (Maliga & Graham, 2004). Murhy (2007) compared various approaches to bio-pharming and state the advantages achieved by mosses.

For example, transgenic varieties of the moss *Physcomitrella patens* have been used by Greenovation Biotechnologie GmbH in Germany to secrete recombinant human growth factor and human serum albumin directly into the culture medium (Baur *et al.*, 2005a, b; Weise *et al.*, 2006), and Saidi *et al.* (2005) expressed several xenoproteins using an inducible heat-shock promoter. Koprivova *et al.* (2003) reported that the *N*-glycosylation patterns in *Physcomitrella patens* were similar to those in higher plants, and hence appropriate for the expression of many animal glycoproteins. A review of the 'moss bioreactor' concept is given in Decker and Reski (2004). Cañizares *et al.* (2006) have used a bipartite method, based on a combined transgene–virus vector system that allows either inducible or constitutive expression of a transgene for the accumulation of high levels of xenoproteins in plants. Another inducible system successfully trialed is a soybean heat-shock promoter driving several recombinant proteins in the moss *Physcomitrella patens* (Saidi *et al.*, 2005).

Althought current production of recombinant pharmaceuticals come mainly from microbial systems like bacteria *Escherichia coli* and yeast *Saccharomyces cerevisiae* or from mamalian systems (*e.g.* Chinses hamster ovary cells), plant systems especially bryophytes have many advanages, combining microbial and mammalian production features.

Plants are higher eukaryotes and can produce multimeric proteins with post-transitional modification closely resembling mammalian modifications (Walsh & Jefferis, 2006). Also, plant produced biopharmaceuticals avoid the risk of product contamination by human pathogens hidden in mammalian cell lines or in complex organic production media.

The advantage of bryophytes over other plants in biopharmaceutical production is that mosses can be maintained easy in *in vitro* controlled condtions in contrast to agriculturing or space and problems in manipulation with vascular plants. Besides, bryophytes posses haploid genome which allows easier genetic manipulations in bio-industry, making them easier to control and more suitable for environmental friendly Genetic Modified Plants (GMP). Tissue culturing offers a cheaper downstream processing, especially when the biopharmaceutical is secreted from the cells or media.

Similarly to mammals, plant can be used to produce N-glycosilated proteins, which is important for the activity, stability and/or immunogenicity (effectivness of antibodies). The problems in plant use for biopharmaceutical production is that plants N-glycolsilate also with some plant specific sugars and that make biopharmaceuticals immunogenic (Gomord *et al.*, 2005). A model moss *Physcomitrella patens* offers solutions for this since it is exceptionally amenable for precise genetic modifications (*e.g.* modification of glycosilation pathway), host for complex biopharmaceuticals and easy low-cost cultivated plant in moss photo-bioreactors (Decker & Reski, 2007). Another *P. patens* advantage is that this moss has fully sequenced genome.

The two plant-specific sugar moieties, a xylose connected to the core mannose residue by beta 1,2-linkage and a fucose, alpha 1,3 linked to the proximal *N*-acetylglucosamine (GlcNAc) are residues of the core glycan, which is the most critical difference between glycosilation in plants and humans. Xylose is unknown in human glycans, while proximal fucose residue in human glycans is linked in a different way (alpha 1,6). Both plant-specific sugars are immunogenic. The foreign protein production is advanced by relatively easier promoter set establishement in *P. patens.*

Moss knockout strains were created to link the enzymes responsible for fucosilation (alpha1,3-fucosyltransferase) and xylosylation (beta1,2-xylosyltransferase) (Koprivova *et al.*, 2004). This double knockout moss strains were shown to be completely devoid of the allergenic sugar residues and were employed to sythesize various pharmaceutical products like: antibodies IgG1 and IgG4 (Gorr & Jost, 2005), erythropoietin (Gorr & Altmann, 2006) and human VEGF (vascular endothelial growth factor; Nechansky *et al.*, 2007).

Moss bioreactors offer safe and efficient producing system for complex modified recombinant biopharmaceuticals under Genetic Modified Plant conditions.

Among the plant biotechnology patents one of the most outstanding is moss patent glyco-engineered antibodies, a method to produce heterologous glycosylated proteins in bryophyte cells.

Also, different *Physcomitrella patens* mutants can be cryoconserved for a long-term period, which allows time and space storage of mutants not in immediate use (Schulte & Reski, 2004).

In contrast to other bryopyhtes, model moss *Physcomitrella patens* is easy to establish, and maintain in axenic culture and to make complete life cycle *in vitro*. Haploid spores germinate giving rise to a filamentous protonemal tissue. On protonema also haploid gametophores develop, bearing both sex organs wich make possible and easy transfer to the next life phase - diploid sporophyte, which develops after fertilization on gametophyte. Certain inductive conditions are need to iniciate pass from gametophyte to sporophyte phase. Liquid *in vitro* condition preferencially gives oportunity for vegetative phase growth. Interestingly, genetic instability of *P. patens* in *in vitro* conditions were never observed in contrast to vascular plants (Decker & Reski, 2007).

P. patens has another high genetically interesting peculiarty: high degree of homologous recombination in its nuclear DNA, greatly facilitating precise and base-specific targeted gene knockout (Decker & Reski, 2007). For example, this moss is able to integrate foreign DNA by homologous recombination at a rate which is several order higher compared to seed plants, allowing a routine for targeted gene knockouts (Schaefer & Zryd, 1997; Schaefer, 2001). Troullier *et al.* (2007) state after comparison between two moss species that efficient gene targeting could be a general mechanism of all bryophyte transformation.

Many novelties are found in bryophytes like that of turgor-responsive aldehyde dehydrogenase (*ALDH7*) from desiccation tolerant moss model system *Syntrichia ruralis*, that play important role in the detoxification of aldehydes generated in response to desiccation, high NaCl, altered light, abscisic acid (ABA) or UV-C levels stresses (Chen *et al.*, 2002). It can be expected that the elucidation and application discovered in this moss will allow the transgenic crops to grow in less hospitable conditions.

Bryophytes are able to survive cryo-preservation in liquid nitrogen without prior pretreatment with abscisic acid (ABA), ABA/proline and or sucrose (Bruch, 2003). The mechanism vary depends on species, but offers the insights into development of the long-term conservation methodology of many crops, crop ancestors or endangered wild plants.

Some of the most exciting uses for mosses are just beginning to emerge. With the capabilities of modern genetic engineering, it is now theoretically possible to manipulate the genomes of plants to endow them with desirable traits for human use. While bryophytes themselves have had limited application, their ability to survive drought and become functional again within 24 h has aroused the imagination of agriculturalists. Furthermore, current research reveals how bryophytes

can withstand freezing while still in a state of hydration, yet recover almost instantly.

The mosses are grown in a bioreactor where only water and minerals, along with light and CO_2, are needed to keep the system active. The moss offers an advantage of requiring no antibiotics during culture, thus avoiding contamination of the final product. Its small size permits lab culturing, reducing the possibility of escape of transgenic plants. Through their long evolutionary history bryophytes have acquired an array of biochemicals that may one day prove to be a substantial source of human medicines or provide a gene bank for making proteins, enzymes, sugars, or fatty acids permitting crop plants to survive drought, cold or infestations.

In the age of health awareness and the increasing concern of using natural products in medicine, bryophytes are increasingly being investigated for their potential pharmaceutical application. Chemical components of some of these plants are known to be biologically active with particular reference for their use in pharmacology, medicine and agriculture for all round benefit of living beings. There are some reports that some bryophyte extract are able to cure or prevent diseases such as AIDS and cancer.

It has been demonstrated that most of the liverworts contain mainly mono-, sesqui- and diterpenoids. The presence of triterpenoids and nitrogen-containing compounds is very rare. Almost all liverworts elaborate α-tocopherol, stigmasterol and squalene. Liverworts are also rich source of aromatic compounds, in particular, bibenzyl and *bis*-bibenzyls which have been found in the Marchantiaceae and Aytoniaceae in the Marchantiales, Lejeuneaceae, Lepidoziaceae and Plagiochilaceae in the Jungermanniales and Blasiaceae, Pelliaceae and Riccardiaceae in the Metzgeriales. Several species elaborate naphthalene, phthalide, isocoumarin, benzoate, cinnamate, acrylate and indole derivatives which constitute the oil bodies. The characteristic components of the mosses are highly unsaturated fatty acids and alkanones, such as 5,8,11,14,17-eicosapentaenoic acid, 7,10,13,16,19-docosapentaenoic acid and 10,13,16-nonadecatrien-7-yn-2-one and triterpenoids. The neolignan is one of the most important chemical markers of the Anthocerotae.

The most characteristic chemical phenomenon of liverworts is that most of sesqui- and diterpenoids are enantiomers of those found in higher plants although there are a few exceptions such as drimane, germacrane and guaianes. It is very noteworthy that the different species of the same genera, like *Frullania tamarisci* and *F. dilatata* (Frullaniaceae) each produces different sesquiterpene enatiomers. Some liverworts, such as *Lepidozia* species (Lepidoziaceae), biosynthesize both enantiomers.

The more detailed chemical constituent of bryophytes (mainly liverworts) is elaborated elsewhere (*e.g.* Zinsmeister & Mues, 1990; Asakawa, 2008).

Macrocyclic bisbibenzyls (phenolic compounds belonging to stilbenoids), a class of secondary metabolites characteristic components produced exclusively in some liverworts, are attracting more and more attention because of their wide range of biological significance, including anti-bacterial, anti-fungus, anti-oxidation and cytotoxicity. These compounds arise biogenetically from lunularic acid or lunularin, which is widely distributed in leafy and thalloid liverworts (Friederich *et al.*, 1999a, b).

The pro-apoptotic effect of marchantin C on human glioma A172 cells shows the inhibition onto cell growth, viability and colony formation ability (Shi, 2008). Morphological observation and DNA laddering assay showed that marchantin C-treated A172 cells displayed outstanding apoptosis characteristics, such as nuclear fragmentation, the appearance of membrane-enclosed apoptotic bodies and DNA laddering fragment. Moreover, flow cytometric detection of phosphatidylserine externalization indicated that marchantin C-induced apoptosis occurred in a dose-dependent manner. RT-PCR and western blot assay further substantiated that marchantin C, as a promising pro-apoptotic agent, had strong effects to induce A172 cell apoptosis, suggesting that the action was achieved through up-regulating Bax and down-regulating Bcl-2.

Also Marchantin C (Fig 2) isolated from *Marchantia polymorpha, Plagiochasma intermedium* and *Asterella angustata* exhibits the potential antifungal activity toward common clinical pathogenic fungus *Candida albicans* (Niu *et al.*, 2006; Xing *et al.*, 2007).

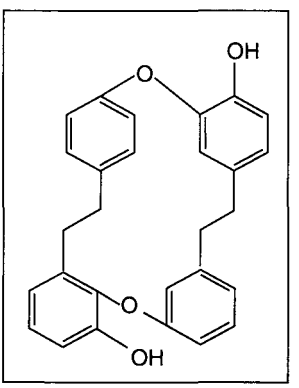


Fig 2. Structure of Marchantin C

Sanionins A and B were isolated from the antarctic moss *Sanionia georgico-uncinata*, collected on the Antarctic Livingston Island (Ivanova *et*

al., 2007). These compounds showed activity against important Gram-positive pathogens, such as mycobacteria, multiresistant staphylococci, and vancomycin resistant enterococci. This activity is combined with anti-inflammatoric activity and low cytotoxicity.

From another Antarctic moss *Polytrichastrum alpinum*, two new benzonaphthoxanthenones, Ohioensins A, C, F and G have been isolated from the MeOH extract. All four compounds showed potent inhibitory activity against therapeutically targeted protein tyrosine phosphatase 1B (PTP1B). Kinetic analysis of PTP1B inhibition by ohioensin F suggested that benzonaphthoxanthenones inhibited PTP1B activity in a non-competitive manner (Seo *et al.*, 2008).

Frahm (2001) stated that only 1% of all bryophytes were phytochemically investigated, but among that 1%, there are many known biological activities whether of complete extract or isloated certain compunds, especially from liverworts. In liverworts, among active substances the most important are sesqui- and diterpenoids or some lipophile aromatic compounds, localized in oli-bodis within cells. These facts place bryophytes to interesting objects for the applied investigation in medical, pharmacological and agriculture sciences.

Ethnobotany of bryophytes is not well known. However, it is known that in some cases North American Indians used bryophytes for the treatment of disease and wounds (Flowers, 1957). In China around 40 bryophyte species were used as medicinal plants to treat hearth diseases, tonsilitis, bronchitis, tympanitis, cystitis, dermatoses or burns (Ding, 1982). Wu (1982) stated that no clinical examinations were performed except for extracts of *Rhodobryum giganteum* on lab animals, which increased blud flux for 30% in crowded capillaries.

The antifungal fraction of liverwort *Palavicinia lyellii* was found to be effective against aspergillosis-induced mortality in immunocompromised mice. Further, there is no report on the immunomodulatory activity of such liverworts. Therefore, the observed protection from aspergillosis-induced mortality is likely to be due to the antifungal activity. This herbal drug appears to be non toxic whereas the antifungal drugs in current use, including ketoconazole, have reported toxic side effects after prolonged use. *P. lyellii* is distributed in India and the biomass can be easily obtained. Thus, *P. lyellii* is likely to be an attractive material for developing invaluable antifungal drugs (Subhisha & Subramoniam, 2006).

Extracts form many bryophytes (mainly liverworts) show antimicrobial and antifungal effects tested in *in vitro* condition. There are a few reports that these extracts can also be used as natural fungicide and for infected plants treatment. Frahm (2001) stated that selected bryophyte extracts express heal effect on tomato plants infected with fungi *Phytophthora infestans*. Sporadically some data on bryophyte extract effects on crop attacking worms and insect can be found as well.

Peat-mosses (*Sphagna*), for example, are known to be colonised by diverse bryophilous ascomycetes, but with no substantial fungal disease (Döbbeler, 1997). The antifungal activity of bacteria that are associated with peat moss natural habitats, especially with bryophytes, is still unclear (Opelt *et al.*, 2007). The study of plant-associated bacteria and their antagonistic potential is important not only for gaining and understanding of their ecological role and the interaction with plants, but also for future biotechnological applications, for example biological control of soil-born plant pathogens or the isolation of bioactive compounds (Bloemberg & Lugtenberg, 2001).

An environmentally friendly alternative to protect roots against fungal pathogens is antagonist-mediated biological control (Weller, 1988; Emmert & Handelsman, 1999; Weller *et al.*, 2002). Antagonists are naturally occurring organisms with traits enabling them to interfere with pathogen growth, survival or infection (Chernin & Chet, 2002).

Knowledge of the indigenous antagonistic potential of each plant species is therefore important for understanding the natural self-protection of plants, as is the detection of new antagonists that could form a basis for biocontrol.

Sphagnum plants form an extreme habitat for microorganisms but they are colonized by specific bacterial populations that are adapted to these special conditions. The high recovery of antagonistic isolates strongly suggests that *Sphagnum* mosses harbour antifungal bacteria, which take part in the pathogen defense. The antifungal bacteria as well as the *Sphagnum* plantlets themselves could be a source for natural fungicides. Moreover, *Sphagnum* plants represent an interesting tool for understanding the natural self-protection of plants and for the detection of new antagonists that could form a basis for biocontrol. They represent an ecological niche not only for diverse and extraordinary microbial populations with a high potential for the biological control of plant pathogens, but also for potentially facultative human pathogens (Opelt *et al.*, 2007).

Extracts from bryophytes *Neckera crispa* and *Porella obtusata* can be used as rapelent against the slug *Arion lusitanicus* (Frahm & Kirchhoff, 2002).

Nozaki *et al.* (2007) found oil body in the moss *Hypnum plumaeforme,* previously exclusively known from liverworts. Ethyl acetate extract from this moss showed potent inhibition of *Arabidopsis thaliana* growth, but not affecting the *H. plumaeforme* itself. These observations suggested that *H. plumaeforme* contains allelochemicals, and that alleleopathy is present within bryophytes as previously believed but not well documented. They isolated two momilactones A and B from oil bodies of *H. plumaeforme* (Fig 3). These two momilactones were previously known only from seed husk of rice (*Oryza sativa*). In rice these compounds increase response to UV irradiation and pathogenesis, and play significant role in plant defence.

Momilactones are accumulated in rice during vegetative growth and realised to the soil, causing growth inhibition of neighbouring plants (Kato-Noguchi & Ino, 2005a, b). Moss *H. plumaeforme* produces a lot of mimilactone B which is probably responsible for allelopathic effect against the growth of other plants. It was isolated 4100 µg/kg dry weight from *H. plumaeforme,* and only 21 and 80 µg/kg from rice roots and shoots fresh weight, respectively (Kato-Noguchi & Ino, 2005a; Nozaki *et al.*, 2007). Nozaki *et al.* (2007) stated that allelochemicals from bryophytes have not been described. Therefore, from this example it is to expect that mosses are potential natural source of herbicides for use in weed control.

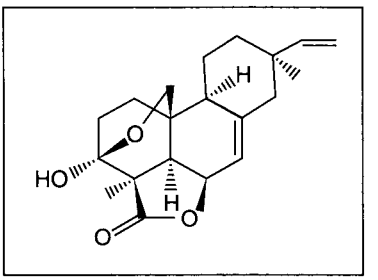


Fig 3. Structure of Momilactone B

Up to date, 150 bryophytes (incl. one hornwort and 25 liverworts) are known in ethnobotanical use (Harris, 2008). Some species are extremely interesting due to medical potential they have. Thus, *Rhodobryum giganteum* and *R. roseum,* used ethno medicinally to cure heart disease, showed significant results in clinical examination or in lab animals (Wu, 1977; Yu & Ma, 1993; Yu *et al.*, 1994, 1995; Yan *et al.*, 1998; Lei *et al.*, 2001a, b; Gao *et al.*, 2004; Zhou *et al.*, 2004; Wang *et al.*, 2005; Dai *et al.*, 2006).

Asakawa (1998) and Frahm (2001) reported that some ether extracts from various liverworts stop the proliferation of tumor cells *in vitro*. They stated that not all are confirmed, but for substances like diplophyllin from *Diplophyllum* spp., marsupellone from *Marsupella emarginata,* plagiochillin from *Plagiochilla* spp. and tulipinoid from *Conocephalum conicum* and *Wiesnerella denudata* are known to effect the tumor cell proliferation. Some sesquiterpenoids and diterpenoids from *Porella* spp. (for example porelladioidin) are known to cure some cancer types.

Some viruses can be cured with moss extracts as well. Klöcking *et al.* (1976) found that at least some peat humic acids posses antiviral activity against herpes simplex virus types 1 and 2, with the most sensitive phase being during the adsorption of viruses to the host cells. Witthauer *et al.* (1976) characterized several antiviral active humic acids in *Sphagnum* and *Camptothecium* extracts that can inhibit growth of polio virus. Some fungi are inhibited by some bryophytes.

Acyclic acetylenic fatty acid and cyclophentenonyl fatty acid extracts from the mosses completely inhibited the growth of the rice blast fungus *Pyricularia oryzae* (Ichikawa *et al.*, 1983).

Banerjee and Sen (1979) found that degree of antibiotic activity in a given species may depend on age of the gametophyte.

For a long time it has been known that some mosses have anti-tumor effects. Belkin *et al.* (1952-1953) found that extracts of *Polytrichum juniperinum* had anticancer activity against Sarcoma 37 in mice. Ohta *et al.* (1977) isolated *ent*-eudesmanolide, diplophylline, from *Diplophyllum albicans* and *D. taxifolium*. Diplophyllin showed significant activity (ED50 4–16 μg/mL) against human epidermoid carcinoma (KB cell culture). Sesquiterpenoids costunolide and tulipinolide, tumor growth-inhibiting substances also known from higher plants, were afterwards isolated from *Conocephalum supradecompositum, Frullania monocera, F. tamarisci, Marchantia polymorpha, Porella japonica,* and *Wiesnerella denudata, Lepidozia vitrea* and *Plagiochila semidecurrens* (Glime, 2007a, b). These substances have demonstrated activity against carcinoma of the nasopharynx, at least in cell culture. Spjut *et al.* (1986) tested 184 species of mosses and 23 species of liverworts for anti-tumor activity. They found that extracts of 43 species were active, while those of 75 species were toxic to the test mice. The best anti-tumor activity was found within representatives of families Brachytheciaceae, Dicranaceae, Grimmiaceae, Hypnaceae, Mniaceae, Neckeraceae, Polytrichaceae, and Thuidiaceae. However, in 1988, this team reported that the anti-tumor activity of the moss *Claopodium crispifolium* was the greatest in samples with the Cyanobacterium *Nostoc* cf. *microscopicum*, and they suggested that the *Nostoc* could be direct source of the activity, or that the activity could be the result of interaction between the species (Spjut *et al.*, 1988). Interaction could result from the transfer of a precursor from the *Nostoc* to the moss and subsequent alteration to the active substance by the moss, or it might result from an allelopathic response of the moss to the presence of the *Nostoc.* In any event, this raises important and intriguing questions, both medically and ecologically.

Several compounds from leafy liverworts exhibit antileukemic activity (Asakawa, 1981). Asakawa (1981) also reported that marchantin A from *Marchantia palacea, M. polymorpha*, and *M. tosana*, riccardin from *Riccardia multifida*, and perrottetin E from *Radula perrottetii* all show cytotoxicity against the KB cells. Peat preparations hold some promise against some types of human cancer (Glime, 2007a, b).

Caution is in order regarding medicinal use of bryophytes, particularly liverworts, because of potential allergic reactions. *Frullania* is well known for its ability to cause contact dermatitis and strong dermatosis (Asakawa, 1998), especially in forest workers and in southern Europe, in olive pickers (Mitchell *et al.*, 1969; Glime, 2007). The active components are sesquiterpene

lactones, also identified in further 24 liverwort species (Asakawa, 1981). Sabovljevic *et al.* (2001) and Sabovljevic & Sabovljevic (2008) reviewed more about bryophytes as a sources of medicinal compounds. However, most data deal with temperate species and there are very few data on tropical or south-hemisphere bryophytes (*e.g.* Pinheiro *et al.*, 1989).

Unfortunately, biologically active substances so far obtained from bryophytes have not proved significant economical in practice. While their pharmaceutical worth seems promising, we lack any understanding of potential harmful side effects.

Bryophytes are known to be very useful in determining the envronmental conditions (Ah-Peng & De Traubenberg, 2004; Zechmeister *et al.*, 2004). Concerning that the bryophytes are very sensitive on microhabitat conditions, Frahm and Klaus (1997, 2001) stated the use of these plants in determination of climate changing.

Bryophytes are beings used in ornamental gardening and horticulture (reviewed by Ando, 1980; Ando & Matsuo, 1984; Iwatsuki & Kodama, 1961; Schenk, 1997). Bryophytes are respected material in cosmetic industry as well (Harris, 2008). Bryophytes are used for such purposes as dyes, lamp-wicks and perfumes (Harris, 2008). In China where gallnut is produced, mosses (*e.g. Plagiomnium maximoviczii*) are grown to encourage the growth of gallnut-producing aphids, which overwinter in mosses (Li & Longton, 1993).

Lately, bryophytes are found to be powerfool tool in forensic science (Virtanen *et al.*, 2007) due to possibility of small piece attachment to the clothes and shoes, combined with the clonal nature of many bryophytes and the ability to extract functional DNA from small plant peaces after long term.

A tiny riparian liverwort *Riccia fluitans* is rich in proteins (27-31%) and posses cation-exchange capacity which prediposed this plant to be used as biological mineral supplement – biological material to which minerals can be bound and be supplied to animals in biologically available form. Chojnacka (2007) shows that native biomass contained only traces of the studied microelements and that the metal binding process inreaseed several fold their content. *Riccia fluitans* bounds metal ions in exchange with alkaline earth metal.

Kobayashi *et al.* (2006) presented a novel treatment system of wastewater contaminated with copper using copper moss *Scopelophila cataractae* that could remove copper from water more efficiently than some other mosses (*Physcomitrella patens* and *Polytrichastrum formosum*). One hundered miligram per liter of copper ion was removed completely for nine days using suspended cultivation system flowing air coupled with intermittent mechanical disruption of the *S. cataractae* protonema filaments made by a homogenizer.

Bleuel *et al.* (2005) showed that two *Fontinalis* (*F. antipyretica* and *F. delecarlica*) species have capacities for biosorption (extracellular adsorption) and bioaccumulation (intracellular uptake) of heavy metals. Although the heavy metal binding potentials of the two mentioned species differ, they are both suitable for monitoring of heavy metals in aquatic habitats.

Some geologist use bryophytes for mineral prospecting. However, not so many data can be found that bryophytes are indicators of specific minerals. The exceptions are copper mosses (*Mielichhoferia elongata, M. mielichhoferi* and *Scopelophila cataractae*) that grow almost exclusively in areas high in copper, particularly in copper sulfate (substrate copper value 30-770 ppm; Glime, 2007b). Although no bryophyte seems to be restricted to substrates containing iron, photosynthesizing bryophytes have the ability to change soluble reduced iron to its insoluble oxidized form and make this molecule visible (Glime, 2007a, b). Certain ecotypes of *Solenostoma crenulatum* (= *Jungermannia gracillima*) significantly increase photosynthesis in presence of Cu, indicating by rich appearance the presence of this metal in substratum (Brown, 1982). According to herbarium data on collection localities of copper mosses subsequent mineral explorations and exploitations are performed (Brooks, 1971). This methodology is called in geology geobotanical prospecting.

It is very interesting that, as a pioneer species, bryophyte from genus *Barbula, Bryum* and *Weissia* are important on the road banks stabilization along the highways in dry areas. Sowing spores and vegetative fragments of bryophytes on bare areas prevent erosion before larger plants become established. In milder climate species from genera *Atrichum, Pogonatum, Pohlia, Trematodon, Blasia* and/or *Nardia* are used.

Bryophyte crusts, endowed with nitrogen-fixing Cyanobacteria, considerably contribute to the other plant available nitrogen in soils. Rao and Burns (1990) reported use of blue–green algae and bryophyte biomass as a source of nitrogen for oil-seed rape. Bryophytes show great promise for cleaning up toxic waste. It is found the moss *Calymperes delessertii* to be an efficient adsorbent for dye, with the rate being determined by a combination of surface adsorption and diffusion within the moss (Lee & Low, 1987).

Some mosses are able to live in the oil presence, which give them advantage to be used for oil clean up. *Racomitrium sudeticum* survived a diesel oil spill and ultimately made the area green again in a subalpine meadow (Belsky, 1982).

Peat mosses (*Sphagna*) are even more suitable than other kinds of mosses (Viraraghavan, 1991) to remove contamination from soil. They are used to divert sewage waste through peat lands, and/or to clean up factory effluents containing acid and toxic heavy metal discharge, detergents, and

dyes. Viraraghavan (1991) suggested peat use not only to remove unwanted metal, but to retrieve metal for reuse by first bringing peat in contact with metal-containing waste, drying the moss by mechanical pressure, and then burning the peat to retrieve the metal. It is claimed that this process is economical for developing countries.

Peat is especially effective at removing nitrogen (96%) and phosphorus (97%) applied from eutrophic river water or sewage (Crum, 1988). Even large oil spills have been contained by floating fences of peat and peat has likewise been used to clean waste water containing oil (Mathavan & Viraraghavan, 1989).

In Canada and Finland, researchers are exploring the possibility of using peat as a filter agent for oily waste in vegetable oil factories (Mathavan & Viraraghavan, 1989).

The highly toxic pentachlorophenol (PCP) is readily adsorbed by *Sphagnum* peat. Tests show that, at concentrations of 1 mg l^{-1}, 91% of the PCP is removed in five hours at the optimum pH of 3–3.5. The adsorption is essentially irreversible, making peat an effective and inexpensive means of removing such toxicants (Virarghavan & Tanjore, 1994). In Poland, peat proved to have a favorable effect on recultivation of brown and hard coal ash, resulting from increased microorganisms and nutrient availability, producing a higher crop yield (Glime, 2007a). *Sphagnum* is also being sold for reclaiming strip-mined land.

Peat has been considered a possible material for filtering water for reuse in space travel (Glime, 2007a). It could be cultivated so that fully used peat could be replaced by new growth. Although it is capable of growing only a few centimeters per month, its tremendous absorptive abilities may compensate for this slow growth limitation.

Most authors consider bryophytes to be unimportant as food sources for animals. Indeed, mosses have low caloric values. However, bryophytes may be the source of specific needs of animals at a time when fresh food is scarce (Glime, 2007a, b). For example, *Barbella pendula* has a high content of vitamin B12, a vitamin that is difficult to obtain on a strictly vegetarian diet. When fed to puppies and chickens, it causes no noticeable side effects (Glime, 2007a). Hog farms take advantage of unique properties of *Sphagnum* to administer vitamins. Piglets are often anemic, and milled peat moss, used as a binder for iron and vitamins, is fed to them.

Prins (1981) suggested that it might keep the foot pads of arctic mice and lemmings from freezing.

Although the moss itself is not eaten, *Sphagnum* contributes to the flavor of Scotch whisky (Glime, 2007a). In the Himalayas, Kumaun Indians use slender bryophytes such as *Anomodon, Entodon, Hypnum, Meteoriopsis, Herbertus,* and *Scapania*, wrapped in a cone of *Rhododendron campanulatum* leaves, to serve as a filter for smoking tobacco (Glime, 2007a).

Bryophytes are used to produce moss-mates which make the surfaces (both horizontal and vertical) green (Fig 4). But the main reason why moss-mates pay attention is novelty that mosses can bind fine dust (in size less than 10 ppm). This moss property plus facility in maintaining bring moss-mates to cover high-way and airport edges in Germany (Frahm & Sabovljevic, 2007).

Fig 4. Moss-mates

A liverwort *Jungermannia vulcanicola* accumulates extraordinary high content of mercury (1.3% of dry-weight) which makes this species a potential hyperaccumulator plant. Satake and Miyasaka (1984a) report the mercury to be deposited in the cell walls as a sulphur-mercury component. Satake *et al.* (1984b) found that *Ectropothecium subobscurum* water moss from New Caledonia accumulates nickel (690 μg gr^{-1} of dry weight), manganese (470 μg gr^{-1} of dry weight), chromium (254 μg gr^{-1} of dry weight) and bromine (210 μg gr^{-1} of dry weight), and liverwort *Lopholejeunea* sp. an incredibly high amount of manganese (15300 μg gr^{-1} of dry weight). Same authors state that in general amounts of nickel, chromium and bromine in plants do not exceed 100 μg gr^{-1} of dry weight. These peculiarities highlighted these bryophytes to be of high interest in ecological engineering.

Thus, bryophytes can attach and store (both physically and chemically) many trace metals without demaging vital function. Exactly this bryophyte characteristic make them useful in biomonitoring (*e.g.* Sabovljevic *et al.*, 2005, 2007; Vukojevic *et al.*, 2005, 2006). Moreover, bryophytes have many advantage in monitoring of radionucleids due to special characteristics to "recycle" radionucleids from old to young tissue and "cation exchange capacity" of the membranes (Bates, 2000).

There are too many unknown features of these plants and lot remains to be studied. However, the potentials of these plants for biotechnological use are high as shown above, significantly higher than those already involved in recent biotechnologies.

While their economic value to date has been limited, there are indications of exciting new uses of bryophytes in the near future.

REFERENCES

Ah-Peng, C. and De Traubenberg, C.R. (2004). Aquatic bryophytes as pollutant accumulators and ecophysiological bioindicators of stress: Bibliographic synthesis. *Cryptogamie Bryologie*, **25**: 205-248.

Ando, H. (1980). Bibliography of the use of bryophytes. *Hikobia*, **8**: 424-448.

Ando, H. and Matsuo, A. (1984). Applied Bryology. *Advances in Bryology*, **2**: 133-229.

Asakawa, Y. (1981). Biologically active substances obtained from bryophytes. *Journal of the Hattori Botanical Laboratory*, **50**: 123-142.

Asakawa, Y. (1998). Biologically active compounds from bryophytes. *Journal of the Hattori Botanical Laboratory*, **84**: 91-104.

Asakawa, Y. (2008). Bryophytes-potential source of medicinal compounds. *Current Pharmaceutical Design*, **14**: 3067-3088.

Banerjee, R.D. and Sen, S.P. (1979). Antibiotic activity of bryophytes. *Bryologist*, **82**: 141-153.

Bates, J.W. (2000). Mineral nutrition, substratum ecology and pollution. *In*: Bryophyte biology. *Ed.* By Show, A.J. and Goffinet, B., Cambridge University Press, pp. 248-311.

Baur, A., Kaufmann, F., Rolli, H., Weise, A., Luethje, R., Berg, B., Braun, M., Baeumer, W., Kietzmann, M., Reski, R. and Gorr, G. (2005a). A fast and flexible PEG-mediated transient expression system in plants for high level expression of secreted recombinant proteins. *J. Biotechnol.*, **119**: 332-342.

Baur, A., Reski, R. and Gorr, G. (2005b). Enhanced recovery of a secreted recombinant human growth factor using stabilizing additives and by co-expression of human serum albumin in the moss *Physcomitrella patens*. *Plant Biotechnol. J.*, **3**: 331-340.

Belkin, M., Fitzgerald, D.B. and Felix, M.D. (1952-1953). Tumor-damaging capacity of plant materials. II. Plants used as diuretics. *J. Nat. Cancer Inst.*, **13**: 741-744.

Belsky, J. (1982). Diesel oil spill in a subalpine meadow: 9 years of recovery. *Can. J. Bot.*, **60**: 906-910.

Bijelovic, A. and Sabovljevic, M. (2003). Callus induction and plant regeneration in the moss *Aloina aloides* (Schultz.) Kindb. (*Pottiaceae, Bryopsida*). *Archives of Biological Sciences*, **55**: 77-80.

Bijelovic, A., Sabovljevic, M., Grubisic, D. and Konjevic, R. (2004). Phytohormone influence on the morphogenesis of two mosses [*Bryum argenteum* Hedw. and *Atrichum undulatum* (Hedw.) P. Beuav.]. *Israel Journal of Plant Sciences*, **52**: 31-36.

Bleuel, C., Wesenberg, D., Sutter, K., Miersch, J., Braha, B., Bärlocher, F. and Krauss, G.J. (2005). The use of the aquatic moss *Fontinalis antipyretica* L. ex Hedw. as a bioindicator for heavy metals 3. Cd^{2+} accumulation capacities and biochemical stress response of two *Fontinalis* species. *Science of the Total Environment*, **345**: 13-21.

Bloemberg, G.V. and Lugtenberg, B.J.J. (2001). Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Curr. Opin. Plant Biology*, **4**: 342-350.

Brooks, R.R. (1971). Bryophytes as a guide to mineralization. *New Zealand Journal of Botany*, **9**: 674-677.

Brown, D.H. (1982). Mineral nutrition. *In*: Bryophyte Ecology. *Ed.* By Smith, A.J.E., London. Chapman & Hall, pp. 383-444.

Bruch, J. (2003). Some mosses survive cryopreservation without prior pretreatment. *Bryologist*, **106**: 270-277.

Cañizares, M.C., Liu, L., Perrin, Y., Tsakiris, E. and Lomonossoff, G.P. (2006). A bipartite system for the constitutive and inducible expression of high levels of foreign proteins in plants. *Plant Biotechnol. J.*, **4**: 183-193.

Chen, X., Zeng, Q. and Wood, A.J. (2002). *Aldh7B6* encodes a turgor-responsive aldehyde dehydrogenase homologue that is constitutively expressed in *Tortula ruralis* gametophytes. *Bryologist*, **105**: 177-184.

Chernin, L. and Chet, I. (2002). Microbial enzymes in biocontrol of plant pathogens and pests. *In*: Enzymes in the environment: activity, ecology and applications. *Ed.* By Burns, R. and Dick, R., Marcel Dekker Inc., New York, NY, pp. 171-225.

Chojnacka, K. (2007). Biosorption and bioaccumulation of microelements by *Riccia fluitans* in single and multi-metal system. *Bıosource Technology* **98**: 2919-2925.

Chopra, S.C. (1994). Moss protonema differentiation. New York: Wiley.

Crum, H.A. (1988). A Focus on Peatlands and Peat Mosses. University of Michigan, Ann Arbor.

Cvetic, T., Sabovljevic, A., Sabovljevic, M. and Grubisic, D. (2007). Development of the moss *Pogonatum urnigerum* (Hedw.) P. Beauv. under *in vitro* culture conditions. *Archives of Biological Sciences*, **59**: 57-61.

Cvetic, T., Sabovljevic, M., Sabovljevic, A. and Grubisic, D. (2005). *In vitro* culture and apogamy – alternative pathway in life cycle of the moss *Amblystegium serpens* (Amblystegiaceae). *Archives of Biological Sciences*, **57**: 267-272.

Dai, C., Liu, P., Liu, C., Wang, B. and Chen, R.Y. (2006). Studies on chemical constituents from moss *Rhodobryum roseum* II. *ZhongguoZhongYaoZaZhi* **31(13)**: 1080-1082.

Decker, E.L. and Reski, R. (2004). The moss bioreactor. *Curr. Opın. Plant Biol.*, **7**: 166-170.

Decker, E.L. and Reski, R. (2007). Moss bioreactors producing biopharmaceuticals. *Current Oppinion in Bıotechnology*, **18**: 393-398.

Ding, H. (1982). Medical spore-bearing plants of China. Schangai, pp. 499.

Döbbeler, P. (1997). Biodiversity of bryophilous ascomycetes. *Biodivers. Conserv.*, **6**: 721-738.

Duckett, J.G., Burch, J., Fletcher, P.W., Matcham, H.W., Read, D.J., Russel, A.J. and Pressel, S. (2004). *In vitro* cultivation of bryophytes: a review of practicalities, problems, progress and promise. *Journal of Bryology*, **26**: 3-20.

Emmert, E.A.B. and Handelsman, J. (1999). Biocontrol of plant disease: A (Gram1) positive perspective. *FEMS Microbiol Lett.*, **171**: 1-9.

Flowers, S. (1957). Ethnobryology of Gosuite Indians of Utah. *Bryologist*, **60**: 11-14.

Frahm, J.P. (2001). Biologie der Moose. Spectrum Akademischer Verlag. Heidelberg, Berlin. pp. 357.

Frahm, J.P. (2004). Recent developments of commercial products from bryophytes. *Bryologist*, **107**: 277-283.

Frahm, J.P. and Klaus, D. (1997). Moose als Indikatoren von Klimafluktuationen. *Erdkunde*, **51**: 181-190.

Frahm, J.P. and Klaus, D. (2001). Bryophytes as indicators for past and present climate fluctuations. *Lindbergia*, **26**: 97-104.

Frahm, J.P. and Kirchhoff, K. (2002). Antifeeding effects of bryopyhte extracts from *Neckera crispa* and *Porella obtusata* against the slug *Arion lusitanicus*. *Cryptogamie Bryologie*, **23**: 271-275.

Frahm, J.P. and Sabovljevic, M. (2007). Feinstaubreduzierung durch Moose. *Immissionsschutz*, **4**: 152-156.

Friederich, S., Maier, U.H., Deus-Neumann, B., Asakawa, Y. and Zenk, M.H. (1999a). Biosynthesis of cyclic *bis*(benzyls) in *Marchantia polymorpha*. *Phytochemistry*, **50**: 589-598.

Friederich, S., Rueffer, M., Asakawa, Y. and Zenk, M.H. (1999b). Cytochromes P-450 catalyse the formation of marchantin A and C in *Marchantia polymorpha*. *Phytochemistry*, **52**: 1195-1202.

Gao, G.Q., Wang, G.W. and Zhang, H.K. (2004). Investigation of the effect, mechanism and activity of *Rhodobryum roseum* on curing lack of blood and cardiovascular disease. *Chinese Journal of Integrated Traditional and Western Medicine*, **24**: 929.

Glime, J.M. (2007a). Economic and ethnic uses of bryophytes. *In*: Flora of North America North of Mexico. Vol. 27. Bryophyta, part 1. Editorial Committee. Oxford University Press, New York. pp. 14-41.

Glime, J.M. (2007b). Bryophyte Ecology. Vol. 1. Physiological Ecology. Ebook sponsored by Michigan Technological University and the International Association of Bryologists. <http://www.bryoecol.mtu.edu/>.

Gomord, V., Chamberlain, P., Jefferis, R. and Faye, L. (2005). Biopharmaceutical production in plants: problems, solutions and opportunities. *Trends in Biotechnology*, **23**: 559-565.

Gorr, G. and Altmann, F. (2006). Glycosylation of recombinant proteins in plants. *In*: Medical plants biotechnology. *Ed.* By Kayser, O. and Quax, W., Wiley-VCH, Weinheim, pp. 345-374.

Gorr, G. and Jost, W. (2005). Glycosilation design in transgenic moss for better product efficiency. *Bioprocess Journal*, **4**: 26-30.

Haltorf, H., Frank, W. and Reski, R. (2004). Applied genomics in *Physcomitrella*. *In*: New frontiers in bryology: Physiology, molecular biology and functional genomics. *Ed.* By Wood, A.J., Oliver, M.J. and Cove, D.J., Kluwer Academic Publisher, the Netherlands, pp. 51-70.

Harris, E.S.J. (2008). Ethnobryology: traditional uses and folk classification of bryophytes. *Bryologist*, **111**: 169-217.

Ichikawa, T., Namikawa, M., Yamada, K., Sakai, K. and Kondo, K. (1983). Novel cyclopentenonyl fatty acids from mosses, *Dicranum scoparium* and *Dicranum japonicum*. *Tetrahedron Lett.*, **24**: 3327-3340.

Ivanova, V., Kolarova, M., Aleksieva, K., Dornberger, K.-J., Haertl, A., Moellmann, U. Dahse, H.-M. and Chipev, N. (2007). Sanionins: Anti-inflammatory and Antibacterial Agents with Weak Cytotoxicity from the Antarctic Moss *Sanionia georgico-uncinata*. *Preparative Biochemistry and Biotechnology*, **37**: 343-352.

Iwatsuki, Z. and Kodama, T. (1961). Mosses in Japanese gardens. *Economic Botany*, **28**: 311-337.

Kato-Noguchi, H. and Ino, T. (2005a). Concentration and release level of momilactone B in the seedlings of eight rice cultivars. *J. Plant. Physiol.*, **162**: 965-969.

Kato-Noguchi, H. and Ino, T. (2005b). Possible involment of momilactone B in the rice allelopathy. *J. Plant Physiol.*, **162**: 718-721.

Kobayashi, F., Kofuji, R., Yamashita, Y. and Nakamura, Y. (2006). A novel treatment system of wastewater contaminated with copper by a moss. *Biochemical Engineering Journal*, **28**: 295-298.

Klöcking, R., Thiel, K.-D. and Sprössig, M. (1976). Antiviral activity of humic acids from peat water. *In*: Proc. 5th Internat. Peat Congr., Poznabn, Poland, Vol. 1. Peat and Peatlands in the Natural Environment Protection, pp. 446-455.

Koprivova, A., Altmann, F., Gorr, G., Kopriva, S., Reski, R. and Decker, E.L. (2003) N-glycosylation in the moss *Physcomitrella patens* is organized similarly to that in higher plants. *Plant Biol.*, **5**: 582-591.

Koprivova, A., Stemmer, C., Altmann, F., Hoffmann, A., Kopriva, S., Gorr, G., Reski, R. and Decker, E.L. (2004). Targeted knockout of *Physcomitrella* lacking plant-specific immunogenic *N*-glycans. *Plant Biotechnology Journal*, **2**: 517-523.

Lee, C.K. and Low, K.S. (1987). The sorption characteristics of lead and copper by the moss, Calymperes delessertii Besch. *Pertanika*, **10**: 321-326.

Lei, X.L., Zhang, R.P., Dong, X.F., Pan, Q., Yan, Q.R., Luo, T.H. and He, G.X. (2001a). Influence of the Yunnan herbal *Rhodobryum roseum* on prostacycline PG12/ tromboxane A2 and in curing cardiovascular disease. *Natural Product Research and Development*, **6**: 63-66.

Lei, X.L., Zhang, R.P., Dong, X.F., Pan, Q., Yan, Q.R., Luo, T.H. and He, G.X. (2001b). Protective effect of Yunnan folk medicine *Rhodobryum roseum* on experimental myocardial ischemia of rat. *Chinese Traditional and Herbal Drugs*, **32**: 1103-1106.

Li, Y.M. and Longton, R.E. (1993). Mosses and the production Chinese gallnuts. *Journal of Bryology*, **17**: 421-430.

Maliga, P. and Graham, I. (2004). Plant Biotechnology: Molecular farming and metabolic engineering a new generation of high-tech crops. *Current Oppinion in Plant Biology*, **7**: 149-151.

Mathavan, G.N. and Viraraghavan, T. (1989). Use of peat in the treatment of oily waters. *Water, Air and Soil Pollution*, **45**: 17-26.

Mitchell, J.C., Schofield, W.B.T., Singh, B. and Towers, G.H.N. (1969). Allergy to *Frullania*, allergic contact dermatitis occurring in forest workers caused by exposure to *Frullania nisquallensis*. *Arch. Dermatol.*, **100**: 46-49.

Murphy, D.J. (2007). Improving containment strategies in biopharming. *Plant Biotechnology Journal*, **5**: 555-569.

Nechansky, A., Schuster, M., Jost, W., Siegel, P., Wiederkum, S., Gorr, G. and Kircheis, R. (2007). Compensation of endogenous IgG mediated inhibition of antibody-dependent cellular cytotoxicity by glycoengineering of therapeutic antibodies. *Molecular Immunology*, **44**: 1826-1828.

Niu, C., Qu, J.B. and Lou, H.X. (2006). Antifungal *bis* (bibenzyls) from the Chinese liverwort *Marchantia polymorpha* L. *Chem. Biodiversity*, **3**: 34-40.

Nozaki, H., Hayashi, K.-I., Nishimura, N., Kawaide, H., Matsuo, A. and Takaoka, D. (2007). Momilactone A and B as allelochmiclas from moss *Hypnum plumaeforme*: First occurrence in bryophytes. *Biosci. Biotechnol. Biochem.*, **71**: 3127-3130.

Ohta, Y., Andersen, N.H. and Liu, C.-B. (1977). Sesquiterpene constituents of two liverworts of genus *Diplophyllum*. Novel eudesmanolides and cytotoxicity studies for enantiomeric methylene lactones. *Tetrahedron*, **33**: 617-628.

Opelt, K., Berg, C. and Berg, G. (2007). The bryophyte genus *Sphagnum* is a reservoir for powerful and extraordinary antagonists and potentially facultative human pathogens. *FEMS Microbial Ecol.*, **61**: 38-53.

Pinheiro, M.F., Lisboa, R.C.L. and Brazao, R.V. (1989). Contribuicao ao estudo de briofitas como fonts de antibioticos. *Acta Amazonica*, **19**: 139-145.

Prins, H.H.T. (1981). Spring grazing and the manipulation of food quality by barnacle geese. *J. Appl. Ecol.*, **18**: 443-453.

Rao, D.L.N. and Burns, R.G. (1990). Use of blue–green algae and bryophyte biomass as a source of nitrogen for oil-seed rape. *Biol. Fertil. Soils*, **10**: 61-64.

Reski, R. (1998). *Physcomitrella* and *Arabidopsis*: The David and Goliath of reverse genetics. *Trends in Plant Sciences*, **3**: 209-210.

Reski, R. (1999). Molecular genetics of *Physcomitrella*. *Planta*, **208**: 301-309.

Rowntree, J. (2006). Development of novel methods for the initiation of *in vitro* bryophyte cultures for conservation. *Plant Cell Tiss Organ Cult*, **87**: 191-201.

Sabovljevic, A. and Sabovljevic, M. (2008). Bryophytes, a source of bioactive and new compounds. *In*: Phytopharmacology and Therapeutic Values IV, the Series "Recent Progress in Medicinal Plants". *Ed.* By Govil, J.N. Studium Press, Houston, Texas, USA. pp. 9-25.

Sabovljevic, M., Bijelovic, A. and Grubisic, D. (2001). Bryophytes as a potential source of medicinal compounds. *Lekovite Sirovine*, **21**: 17-29.

Sabovljevic, M., Bijelovic, A. and Dragicevic, I. (2002). Effective and easy way of establishing *in vitro* culture of mosses, *Bryum argenteum* Hedw. and *Bryum capillare* Hedw. (*Bryaceae*). *Archive of Biological Sciences*, **54**: 7P-8P.

Sabovljevic, M., Bijelovic, A. and Dragicevic, I. (2003). *In vitro* culture of mosses: *Aloina aloides* (K.F. Schultz) Kindb., *Brachythecium velutinum* (Hedw.) B.S. & G., *Ceratodon purpureus* (Hedw.) Brid., *Eurhynchium praelongum* (Hedw.) B.S. & G. and *Grimmia pulvinata* (Hedw.) Sm. *Turkish Journal of Botany*, **27**: 441-446.

Sabovljevic, A., Cvetic, T. and Sabovljevic, M. (2006). The establishment and development of the Catherine's moss *Atrichum undulatum* (Hedw.) P. Beauv. (Polytrichaceae) in *in vitro* conditions. *Archives of Biological Sciences*, **58**: 87-93.

Sabovljevic, A., Sabovljevic, M., Grubisic, D. and Konjevic, R. (2005). The effect of sugars on development of two moss species (*Bryum argenteum* and *Atrichum undulatum*) during *in vitro* culture. *Belgian Journal of Botany*, **138**: 79-84.

Sabovljevic, A., Sokovic, M., Sabovljevic, M. and Grubisic, D. (2006). Antimicrobial activity of *Bryum argenteum*. *Fitoterapia*, **77**: 144-145.

Sabovljevic, M., Vukojevic, V., Mihajlovic, N., Drazic, G. and Vucinic, Z. (2005). Determination of heavy metal deposition in the county of Obrenovac (Serbia) using mosses as bioindicators. I: Aluminium (Al), Arsenic (As) and Boron (B). *Archives of Biological Sciences*, **57**: 205-212.

Sabovljevic, M., Vukojevic, V., Sabovljevic, A., Mihajlovic, N., Drazic, G. and Vucinic, Z. (2007). Determination of heavy metal deposition in the county of Obrenovac (Serbia) using mosses as bioindicators III. Copper (Cu), Iron (Fe) and Mercury (Hg). *Archives of Biological Sciences*, **59**: 351-361.

Saidi, Y., Finka, A., Chakhporanian, M., Zryd, J.P., Schaefer, D.G. and Goloubinoff, P. (2005). Controlled expression of recombinant proteins in *Physcomitrella patens* by a conditional heat-shock promoter: A tool for plant research and biotechnology. *Plant Mol. Biol.*, **59**: 697-711.

Satake, K. and Miyasaka, K. (1984a). Evidence of high mercury accumulation in the cell wall of the liverwort *Jungermannia vulcanicola* Steph. To form particles of a mercury-sulphur compound. *Journal of Bryology*, **13**: 101-105.

Satake, K., Ieatsuki, Z. and Nishikawa, M. (1984b). Inorganic elements in some aquatic bryophytes from streams in New Caledonia. *Journal of the Hattori Botanical Laboratory*, **57**: 71-82.

Saxena, D. and Harinder, K. (2004). Uses of bryophytes. *Resonance*, **9**: 56-65.

Schaefer, D.G. (2001). Gene targeting in *Physcomitrella patens*. *Current Oppinion in Plant Biology*, **4**: 143-150.

Schaefer, D.G. and Zryd, J.P. (1997). Efficient gene targeting in the moss *Physcomitrella patens*. *Plant Journal*, **11**: 1195-1206.

Schaefer, D. and Zryd, J.-P. (2004). Principles of targeted mutagenesis in the moss *Physcomitrella patens*. *In*: New frontiers in bryology: Physiology, molecular biology and functional genomics. *Ed.* By Wood, A.J., Oliver, M.J. and Cove, D.J., Kluwer Academic Publisher, the Netherlands, pp. 37-49.

Schenk, G. (1997). Moss Gardening, including lichens, liverworts and other miniatures. Timner Press, Portland.

Schulte, J. and Reski, R. (2004). High troughput cryopreservation of 140000 *Physcomitrella patens* mutants. *Plant Biology*, **6**: 119-127.

Seo, C., Choi, Y.-H., Sohn, J.H., Ahn, J.S., Yim, J.H., Lee, H.K. and Oh, H. (2008). Ohioensins F and G: Protein tyrosine phosphatase 1B inhibitory enzonaphthoxanthenones from the Antarctic moss *Polytrichastrum alpinum*. *Bioorganic & Medicinal Chemistry Letters*, **18**: 772-775.

Servettaz, C. (1913). Reserches experimentales sur le development et la nutrition des mousses en milieux sterilise. *Ann. Sci. Nat., Bot. Biol. Veg.*, **17**: 111-223.

Shi, Y.-S., Liao, Y.X., Qu, X.-J., Yian, H.-Q., Li, S., Qu, J.-B. and Lou, H.-X. (2008). Marchantin C, a macrocyclic bisbibenzyl, induces apoptosis of human glioma A cells. *Cancer Letters*, **262**: 173-182.

Spjut, R.W., Suffness, M., Cragg, G.M. and Norris, D.H. (1986). Mosses, liverworts, and hornworts screened for antitumor agents. *Econ. Bot.*, **40**: 310-338.

Spjut, R.W., Cassady, J.M., McCloud, T., Suffness, M., Norris, D.H., Cragg, G.M. and Edson, C.F. (1988). Variation in cytotoxicity and antitumor activity among samples of the moss *Claopodium crispifolium* (Thuidiaceae). *Econ. Bot.*, **42**: 62-72.

Subhisha, S. and Subramoniam, A. (2006). *In vivo* efficacy of an antifungal fraction from *Palavicinia lyellii*, a liverwort. *Indian Journal of Pharmacology*, **38**: 211-212.

Thieret, J.W. (1956). Bryophytes as economic plants. *Economic Botany*, **10**: 75-91.

Trouiller, B., Charlot, F., Choinard, S., Schaefer, D.G. and Nogué, F. (2007). Comparison of gene targeting efficiencies in two mosses suggests that it is a conserved feature of bryophyte transformation. *Biotechnology Letters*, **29**: 1591-1598.

Viraraghavan, T. (1991). Use of peat in pollution control. *International Journal of Environmental Studies*, **37**: 163-169.

Viraraghavan, T. and Tanjore, S. (1994). Removal of pentachlorophenol from wastewater using peat. *Hazardous Waste Hazardous Materials*, **11**: 423-433.

Virtanen, V., Korpelainen, H. and Kostamo, K. (2007). Forensic botany: Usability meterial in forensic studies. *Forensic Science International*, **172**: 161-163.

Vukojevic, V., Sabovljevic, A. and Sabovljevic, M. (2004). Effect of ferri(III)citrate and potassium hexacyanoferrate(III) on growth of the moss *Bryum argenteum* Hedw *in vitro*. *Archives of Biological Sciences*, **56**: 75-78.

Vukojevic, V., Sabovljevic, M. and Jovanovic, S. (2005). Mosses accumulate heavy metals from the substracta of coal ash. *Archives of Biological Sciences*, **57**: 101-106.

Vukojevic, V., Sabovljevic, M., Sabovljevic, A., Mihajlovic, N., Drazic, G. and Vucinic, Z. (2006). Determination of heavy metal deposition in the county of Obrenovac (Serbia) using mosses as bioindicators II: Cadmium (Cd), Cobalt (Co) and Chromium (Cr). *Archives of Biological Sciences*, **58**: 95-104.

Walsh, G. and Jefferis, R. (2006). Post-transitional modifications in the context of therapeutic proteins. *Nat. Biotechnol*, **24**: 1241-1252.

Wang, B., Liu, P., Shenm Y.M. and Dai, C. (2005). Studies on the chemical constituents from herb of *Rhodobryum roseum*. *Zhongguo Zhong YaoZa Zhi* **30**: 895-897.

Weise, A., Rodriguez-Franco, M., Timm, B., Hermann, M., Link, S., Jost, W. and Gorr, G. (2006). Use of *Physcomitrella patens* actin 5′ regions for high transgene expression: importance of 52†introns. *Appl. Microbiol. Biotechnol.*, **70**: 337–345.

Weller, D.M. (1988). Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol*, **26**: 379-407.

Weller, D.M., Raaijmakers, J.M., Gardener, B.B. and Tomashow, L.S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu Rev Phytopathol.*, **40**: 309-348.

Witthauer, J., Klöcking, R., Helbig, B. and Drabke, P. (1976). Chemical and physicochemical characterization of antivirally active humic acids. *In*: Proc. 5th Internat. Peat Congr., Poznabn, Poland, Vol. 1. Peat and Peatlands in the Natural Environment Protection. pp. 456-466.

Wu, P.C. (1977). *Rhodobryum giganteum* (Schwaegr.) Par. Can be used for curing cardiovascular desease. *Acta Phytotaxonomica Sinica*, **15**: 93.

Wu, P.C. (1982). Some uses of mosses in China. *Bryological Times*, **13**: 5.

Xing, J., Xie, C.F., Qu, J.B., Guo, H.F., Lv, B.B. and Lou, H.X. (2007). Rapid screening for bisbenzyls in bryophyte crude extracts using liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrometry*, **21**: 2467-2476.

Yan, Q.X., He, G.X., Zhang, R.P., Lei, X.L., Luo, T.H. and Liang, X.Y. (1998). Pharmaceutical research on *Rhodobryum roseum*. *Journal of the Yunnan College of Traditional Chinese Medicine*, **21**: 5-7.

Yu, Y.M. and Ma, Y. (1993). Effects of *Rhodobryum roseum* on hemorheology following acute coronary occlusion in dogs. *Chinese Journal of Integrated Traditional and Western Medicine*, **13**: 672-674.

Yu, Y.M., Ma, Y., Xia, T. and Ma, S.P. (1995). Studies of action of *Rhodobryum roseum* Limpr. On changes of red cell aggregation and yield-shear stress in dogs with acute experimental coronary occlusion. *China Journal of Chinese Materia Medica*, **20**: 429-431.

Yu, Y.M., Ma, Y., Xia, T., Wei, H. and Han, M.Y. (1994). Experiments and research on the prevention of artherosclerosis in rabbit by *Rhodobryum roseum*. *Shaanxi Chinese Medicine*, **15**: 562-563.

Zechmeister, H.G., Riss, A. and Hanus-Illnar, A. (2004). Biomonitoring of atmospheric heavy metal deposition by mosses in the vicinity of industrial sites. *Journal of Atmospheric Chemistry,* **49**: 461-477.

Zhou, J., Sun, X.L., Wang, S.W. and Sun, H.B. (2004). Summarization of research on the chemical composition, pharmacological activities and clinical application of *Rhodobryum roseum*. *China New Medicine,* **3**: 79-80.

Zinsmeister, H.D. and Mues, R. (1990). Bryophytes, their chemistry and chemical taxonomy. Clarendon Press, Oxford. pp. 470.

"This page is Intentionally Left Blank"

10

Review: Molecular Biology Approach to Viper Venoms

PONLAPAT ROJNUCKARIN[1]*, CHUANCHOM MUANPASITPORN[1], PON SINGHAMATR[1] AND JARADPONG ARPIJUNTARANGKOON[1]

ABSTRACT

Viper venoms comprise a large array of proteins affecting endothelium, platelets, blood coagulation and anti-coagulation, fibrinolysis, blood circulation, extracellular matrix, as well as cancer biology. Surprisingly, this plethora of functions is generated from limited molecular structures, e.g. snake venom serine protease, snake venom metalloprotease / disintegrin, C-type lectin-like protein and phospholipase A_2. Venom gland accelerated evolution greatly diversifies their activities to fit a wide variety of preys. Several derivatives of these components have been, or potentially are, useful diagnostic or therapeutic agents. Molecular cloning and recombinant expression of these peptides give us complete and accurate amino acid sequences to correlate with their functions and to compare with related proteins. This will provide the deeper insights in pathogenesis of snakebites and the molecular mechanisms of protein functions. The cDNA allows us to make a large amount of pure recombinant proteins and perform mutagenesis for detailed structure-function studies. Therefore, current molecular technology may be able to mimic the 'nature experiments' by engineering proteins with desirable activities from these molecular platforms for clinical and / or research uses.

Key words : Viper venom, molecular cloning, serine protease, snake venom metalloproteinase, disintegrin, C-type lectin-like protein, phospholipase A_2

1. Snake Venom and Snakebite Research Unit, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.

* *Corresponding author* : E-mail : rojnuckarinp@gmail.com

INTRODUCTION

Venom glands evolved very early before snakes phylogenetically separated from lizards. Advance snakes recruited a number of genes, *e.g.* a digestive enzyme, A Disintegrin and Metalloprotease (ADAM) protein and phospholipase A_2, to be expressed in glandular tissue and acquire their toxicities (Fry, 2006). On these limited genes, venom proteins underwent accelerated evolution by gene duplications and substitutions in coding regions to maximize toxic effects to a wide variety of preys in various geographies (Nakashima, 1993; Ogawa, 1996). As a result, although venom proteins show a high degree of sequence homology, they surprisingly display a myriad of unique functions (Matsui, 2000). Therefore, the molecular cloning is an intriguing approach, as we can clone novel proteins with distinct functions using information from known homologs. How the snakes can greatly diversify, specifically, the venoms is still an enigma.

VIPER VENOMS

The 2 major families of highly venomous (Front-fanged) snakes are the elapids that possess principally paralyzing neurotoxin and the vipers that exert mainly hemorrhagic toxins. The effects of viper envenomation can be divided into local and systemic symptoms. Local damages include edema, blisters, muscle toxicity and gangrene. Both catalytically active and inactive classes of phospholipases A_2 (PLA_2) in snake venoms can induce local edema in animals (Liu, 1991). Snake venom metalloproteinases (SVMPs) are implicated in tissue necrosis and dermo-epidermal separation (Blister) after viper bites. SVMPs rapidly degrade basement membrane components and endothelial adhesion proteins resulting in microvascular disruption and vascular wall weakness susceptible to injury by blood flow (Gutierrez, 2005). Furthermore, SVMPs can up-regulate several inflammatory cytokines enhancing local tissue damages (Laing, 2003). Although current antivenoms can reverse systemic effects of vipers, they are relatively ineffective for local tissue injury (Rojnuckarin, 2006; Chotenimitkhun, 2008). In animal models, antivenoms could not prevent local effects, although it was administered very shortly after, or even before, venoms (Russell, 1973; Gutierrez, 1998). Novel treatment modalities are really needed. Studies of the molecular mechanisms of local tissue damages are essential to solve this clinical problem.

The prominent symptom of systemic envenomation of vipers is bleeding. Viper venoms affect every aspect of hemostasis from blood vessel wall, platelet, blood coagulation/anticoagulation and fibrinolysis. Some venom components directly impede hemostatic processes, while others contain activating activities. However, these activating proteins paradoxically inhibit coagulation *in vivo*. For example, thrombin-like serine proteases can clot fibrinogen *in vitro*, but these friable clots are subjected to fibrinolysis

resulting in hypofibrinogenemia in humans (Rojnuckarin, 1999). In addition, platelets activated by venom are rapidly cleared by phagocytic system resulting in thrombocytopenia. Therefore, these venom proteins have potentials to be useful diagnostic agents, therapeutic anti-coagulants or reagents to elucidate mechanisms of hemostasis in health and diseases.

In post-genomic era, molecular cloning of venom proteins is a more attractive alternative compared with conventional protein purification for investigating the venom components. The deduced protein sequences are complete and accurate, providing data for a bioinformatic analysis including functional prediction (Rojnuckarin, 2006b). The impacts of these studies are far beyond the tropical medicine arena, as viper venom is an exceptional source of proteins that have potentials to be not only agents affecting hemostasis, but also novel anticancer therapy. The cDNA allows us to make a large amount of pure recombinant proteins and perform mutagenesis for structure-function studies. Utilizing this technology, we can engineer proteins with desirable effects for future clinical or research uses.

The following sections will briefly review selected proteins from viper venoms to illustrate the concept. There are other venom protein families, *e.g.* L-amino acid oxidase, venom-derived vascular endothelial growth factor (VEGF), bradykinin-potentiating peptide (BPP) and cysteine-rich secretory proteins (CRISP), which will not be discussed here.

STRUCTURES, FUNCTIONS AND POTENTIAL USES OF VIPER VENOM PROTEINS

1. Snake venom serine protease (SVSP) is a family of proteins that contain His, Asp and Ser residues on catalytic sites and are inhibited by di-isopropyl fluorophosphates (DFP) and phenylmethylsulfonyl fluorophosphates (PMSF). Examples of mammalian proteases in this family are intestinal digestive enzymes, trypsin and chymotrypsin, and almost all enzymes in coagulation and fibrinolytic system, *e.g.* factor II, VII, IX, X, plasmin and plasminogen activator.

Activities of SVSPs

1.1 Snake venom thrombin-like enzymes (SVTLE)

Thrombin plays central roles in hemostasis. It clots fibrinogen through cleavages of fibrinopeptide (Fp) A and, then, FpB from Aα and Bβ chains of fibrinogen, respectively, resulting in fibrin polymerization. In addition, it also activates factor V, VIII, XI and XIII to enhance fibrin strength and stimulates platelets *via* protease-activated receptors (PARs). Binding of thrombin to platelet glycoprotein (Gp) Ib is essential for factor XI and PAR activation. Furthermore, in the presence of the allosteric regulator, thrombomodulin (TM), thrombin also activates protein C and thrombin-activatable fibrinolysis inhibitor (TAFI) mediating anti-coagulation and anti-

fibrinolysis, respectively. The molecular mechanisms of multi-functionality of thrombin have been extensively studied. Crystal structures of thrombin revealed that not only the active site canyon across the molecule, but also the 2 exosites (ABEs) on both sides (Fig 1) that are essential for substrate, regulator and inhibitor specificities (Bode, 2005). The hydrophobic exosite I binds fibrinogen, factor V, VIII, XI, PAR, 5^{th} and 6^{th} epidermal growth factor (EGF) domains of TM, heparin cofactor II and hirudin, while the anion binding exosite II binds platelet Gp Ib, chondroitin sulfate moiety of TM, fibrinogen γchain, heparin and hemadin (Huntington, 2005).

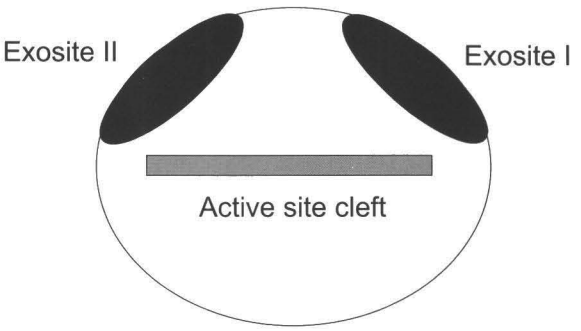


Fig 1. Structure of thrombin

SVTLEs are 233 ± 10-residue peptides with 6 conserved disulfide bonds (Castro, 2004). Only partial functions of thrombin can be performed by each SVTLE. A sequence analysis of SVTLEs suggested the presence of exosites I and II, similar to thrombin (Maroun, 2004). However, our sequence analysis of *Cryptelytrops albolabris* SVTLEs could not identify the putative exosites (Rojnuckarin, 2006b). Correlations of sequences and functional activities of them require further studies (Serrano, 2005).

1.1.1 *Venombin A* is the largest group. They cleave only FpA from fibrinogen, *e.g.* ancrod from Malayan pit viper, *Calloselasma rhodostoma*, (Au, 1993) flavoxobin from habu snake, *Trimeresurus flavovirides*, (Shieh, 1988) stejnobin from chinese tree viper, *Viridovipera stejnegeri,* (Zhang, 1998) and GPV-TL1 and GPV-TL2 from green pit viper, *C. albolabris* (Ronuckarin, 2006).

1.1.2 *Venombin B* releases only FpB, *e.g.* TM-VIG from Chinese habu, *Protrobothrops Mucrosquamatus,* (Hung, 1994) and contortrixobin from copperhead snake, *Agkistrodon contortrix contortrix* (Amiconi, 2000).

1.1.3 *Venombin AB* cleaves both FpA and FpB, *e.g.* bilineobin from cantil, *Agkistrodon bilineatus* (Komori, 1993). However, bilineobin preferentially cleaves FpB.

1.1.4 *Platelet protease-activated receptor (PAR)* activation results in platelet aggregation, *e.g.* PA-BJ from jararaca, *Bothrops jararaca,* (Serrano, 1995).

1.1.5 *Kallikrein-like activity* releases bradykinin from kininogen causing hypotension in biting victims, *e.g.* halystase from halys viper, *Gloydius halys, blomhoffii,* (Matsui, 1998) and KN-BJ from *B. jararaca*.

In contrast to thrombin, SVTLEs are not inhibited by heparin. Therefore, batroxobin (Defibrase®) from fer-de-lance, *Bothrops atrox,* can be used to determine fibrinogen levels with contaminating heparin (Reptilase time, Funk, 1971). Reptilase has been used to test abnormalities of fibrinogen functions or dysfibrinogenemia (Cunningham, 2002). Venombin A and venombin B were used to investigate the molecular consequences of FpA and FpB cleavage, respectively, in fibrin polymerization process (Geer, 2007). Furthermore SVTLEs were used for removal of fibrinogen from the samples to test for other thrombin-dependent functions (Mullin, 2000).

High fibrinogen levels are associated with occurrences of thrombosis in population and poor outcomes in stroke. Recombinant ancrod, a defibrinating venombin A, significantly improved outcomes after cerebral infarction, if given within 3 h after onsets (Sherman, 2000). In a meta-analysis of randomized, placebo-controlled trials in cerebral infarction, fibrinogen-depleting agents decreased death and disability from 42% to 37%, compared with placebo ($p=0.02$, Liu, 2003) with the incidence of intracranial hemorrhage of 1 and 4%, respectively ($p=0.06$). Furthermore, ancrod was shown to be cost-effective with this degree of efficacy (Samsa, 2002). Ancrod is also an alternative anticoagulant for vascular injury when heparin is contra-indicated, *e.g.* in patients suffering from heparin-induced thrombocytopenia (Cole, 1993).

Furthermore, defibrase has been investigated in China and shown to improve neurological functions of ischemic stroke, if given within 12 h (CGRD 2005). Defibrase has also been injected every 3 months for 1 year in stroke patients with hyperfibrinogenemia. This study found that the recurrences were fewer than those of the historical controls (Xu, 2007). Other SVTLEs should also be further investigated to be novel anti-coagulants.

1.2 Fibrinogenolytic enzymes: They may preferentially degrade Aα or Bβ chain of fibrinogen/fibrin and, thus, are termed α or β fibrogenases, respectively (Swenson, 2005). The examples are albofibrase, an α fibrigenase from *C. albolabris* (Muanpasitporn, 2007) and stejnefibrase 2, aβ fibrogenase from *V. stejnegeri* (Gao, 1998). These agents have potentials to be fibrinogen-depleting and fibrinolytic agents for clinical thrombotic disorders.

1.3 Plasminogen activators (PA): TSV-PA was isolated from *V. stejnegeri* and found to cleave the same site on plasminogen as mammalian PA (Zhang, 1995, 1997). More PA have been identified from snake venoms, *e.g.* Haly-PA from *G. halys* (Park, 1998), LV-PA from South American bushmaster,

Lachesis muta muta, (Sanchez, 2000) and finally GPV-PA from *C. albolabris* (Rojnuckarin, 2006b). They may be useful clinically as thrombolytic agents.

1.4 Protein C activator: ACC-C was isolated from *A. contortrix contortrix* (Kisiel, 1987). The venom has been used as a reagent in laboratory assays for thrombosis-prone conditions that are defective in protein C pathway, *e.g.* activated protein C resistance due to factor V mutation (Factor V Leiden) or protein C or its cofactor, protein S, deficiencies (Marsh, 2005).

1.5. Factor V activator: RVV-V was purified from Russell's viper, *Daboia russelii* (Tokunaga, 1988). Russell's viper venom is widely used as a clotting assay to detect lupus anticoagulant, a laboratory marker of autoimmune thrombosis, because the venom activates the common pathway of blood coagulation and not interfered by factor VIII inhibitor, the intrinsic pathway disorder.

2. Snake venom metalloproteinases (SVMP) and disintegrins

SVMPs join the mammalian hemolog, A disintegrin and metalloprotease (ADAM) family of proteins, in the 'reprolysin' family of metalloproteases. As names imply, they depend on Zn^{2+} ion and the chelating agent, EDTA, abolishes their activity. SVMPs are multi-domain proteins comprising, at least, a signal peptide (SP), a pro-sequence, and a metalloproteinase domain (Class P-I). While class P-II contains additional disintegrins, P-III class possesses disintegrin-like and cysteine-rich (CR) domains (Fig 2). Class P-IV SVMPs contains the heterodimeric C-type lectin-like domains that joined the C-terminus of proteins by disulfide bonds. The prototype of this class is RVV-X, a factor X activator from Russell's viper. The structure of this protein showed that the lectin domain bound the γ-carboxyglutamic acid (Gla) domain of factor X facilitating the enzymatic action (Takeda, 2007). In addition to these domains in SVMPs, the mammalian ADAM also contains an epidermal growth factor-like, a transmembrane and a cytoplasmic domain. The ADAM proteins play important roles in fertilization, protein ectodomain processing, notch-mediated signaling, neurogensis, and muscle fusion (Schlondoeff, 1999).

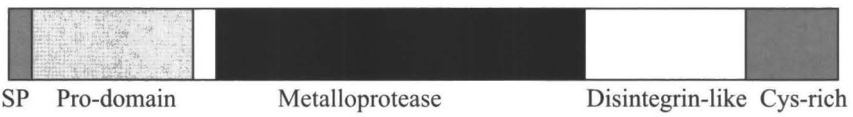


Fig 2. Domain structure of class P-III snake venom metalloproteinases, SP = signal peptide

The pro-region of the proteins prevents the activity of the catalytic domain. The enzymes are activated via cleavage of this region either by auto-activation or by other proteases. The 200-residue metalloprotease domain contains zinc-binding sequences, HEXXHXXGXXHD...X_{10}...CI/VM

(X is any amino acid). A 21-residue spacer region follows this domain. The disintegrin domains are usually released from the mature proteins. Free disintegrins interfere with cell-matrix interaction via integrin binding, while attached disintegrin-like or disintegrin domains target the enzymes to the sites of actions (Laing, 2005).

More recently, variants of these SVMP subtypes have been discovered including P-II SVMPs with disintegrin domains remain attached, termed P-IIa and P-III SVMPs with disintegrin-like domains free from mature proteins, termed P-IIIa. There are also homodimeric SVMPs, called P-IIb and P-IIIb. The variant structures can be predicted by cysteine residue arrangements. For example, extra cysteines at 222 and 241, and lacking cysteine 195 indicate P-IIa and P-IIIa SVMPs, respectively (Fox, 2005).

Disintegrins are small peptides that bind and block functions of integrins. Integrins are cell surface proteins playing critical roles in cell-extracellular matix or cell-cell interactions. They not only perform adhesive functions, but also transmit survival, proliferation, differentiation and/or activation signals to integrin-bearing cells. The integrin's common feature is a heterodimer comprising α and β transmembrane subunits. Nineteen different α and 8 β subunits have been characterized in mammals. Permutation of these subunits generates several distinct integrins. Examples of extracellular matix binding integrins (and their ligands) are $\alpha_1\beta_1$ (Collagen type IV), $\alpha_2\beta_1$ (Collagen type I), $\alpha_5\beta_1$ (Fibronectin), $\alpha_6\beta_1$ (Laminin), $\alpha_8\beta_1$ and $\alpha_9\beta_1$ (Tenascin), $\alpha_V\beta_1$ and $\alpha_V\beta_3$ (Vitronectin), as well as $\alpha_{IIB}\beta_3$ or Gp IIb/IIIa (The major fibrinogen receptor mediating platelet aggregation). In addition, some integrins also bind cellular adhesion molecules, such as $\alpha_4\beta_1$ integrin on hematopoietic cells that binds VCAM1 and β_2 integrins on leukocytes that mediate cell migration and homing.

Disintegrins are low-molecular-weight (40-100 amino acids), cysteine-rich proteins purified from viper venoms. Molecular cloning showed that they are usually parts of SVMP genes before post-translational release. An exception is the a subunit of a dimeric disintegrin acostatin that is derived from a transcript devoid of SVMP metalloprotease domain (Okuda, 2002). Disintegrins can be classified into long (7 disulfide bonds), medium (6 disulfide bonds), short (4 disulfide bonds) and dimeric dinintegrins (4 intra-chain and 2 inter-chain disulfide bridges). The hallmark of disintegrins is the tri-peptide integrin-binding motifs, typically RGD or KGD. However, other variations (WGD, VGD, MGD, MLD, RTS and KTS) have later been characterized. The different motifs contribute to integrin-binding specificity. For examples, KGD binds more specifically to GpIIb/IIIa (Scarborough, 1991; Suehiro, 1996), while RGD binds both GpIIb/IIIa and $\alpha_V\beta_3$. In addition, the novel KTS or RTS disintegrins preferably bind $\alpha_1\beta_1$ integrin. However, we found that albolatin, the recombinant KGD-containing dimeric disintegrin from *C. albolabris*, inhibited platelet aggregation induced by collagen, but not ADP (Singhamatr, 2008) suggesting that the sequences outside the integrin-

binding motif also determine the integrin specificity. Previous reports indicated that the C-terminal tail of disintegrin is also essential for binding (Marcinkiewicz, 1997).

Three-dimensional structures of several disintegrins revealed the integrin-binding loop with the tripeptide motif near its tip (Calvete, 2005; Fig 3). In addition, there is the protruding C-terminal tail. The structure of dimeric disintegrins revealed the attached N-termini and the divergent integrin-binding loops and C termini (Fig 3). Notably, the crystal structure of the complex between integrin $\alpha_V\beta_3$ and RGD disintegrin showed that Arg and Gly (RG) residues interacted with α_V, while Asp (D) residue bound integrin β subunit.

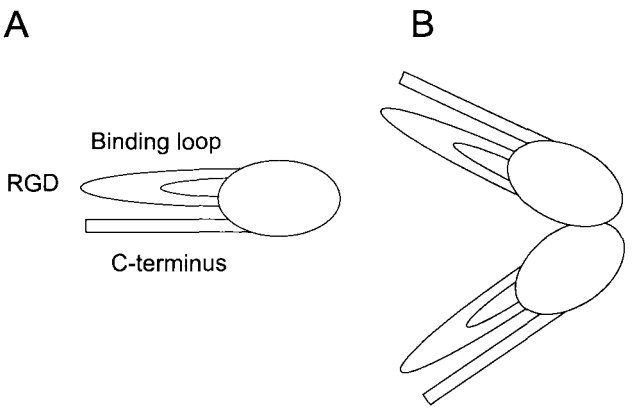


Fig 3. Structures of disintegrins (A) and homodimeric disintegrins (B)

The disintegrin-like domains can be distinguished from the disintegrins by the facts that they contain XCD, a putative integrin-binding motif, and exist with a CR domain. Most cysteine residues of the CR domain probably form intra-domain disulfide bonds (Jia, 1996). Recent crystal structures of vascular apoptosis-inducing proteins form Western diamondback rattlesnake (*Crotalus atrox*), VAP1 (Takeda, 2006) and VAP2 (Igarashi, 2007), revealed that the disintegrin-like domains had very different 3-D structure from disintegrins and the XCD motif was unlikely to bind integrins as previously assumed. Interestingly, the disintrigin-like and CR domains form a C-shaped structure and bind ligands via the terminal hypervariable region (HVR), targeting the catalytic domain to specific substrates (Fig 4).

2.1 *In vitro* and *in vivo* activities of SVMPS

2.1.1 *Tissue damage*: Most SVMPs possess hemorrhagic and necrotic activity caused primary by extracellular matrix degradation and vascular damages as discussed above. BaP1 from terciopelo, *Bothrops asper,* was also found to induce expression of endogenous matrix metalloproteases in the victims

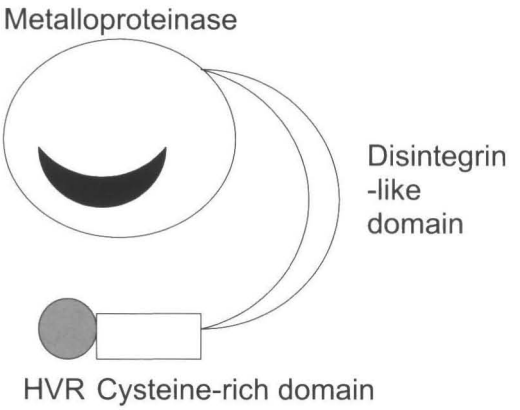


Fig 4. Structure of snake venom metalloproteinase, Vascular apoptosis-inducing proteins, HVR = Hyper-variable region

(Rucavado, 1998). Apart from the hemorrhagic activity, bilitoxin from *A. bilineatus* can cause muscle damage (Ownby, 1990).

2.1.2 *Effects on vascular biology*: Adult angiogenesis, the blood vessel formation process, is the subject under extensive research. As it is essential for tissue repair, pathological angiogenesis plays pivotal roles in diabetic retinopathy, some inflammatory diseases and, notably, cancer growth.

As cell-extracellular matrix interactions via integrins and cell-cell interactions are critical for survival, dissociation of cells from their environment results in death, a process called anoikis. Several SVMPs including BaP1 from *B. asper* (Díaz, 2005), HV-1 from *T. flavovirides* (Masuda, 2001), VLAIP from blunt-nose viper, *Macrovipera lebetina* (Trummal, 2005) and Halysase from *G. halys* (You, 2003) are found to cause endothelial cell apoptosis. The possible mechanisms can be the combination of the degradation of integrins or extracellular matrix or adhesion molecules, blocking integrins, directly activating endothelial cells and releasing antiangiogenic molecules, such as angiostatin, endostatin and tumstatin from plasminogen, collagen type XVIII and type IV, respectively (Moura-da-Silva, 2007).

2.1.3 *Fibrinolytic activity*: is direct degradation of fibrinogen by SVMPs. The examples are TM-1, TM-2 and TM-3 from *P. mucrosquamatus* (Huang, 1995) and fibrolase from *A. contortrix contortrix* venom (Guan, 1991).

Fibrolase preferentially cleaves Aα of fibrinogen and has been shown to rapidly lyse thrombi in arteries and veins of experimental animals (Markland, 1996). The recombinant truncated form of fibrolase, termed alfimeprase, has been developed for clinical uses as a fibrinolytic agent. One major advantage of alfimeprase is that thrombus lysis is six times faster than that of the current thrombolytic agent, rt-PA (Toombs, 2001) meaning better organ salvage. In addition, the plasma naturally occurring

protease inhibitor, α_2 macroglobulin, can neutralize excessive alfimeprase. Therefore, systemic bleeding side effect is expected to be less. In animal studies, alfimeprase is more effective in lysis of large and organized clots than urokinase. Recently, a phase I/II clinical trial of alfimeprase was initiated in patients with arterial thrombosis of lower extremities using intra-vascular injection showing that the drug was effective and well tolerated (Swenson, 2005). However, the phase III trial result is dissappointing.

2.1.4 *Targeting platelet membrane receptors and their ligands*: A SVMP, alborrhagin, was purified from *C. albolabris* venom and found to activate the collagen receptor, Gp VI, on platelet surface resulting in platelet activation via tyrosine phosphorylation (Andrew, 2001). This protein is useful for dissecting the mechanism of platelet activation. However, the sequence of the protein and the mechanisms of activation remain to be elucidated.

Furthermore, SVMPs may degrade platelet receptors and their ligands inhibiting platelet functions. For example, jararhagin from *B. jararaca* can degrade platelet collagen receptor and von Willbrand factor (vWF) contributing to bleeding in patients (Kamiguti, 1996). Triflamp, a P-I SVMP from *T. flavoviridis*, cleaves platelet Gp Ib, the vWF receptor, and PSGL1, the P-selectin ligand (Tseng, 2004). Crotalin from *C. atrox* (Wu, 2001) and kistomin from *C. rhodostoma* (Hsu, 2007) degrade both vWF and its receptor Gp Ib. Furthermore, crovisidin from prairie rattlesnake, *C. viridis,* (Liu, 1997) and catrocollastatin from *C. atrox* (Zhou, 1996) bind collagen preventing platelet activation.

2.1.5 *Coagulation factor activation*: Ecarin from saw scaled viper, *Echis carinatus*, is a prothrombin activator (Nishida, 1995) without requirement of any cofactor. Therefore, it is classified as a class A prothrombin activator. It has been used in a clotting assay (Ecarin time) for monitoring thrombin-inhibiting anticoagulants, *e.g.* ximelagatran, and hirudin. RVV-X from *D. russelii* is a clotting factor X activator. The venom is used for detecting lupus anticoagulant.

2.2 Activities of disintegrins

2.2.1 *Inhibition of platelet aggregation*: This family of proteins contains RGD or KGD or WGD motif crucial for binding to platelet Gp IIb/IIIa preventing platelet aggregation. Therefore, several disintegrins or their peptide derivatives have been purified for potential clinical uses. Currently, eptifibatide, a derivative of the disintegrin, barbourin, from dusky pygmy rattlesnake, *Sitrurus miliarius barbouri*, is commercially available for treatments of thromboembolic diseases. It was shown to reduce death and myocardial infarction in patients presented with acute coronary syndrome (PURSUIT trial investigators, 1998). Furthermore, eptifibatide is shown to

prevent platelet aggregation in patients undergoing coronary stenting (O'Shea, 2002; Gurbel, 2005).

2.2.3 *Antitumor activities*: In addition to Gp IIb/IIIa blockade, disintegrins can bind other integrins disrupting tumor cell-matrix association (Pfaff, 1994). In cancer biology, this interaction is important for cell migration and metastatic process. Rhodostomin from *C. rhodostoma* and trigramin from *T. flavovrtidis* were able to inhibit cancer cell migration and invasion both in osteoblast culture and in a nude mouse model (Yang, 2005). In addition, albolabrin, a disintegrin previously purified from *C. albolabris* venom, successfully inhibits melanoma cell metastasis in a mouse model (Soszka, 1991; Beviglia, 1995).

New blood vessel generation, is critical for tumor growth over a certain size, the stage called angiogenic switch. Endothelial cells survival, proliferation, migration and tube formation require signals from integrins, especially $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_1\beta_1$, $\alpha_2\beta_3$, $\alpha_5\beta_1$ and $\alpha_6\beta_4$ (Calvete, 2007). Disruption of matrix interactions can inhibit tumor angiogenesis.

Salmosin, a disintegrin from *G. halys*, was found to inhibit basic fibroblast growth factor (bFGF)-induced angiogenesis both *in vitro* and in a mouse model (Kang, 1999). Contortrostatin, a homodimeric RGD-disintegrin from *A. contortrix contortrix*, binds and prevents endothelial contact with $\alpha_V\beta_3$ integrin resulting in vascular apoptosis (Zhou, 1999). Furthermore, it strongly inhibits angiogenesis in a mouse model (Golubkov, 2003). Contortrostatin was recently reported to successfully inhibit breast cancer cell progression in a mouse model using a liposomal delivery system. Disintegrins encapsulated in liposomes showed significantly prolonged half-life and accumulated in cancer cells without interaction with immune system or platelets (Swenson, 2004). Therefore, liposomal disintegrins may be a mode of choice to administer these agents to patients in the future (Swenson, 2007). In addition, anti-angiogenic, KTS-disintegrins, *e.g.* obtustatin from *V. labetina* obtusa, viperistatin from *V. palestinae* and lebestatin from *M. labetina transmediterranea*, have been recently described (Calvete, 2007).

4. The C-type lectin-like proteins (CLPs)

Amino acid sequences of CLPs subunits are 15–40% identical to the carbohydrate recognition domain of C-type lectins, such as mannose-binding protein (MBP) (Weis, 1991). However, CLP are neither C-type (Calcium dependent) nor lectin (Carbohydrate binding) proteins (Drickamer, 1999; Morita, 2004) and they are found only in snake venoms.

A prominent feature of CLPs is that they are heterodimers or oligomeric complexes of heterodimers including 2 homologous subunits: subunit a (α chain) of 14–15 kDa and subunit b (β chain) of 13–14 kDa linked by interchain disulfide bond. In contrast, C-type lectins are exclusively homodimers or homo-oligomers. CLPs present in a variety of oligomeric

forms, including αβ, $(\alpha\beta)_2$, $(\alpha\beta)_4$ or $\alpha_1\alpha_2\beta_1\beta_2$ (Atoda, 1991; Kowalska, 1998; Murakami, 2003).

The three dimensional structures of several lectins have been solved using X-ray crystallography (Morita, 2005). The basic building block, dimer, provides two convex surfaces, intervened by the area between the αβ dimer providing one concave surface (Fig 5A). The area between two subunits is characterized by a domain swap, *i.e.* a loop of one subunit protrudes into the other and *vice versa*. This concave interface serves as a binding site for various target proteins including platelet Gp Ib, vWF and clotting factors (Morita, 1996). The structure of higher multimer of αβ dimer has also been solved (Fukuda, 2000; Fig 5B).

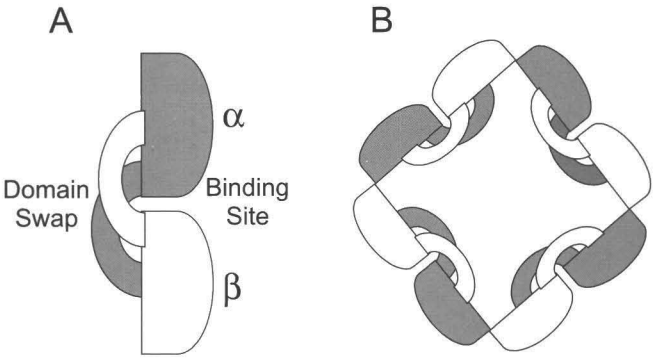


Fig 5. Structures of heterodimeric C-type lectin-like proteins (A) and a higher multimeric form (B)

Activities of CLPs

4.1 Anticoagulants: Anticoagulant CLPs have been classified into 3 types.

4.1.1. *Coagulation factor* IX/X-binding proteins, such as jararaca IX/X-binding protein from *B. jararaca* (Sekiya, 1993), habu IX/X-binding protein from *T. flavoviridis* (Atoda, 1991), agkisacutacin from hundred-pace viper, *Deinagkistrodon acutus*, venom (Li, 2005).

4.1.2. *Coagulation factor* IX-binding proteins, such as habu IX-binding protein from *T. flavoviridis* (Atoda, 1995), TSV FIX-binding protein from *V. stejnegeri* (Lee, 2003).

4.1.3. *Coagulation factor* X-binding proteins, such as acutus X-binding protein from *D. acutus* (Atoda, 1998).

In the presence of Ca^{2+}, these proteins bind to the Gla domains at the N-termini of coagulation factors IX and X (Atoda, 1994, 1995, 1998).

4.2 Platelet inhibition and activation

Glycoprotein receptors on platelets are also targets for snake venom CLPs. Physiologically, a primary platelet plug is initiated by the interactions

between the platelet Gp Ib-IX-V complex and vWF, as well as platelet collagen receptors, Gp VI and integrin $\alpha_1\beta_2$ (GpIa/IIa) and subendothelial collagen resulting in platelet activation. GpIIb/IIIa, then, changes conformation to bind fibrinogen resulting in aggregation.

4.2.1 *Platelet inhibition*: The majority of GpIb-binding CLPs, such as flavocetins A and B, high-molecular-weight CLPs from *T. flavovirides* (Fukuda, 2000) or CHH-A and B from timber rattlesnake, *Crotalus horridus horridus* (Andrews, 1996), blocks vWF binding and inhibits platelet aggregation. On the other hand, 3 dimeric Gp Ib-binding CLPs, mamushigin from *G. halys blomhoffii* (Sakurai, 1998), alboaggregin B from *C. albolabris* (Peng, 1991) and TSV-GPIb-BP from *V. stejnegeri* (Lee, 2003) bind platelet GpIb complex, and directly agglutinate platelets. Molecular cloning, sequence analysis and structural modeling found that the β subunits of these 3 CLPs distinctively contain a conserved motif SRTY at positions 112-115 and hydrophilic patches that may contribute to the bivalent binding of two GpIb on nearby platelets causing agglutination (Arpijuntarangkoon, 2007). Although these CLPs aggregate platelet *in vitro*, the interaction is not as strong as the endogenous ligand, vWF, unable to withstand shear stress *in vivo*. Competing with vWF binding, they probably inhibit platelet function in animals.

4.2.2 *Platelet activation*: Several platelet-activating CLPs are higher multimers and bind Gp VI, with or without other receptors, stimulating signal transduction and tyrosine phosphorylation via Fcγ receptor, the signal molecule of Gp VI. Interestingly when the dimeric platelet-inhibiting echicetin is multimerized by IgM, it now activates platelets (Navdaev, 2001) suggesting that higher multimer structure is required for receptor crosslink resulting in activation instead of inhibition. Alboaggregin A ($\alpha_1\alpha_2\beta_1\beta_2$) and alboluxin $(\alpha\beta)_3$, platelet agonists from *C. albolabiris* venom, activate platelets by binding to GpVI and Gp Ib (Andrews, 1996; Kowalska, 1998; Dormann, 2001; Du, 2002). Convulxin $(\alpha\beta)_4$ from South American rattlesnake, *Crotalus durissus terifficus,* venom activates platelets by a mechanism involving both collagen receptors, Gp VI and Gp Ia/IIa. Stejnulxin (120 kDa) form *V. stejnegeri* (Lee, 2003) and trowaglerix (175 kDa) from temple pit viper, *Tropedolaemus wagleri,* can activate only Gp VI. Although trowaglerix initially activates platelets, it causes subsequent Gp VI shedding by matrix metalloprotease and finally platelet inhibition (Chang, 2008).

Other platelet activation mechanisms of CLPs are studied in botrocetin from *B. jararaca* (Read, 1989; Andrew, 1989) and bitiscetin from puff adder, *Bitis arientans,* (Hamako, 1996). They bind directly to vWF (Botrocetin) and vWF and Gp Ib (Bitiscetin) resulting in platelet aggregation (Maita, 2003).

4.3 (Pro)-thrombin inhibitor: Bothrojaracin is a dimeric CLP from *Bothrops* spp. It binds to (pro) thrombin exosite I and II, not to catalytic site

preventing coagulant and platelet-activating activities of thrombin. Bothrojaracin is successful in treating venous thrombosis in a rat model (Arocas, 1996; Zingali, 2005).

4.4 Promote angiogenesis: A heterotetrameric ($\alpha_1\alpha_2\beta_1\beta_2$) CLP, agglucetin, from *D. acutus* (Formosan pit viper) was found to induce GpIb-mediated platelet agglutination. In addition, it can promote angiogenesis via integrin $\alpha_V\beta_3$ activation (Wang, 2003, 2008).

5. Phospholipases A_2 (PLA_2)

PLA_2 are enzymes that digest phospholipids at sn-2 position releasing free fatty acids from the glycerol backbone. In animals, there are cytosolic PLA_2 that mediate cellular signal transduction and pancreatic enzymes and secretory PLA_2 that function in gastrointestinal digestion and inflammation, respectively. Snake venom PLA_2 are homologous to the latter 2 classes. Some secretory PLA_2 enhance inflammatory reactions and some display antimicrobial activities (Nevalainen, 2008).

PLA_2 are approximately 120-residue peptides with 6-7 disulfide bonds (Kini, 2005). The 3-D structures of venom PLA_2 revealed 3 alpha helices intervened by a Ca^{2+} binding loop and a β wing (Fig 6). The active site HD residues (Asp49) are essential for Ca^{2+} binding and, thus, for enzymatic activity. However, several inactive PLA_2 were described to contain HK (Lys49), HN (Asn49) or HS (Ser49) at their active sites. The catalytically inactive, HK-PLA_2s were found to have long hydrophobic C-terminal tails that are toxic to membrane causing myotoxicity (Lomonte, 2003).

PLA_2 is an example of the family of proteins with common 3-D structure, but extremely diverse functions. They have been found in almost all kinds of venomous snakes and contain numerous activities including edema formation, myotoxicity, neuromuscular blocking, cardiotoxicity, hemolysis, and convulsive, as well as antiplatelet and anticoagulant activities.

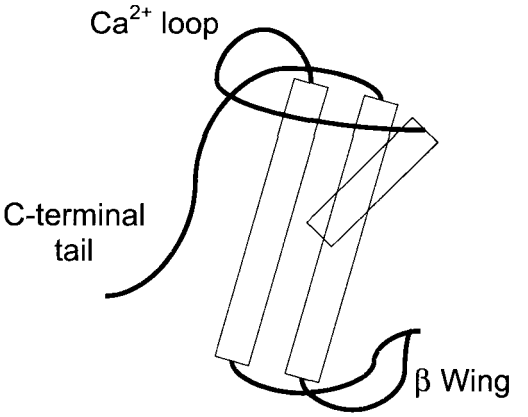


Fig 6. Structure of phospholipase A_2

Three anticoagulant proteins, CM-I, CM-II and CM-IV, are isolated from black neck spitting cobra, *Naja nigricollis* (Kini, 2005) CM-IV inhibits prothrombinase complex (Factor Xa/Va/ Ca^{2+}/Phospholipid complex) by direct binding with factor Xa. Furthermore, PLA_2 from Neuwied's lancehead, *Bothrops neuwiedi pauloensis*, possess anti-microbial activity (Rodrigues, 2004).

CONCLUSIONS

There are greatly expanding venom proteins being, and remaining to be, cloned and characterized. Millions years of snake evolution provide us these agents affecting innumerable physiologic and pathologic processes. Hopefully, current technology will transform these harmful proteins to become helpful medical compounds.

REFERENCES

Amiconi, G., Amoresano, A., Boumis, G., Brancaccio, A., De Cristofaro, R., De Pascalis, A., Di Girolamo, S., Maras, B. and Scaloni, A. (2000). A novel venombin B from *Agkistrodon contortrix contortrix*: evidence for recognition properties in the surface around the primary specificity pocket different from thrombin. *Biochemistry,* **39**: 10294-10308.

Andrews, R.K., Kroll, M.H., Ward, C.M., Rose, J.W., Scarborough, R.M., Smith, A.I., López, J.A. and Berndt, M.C. (1996). Binding of a novel 50-kilodalton alboaggregin from *Trimeresurus albolabris* and related viper venom proteins to the platelet membrane glycoprotein Ib-IX-V complex. Effect on platelet aggregation and glycoprotein Ib-mediated platelet activation. *Biochemistry*, **35**: 12629-12639.

Andrews, R.K., Gardiner, E.E., Asazuma, N., Berlanga, O., Tulasne, D., Nieswandt, B., Smith, A.I., Berndt, M.C. and Watson, S.P. (2001). A novel viper venom metalloproteinase, alborhagin, is an agonist at the platelet collagen receptor GPVI. *Journal of Biological Chemistry*, **276**: 28092-28097.

Arocas, V., Zingali, R.B., Guillin, M.C., Bon, C. and Jandrot-Perrus, M. (1996). Bothrojaracin: A potent two-site-directed thrombin inhibitor. *Biochemistry*, **35**: 9083-9089.

Arpijuntarangkoon, J., Rojnuckarin, P., Muanpasitporn, C., Kaeothip, S., Sangvanich, P. and Intragumtornchai, T. (2007). Molecular cloning and sequence analysis of alboaggregin B. *Platelets*, **18**: 266-72.

Atoda, H., Hyuga, M. and Morita, T. (1991). The primary structure of coagulation factor IX/factor X-binding protein isolated from the venom of *Trimeresurus flavoviridis*. Homology with asialoglycoprotein receptors, proteoglycan core protein, tetranectin, and lymphocyte Fc epsilon receptor for immunoglobulin E. *Journal of Biological Chemistry*, **266**: 14903-14911.

Atoda, H., Yoshida, N., Ishikawa, M. and Morita, T. (1994). Binding properties of the coagulation factor IX/factor X-binding protein isolated from the venom of *Trimeresurus flavoviridis*. *European Journal of Biochemistry*, **224**: 703-708.

Atoda, H., Ishikawa, M., Yoshihara, E., Sekiya, F. and Morita, T. (1995). Blood coagulation factor IX-binding protein from the venom of *Trimeresurus flavoviridis*: Purification and characterization. *Journal of Biochemistry*, **118**: 965-973.

Atoda, H., Ishikawa, M., Mizuno, H. and Morita, T. (1998). Coagulation factor X-binding protein from *Deinagkistrodon acutus* venom is a Gla domain-binding protein. *Biochemistry,* **37**: 17361-17370.

Au, L.C., Lin, S., Chou, J., Teh, G., Chang, K. and Shih, C. (1993). Molecular cloning and sequence analysis of cDNA for ancrod, a thrombin-like enzyme of the venom of *Calloselasma rhodostoma*. *Biochemical Journal*, **294**: 387-390.

Beviglia, L., Stewart, G.J. and Niewiarowski, S. (1995). Effect of four disintegrins on the adhesive and metastatic properties of B16F10 melanoma cells in a murine model. *Oncology Research*, **7**: 7-20.

Bode, W. (2005). The structure of thrombin, a chameleon-like proteinase. *Journal of Thrombosis and Haemostasis*, **3**: 2379-2388.

Calvete, J.J., Marcinkiewicz, C., Monleon, D., Esteve, V., Celda, B., Juarez, P. and Sanz, L. (2005). Snake venom disintegrins: Evolution of structure and function. *Toxicon*, **15**: 1063-1074.

Calvete, J.J., Marcinkiewicz, C. and Sanz, L. (2007). KTS and RTS-disintegrins: Anti-angiogenic viper venom peptides specifically targeting the α_1 β_1 integrin. *Current Pharmaceutical Design*, **13**: 2853-2859.

Castro, H.C., Zingali, R.B., Albuquerque, M.G., Pujol-Luz, M. and Rodrigues, C.R. (2004). Snake venom thrombin-like enzymes: from reptilase to now. *Cellular and Molecular Life Sciences*, **61**: 843-856.

Chang, C.H., Chung, C.H., Kuo, H.L., Hsu, C.C. and Huang, T.F. (2008). The highly specific platelet glycoprotein (GP) VI agonist trowaglerix impaired collagen-induced platelet aggregation *ex vivo* through matrix metalloproteinase-dependent GPVI shedding. *Journal of Thrombosis and Haemostasis*, **6**: 669-676.

Chotenimitkhun, R. and Rojnuckarin, P. (2008). Systemic antivenom and skin necrosis after green pit viper bites. *Clinical Toxicology*, **46**: 122-125.

Cole, C.W., Bormanis, J., Luna, G.K., Hajjar, G., Barber, G.G., Harris, K.A. and Brien, W.F. (1993). Ancrod versus heparin for anticoagulation during vascular surgical procedures. *Journal of Vascular Surgery*, **17**: 288-292.

The Cooperative Group for Reassessment of Defibrase. (2005). Reassessment of defibrase in treatment of acute cerebral infarction: A multicenter, randomized, double-blind, placebo-controlled trial. *Chinese Medical Sciences Journal*, **20**: 15-158.

Cunningham, M.T., Brandt, J.T., Laposata, M. and Olson, J.D. (2002). Laboratory diagnosis of dysfibrinogenemia. *Archives of Pathology and Laboratory Medicine*, **126**: 499-505.

Díaz, C., Valverde, L., Brenes, O., Rucavado, A. and Gutiérrez, J.M. (2005). Characterization of events associated with apoptosis/anoikis induced by snake venom metalloproteinase BaP1 on human endothelial cells. *Journal of Cellular Biochemistry*, **94**: 520-528.

Dormann, D., Clemetson, J.M., Navdaev, A., Kehrel, B.E. and Clemetson, K.J. (2001). Alboaggregin a activates platelets by a mechanism involving glycoprotein VI as well as glycoprotein Ib. *Blood*, **97**: 929-936.

Du, X.Y., Magnenat, E., Wells, T.N. and Clemetson, K.J. (2002). Alboluxin, a snake C-type lectin from *Trimeresurus albolabris* venom is a potent platelet agonist acting via GPIb and GPVI. *Thrombosis and Haemostasis*, **87**: 692-698.

Fox, J.W. and Serrano, S.M. (2005). Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. *Toxicon*, **45**: 969-985.

Fry, B.G., Vidal, N., Norman, J.A., Vonk, F.J., Scheib, H., Ramjan, S.F., Kuruppu, S., Fung, K., Hedges, S.B., Richardson, M.K., Hodgson, W.C., Ignjatovic, V., Summerhayes, R. and Kochva, E. (2006). Early evolution of the venom system in lizards and snakes. *Nature*, **439**: 584-588.

Fry, B.G., Scheib, H., van der Weerd, L., Young, B., McNaughtan, J., Ramjan, S.F., Vidal, N., Poelmann, R.E. and Norman, J.A. (2008). Evolution of an arsenal: Structural and functional diversification of the venom system in the advanced snakes (Caenophidia). *Molecular and Cellular Proteomics*, **7**: 215-246.

Fukuda, K., Mizuno, H., Atoda, H. and Morita, T. (2000). Crystal structure of flavocetin-A, a platelet glycoprotein Ib-binding protein, reveals a novel cyclic tetramer of C-type lectin-like heterodimers. *Biochemistry*, **39**: 1915-1923.

Funk, C., Gmur, J., Herold, R. and Straub, P.W. (1971). Reptilase^R—a new reagent in blood coagulation. *British Journal of Haematology*, **21**: 43-52.

Gao, R., Zhang, Y., Meng, Q.X., Lee, W.H., Li, D.S., Xiong, Y.L. and Wang, W.Y. (1998). Characterization of three fibrinogenolytic enzymes from Chinese green tree viper (*Trimeresurus stejnegeri*) venom. *Toxicon*, **36**: 457-467.

Golubkov, V., Hawes, D. and Markland, F.S. (2003). Anti-angiogenic activity of contortrostatin, a disintegrin from *Agkistrodon contortrix contortrix* snake venom. *Angiogenesis*, **6**: 213-224.

Geer, C.B., Tripathy, A., Schoenfisch, M.H., Lord, S.T. and Gorkun, O.V. (2007). Role of 'B-b' knob-hole interactions in fibrin binding to adsorbed fibrinogen. *Journal of Thrombosis and Haemostasis*, **5**: 2344-2351.

Guan, A.L., Retzios, A.D., Henderson, G.N. and Markland, F.S. Jr. (1991). Purification and characterization of a fibrinolytic enzyme from venom of the southern copperhead snake (*Agkistrodon contortrix contortrix*). *Archives of Biochemistry and Biophysics*, **289**: 197-207.

Gurbel, P.A., Bliden, K.P., Zaman, K.A., Yoho, J.A., Hayes, K.M. and Tantry, U.S. (2005). Clopidogrel loading with eptifibatide to arrest the reactivity of platelets: Results of the clopidogrel loading with eptifibatide to arrest the reactivity of platelets (CLEAR PLATELETS) study. *Circulation*, **111**: 1153-1159.

Gutierrez, J.M., Leon, G., Rojas, G., Lomonte, B., Rucavado, A. and Chaves, F. (1998). Neutralization of local tissue damage induced by *Bothrops asper* (terciopelo) snake venom. *Toxicon*, **36**: 1529-1538.

Gutierrez, J.M., Rucavado, A., Escalante, T. and Diaz, C. (2005). Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanisms involved in microvessel damage. *Toxicon*, **45**: 997-1011.

Hamako, J., Matsui, T., Suzuki, M., Ito, M., Makita, K., Fujimura, Y., Ozeki, Y. and Titani, K. (1996). Purification and characterization of bitiscetin, a novel von Willebrand factor modulator protein from *Bitis arietans* snake venom. *Biochemical Biophysical Research Communications*, **226**: 273-279.

Hsu, C.C., Wu, W.B., Chang, Y.H., Kuo, H.L. and Huang, T.F. (2007). Antithrombotic effect of a protein-type I class snake venom metalloproteinase, kistomin, is mediated by affecting glycoprotein Ib-von Willebrand factor interaction. *Molecular Pharmacology*, **72**: 984-992.

Huang, K.F., Hung, C.C., Pan, L.P., Chow, L.P., Tsugita, A. and Chiou, S.H. (1995). Characterization of multiple metalloproteinases with fibrinogenolytic activity from the venom of Taiwan Habu (*Trimeresurus mucroaquamatus*): protein micro-sequencing couples with cDNA sequence analysis. *Biochemical Biophysical Research Communications*, **216**: 223-233.

Hung, C.C., Huang, K.F. and Chiou, S.H. (1994). Characterization of one novel venom protease with beta-fibrogenase activity from the Taiwan habu (*Trimeresurus mucrosquamatus*): purification and cDNA sequence analysis. *Biochemical Biophysical Research Communications*, **205**: 1707-1715.

Huntington, J.A. (2005). Molecular recognition mechanisms of thrombin. *Journal of Thrombosis and Haemostasis*, **3**: 1861-1872.

Igarashi, T., Araki, S., Mori, H. and Takeda, S. (2007). Crystal structures of catrocollastatin/VAP2B reveal a dynamic, modular architecture of ADAM/adamalysin/reprolysin family proteins. *FEBS Letters*, **581**: 2416-2422.

Jia, L.G., Shimokawa, K., Bjarnason, J.B. and Fox, J.W. (1996). Snake venom metalloproteinases: structure, function and relationship to the ADAMS family of proteins. *Toxicon*, **34**: 1269-1276.

Kamiguti, A.S., Hay, C.R.M., Theakston, R.D.G. and Zuzel, M. (1996). Insight into the mechanism of hemorrhage caused by snake venom metalloproteinases. *Toxicon*, **34**: 627-642.

Kang, I.C., Lee, Y.D. and Kim, D.S. (1999). A novel disintegrin salmosin inhibits tumor angiogenesis. *Cancer Research*, **59**: 3754-3760.

Kini, R.M. (2005). Structure-function relationships and mechanism of anticoagulant phospholipase A_2 enzymes from snake venoms. *Toxicon*, **45**: 1147-1161.

Kisiel, W., Kondo, S., Smith, K.J., McMullen, B.A. and Smith, L.F. (1987). Characterization of a protein C activator from *Agkistrodon contortrix contortrix* venom. *Journal of Biological Chemistry*, **270**: 12607-12613.

Komori, Y., Nikai, T., Ohara, A., Yagihashi, S. and Sugihara, H. (1993). Effect of bilineobin, a thrombin-like proteinase from the venom of common cantil (*Agkistrodon bilineatus*). *Toxicon*, **31**: 257-270.

Kowalska, M.A., Tan, L., Holt, J.C., Peng, M., Karczewski, J., Calvete, J.J. and Niewiarowski, S. (1998). Alboaggregins A and B. Structure and interaction with human platelets. *Thrombosis and Haemostasis*, **79**: 609-613.

Laing, G.D., Clissa, P.B., Theakston, R.D., Moura-da-Silva, A.M. and Taylor, M.J. (2003). Inflammatory pathogenesis of snake venom metalloproteinase-induced skin necrosis. *European Journal of Immunology*, **33**: 3458-3463.

Laing, G.D. and Moura-da-Silva, A.M. (2005). Jararhagin and its multiple effects on hemostasis. *Toxicon*, **45**: 987-996.

Lee, W.H., Zhuang, Q.Y. and Zhang, Y. (2003). Cloning and characterization of a blood coagulation factor IX-binding protein from the venom of *Trimeresurus stejnegeri*. *Toxicon*, **41**: 765-772.

Li, W.F., Chen, L., Li, X.M. and Liu, J. (2005). A C-type lectin-like protein from *Agkistrodon acutus* venom binds to both platelet glycoprotein Ib and coagulation factor IX/factor X. *Biochemical Biophysical Research Communications*, **332**: 904-912.

Liu, C.S., Chen, J.M., Chang, C.H., Chen, S.W., Teng, C.M. and Tsai, I.H. (1991). The amino acid sequence and properties of an edema-inducing Lys-49 phospholipase A2 homolog from the venom of *Trimeresurus mucrosquamatus*. *Biochimica et Biophysica Acta*, **1077**: 362-370.

Liu, C.Z. and Huang, T.F. (1997). Crovidisin, a collagen-binding protein isolated from snake venom of *Crotalus viridis*, prevents platelet-collagen interaction. *Archives of Biochemistry and Biophysics*, **337**: 291-299.

Lomonte, B., Angulo, Y. and Calderon, L. (2003). An overview of lysine-49 phospholipase A_2 myotoxins from crotalid snake venoms and their structural determinants of myotoxic action. *Toxicon*, **42**: 885-901.

Maita, N., Nishio, K., Nishimoto, E., Matsui, T., Shikamoto, Y., Morita, T., Sadler, J.E. and Mizuno, H. (2003). Crystal structure of von Willebrand factor A1 domain complexed with snake venom, bitiscetin: Insight into glycoprotein Ibalpha binding mechanism induced by snake venom proteins. *Journal of Biological Chemistry*, **278**: 37777-37781.

Markland, F.S. (1996). Fibrolase, an active thrombolytic enzyme in arterial and venous thrombosis model systems. *Advances in Experimental Medicine and Biology*, **391**: 427-438.

Maroun, R.C. and Serrano, S.M. (2004). Identification of the substrate-binding exosites of two snake venom serine proteinases: Molecular basis for the partition of two essential functions of thrombin. *Journal of Molecular Recognition*, **17**: 51-61.

Marsh, N. and Williams, V. (2005). Practical applications of snake venom toxins in haemostasis. *Toxicon*, **45**: 1171-1181.

Masuda, S., Hayashi, H., Atoda, H., Morita, T. and Araki, S. (2001). Purification, cDNA cloning and characterization of the vascular apoptosis-inducing protein, HV1, from Trimeresurus flavoviridis. *European Journal of Biochemistry*, **268**: 3339-3345.

Matsui, T., Sakurai, Y., Fujimura, Y. *et al.* (1998). Purification and amino acid sequence of halystase from snake venom of *Agkistrodon halys blomhoffii*, a serine protease that cleaves specifically fibrinogen and kininogen. *European Journal of Biochemistry*, **252**: 569-575.

Matsui, T., Fujimura, Y., Titani, K., Hayashi, I., Ohishi, S., Suzuki, M., Hamako, J., Yamamoto, Y., Yamazaki, J., Kinoshita, M. and Titani, K. (2000). Snake venom proteases affecting hemostasis and thrombosis. *Biochimica et Biophysica Acta*, **1477**: 146-156.

Marcinkiewicz, C., Vijay-Kumar, S., McLane, M.A. and Niewiarowski, S. (1997). Significance of RGD loop and C-terminal domain of echistatin for recognition of alphaIIb beta3 and alpha (v) beta3 integrins and expression of ligand-induced binding site. *Blood*, **90**: 1565-1575.

Morita, T., Atoda, H. and Sekiya, F. (1996). Structure and functions of coagulation factor IX/factor X-binding protein isolated from the venom of *Trimeresurus flavoviridis*. *Advances in Experimental Medicine and Biology*, **391**: 187-196.

Morita, T. (2004). C-type lectin-related proteins from snake venoms. *Current Drug Targets—Cardiovascular & Haematological Disorders*, **4**: 357-373.

Morita, T. (2005). Structures and functions of snake venom CLPs (C-type lectin-like proteins) with anticoagulant-, procoagulant-, and platelet-modulating activities. *Toxicon*, **45**: 1099-1114.

Moura-da-Silva, A.M., Butera, D. and Tanjoni, I. (2007). Importance of snake venom metalloproteinases in cell biology: effects on platelets, inflammatory and endothelial cells. *Current Pharmaceutical Design*, **13**: 2893-2905.

Muanpasitporn, C. and Rojnuckarin, P. (2007). Expression and characterization of a recombinant fibrinogenolytic serine protease from green pit viper (*Trimeresurus albolabris*) venom. *Toxicon*, **49**: 1083-1089.

Mullin, J.L., Gorkun, O.V. and Lord, S.T. (2000). Decreased lateral aggregation of a variant recombinant fibrinogen provides insight into the polymerization mechanism. *Biochemistry*, **39**: 9843-9849.

Murakami, M.T., Zela, S.P., Gava, L.M., Michelan-Duarte, S., Cintra, A.C. and Arni, R.K. (2003). Crystal structure of the platelet activator convulxin, a disulfide-linked alpha4beta4 cyclic tetramer from the venom of *Crotalus durissus terrificus*. *Biochemical Biophysical Research Communications*, **310**: 478-482.

Nakashima, K., Ogawa, T., Oda, N., Hattori, M., Sakaki, Y., Kihara, H. and Ohno, M. (1993). Accelerated evolution of *Trimeresurus flavoviridis* venom gland phospholipase A_2 isozymes. *Proceedings of National Academy of Sciences of the United States of America*, **90**: 5964-5968.

Nevalainen, T.J., Graham, G.G. and Scott, K.F. (2008). Antibacterial actions of secreted phospholipases A_2. *Biochimica et Biophysica Acta*, **1781**: 1-9.

Nishida, S., Fujita, T., Kohno, N., Atoda, H., Morita, T., Takeya, H., Kido, I., Paine, M.J., Kawabata, S. and Iwanaga, S. (1995). cDNA cloning and deduced amino acid sequence of prothrombin activator (Ecarin) from Kenyan *Echis carinatus* venom. *Biochemistry*, **34**: 1771-1778.

O'Shea, J.C., Buller, C.E., Cantor, W.J., Chandler, A.B., Cohen, E.A., Cohen, D.J., Gilchrist, I.C., Kleiman, N.S., Labinaz, M., Madan, M., Hafley, G.E., Califf, R.M., Kitt, M.M., Strony, J. and Tcheng, J.E., ESPRIT Investigators. (2002). Long-term efficacy of platelet glycoprotein IIb/IIIa integrin blockade with eptifibatide in coronary stent intervention. *JAMA*, **287**: 618-621.

Ogawa, T., Nakashima, K., Nobuhisa, I., Deshimaru, M., Shimohigashi, Y., Fukumaki, Y., Sakaki, Y., Hattori, S. and Ohno, M. (1996). Accelerated evolution of snake venom phospholipase A_2 isozymes for acquisition of diverse physiological functions. *Toxicon*, **34**: 1229-1236.

Okuda, D., Koike, H. and Morita, T. (2002). A new gene structure of the disintegrin family: a subunit of dimeric disintegrin has a short coding region. *Biochemistry*, **41**: 14248-14254.

Ownby, C.L., Nikai, T., Imai, K. and Sugihara, H. (1990). Pathogenesis of hemorrhage induced by bilitoxin, a hemorrhagic toxin isolated from venom of the common cantil (*Agkistrodon bilineatus bilineatus*). *Toxicon*, **28**: 837-846.

Park, D., Kim, H., Chung, K., Kim, D.S. and Yun, Y. (1998). Expression and characterization of a novel plasminogen activator from *Agkistrodon halys* venom. *Toxicon*, **36**: 1807-1819.

Peng, M., Lu, W. and Kirby, E.P. (1991). Alboaggregin-B: A new platelet agonist that binds to platelet membrane glycoprotein Ib. *Biochemistry*, **30**: 11529-11536.

Pfaff, M., McLane, M.A., Beviglia, L., Niewiarowski, S. and Timpl, R. (1994). Comparison of disintegrins with limited variation in the RGD loop in their binding to purified integrins alpha IIb beta 3, alpha V beta 3 and alpha 5 beta 1 and in cell adhesion inhibition. *Cell Adhesion and Communication*, **2**: 491-501.

The PURSUIT Trial Investigators, (1998). Inhibition of platelet glycoprotein IIb/IIIa with eptifibatide in patients with acute coronary syndromes. *New England Journal of Medicine*, **339**: 436-443.

Read, M.S., Smith, S.V., Lamb, M.A. and Brinkhous, K.M. (1989). Role of botrocetin in platelet agglutination: formation of an activated complex of botrocetin and von Willebrand factor. *Blood*, **74**: 1031–1035.

Rodrigues, V.M., Marcussi, S., Cambraia, R.S., de Araújo, A.L., Malta-Neto, N.R., Hamaguchi, A., Ferro, E.A., Homsi-Brandeburgo, M.I., Giglio, J.R. and Soares, A.M. (2004). Bactericidal and neurotoxic activities of two myotoxic phospholipases A_2 from *Bothrops neuwiedi pauloensis* snake venom. *Toxicon*, **44**: 305-314.

Rojnuckarin, P., Intragumtornchai, T., Sattapiboon, R., Muanpasitporn, C., Pakmanee, N., Khow, O. and Swasdikul, D. (1999). The effects of green pit viper (*Trimeresurus albolabris* and *Trimeresurus macrops*) venom on the fibrinolytic system in human. *Toxicon*, **37**: 743-755.

Rojnuckarin, P., Chanthawibun, W., Noiphrom, J., Pakmanee, N. and Intragumtornchai, T. (2006). A randomized, double-blind, placebo-controlled trial of antivenom for local effects of green pit viper bites. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **100**: 879-84.

Rojnuckarin, P., Muanpasitporn, C., Chanhome, L., Arpijuntarangkoon, J. and Intragumtornchai, T. (2006b). Molecular cloning of novel serine proteases and phospholipases A2 from green pit viper (*Trimeresurus albolabris*) venom gland cDNA library. *Toxicon*, **47**: 279-287.

Rucavado, A., Nunez, J. and Gutierrez, J.M. (1998). Blister formation and skin damage induced by BaP1, a hemorrhagic metalloproteinase from the venom of the snake *Bothrops asper*. *International Journal of Experimental Pathology*, **79**: 245-254.

Russell, F.E., Ruzic, N. and Gonzalez, H. (1973). Effectiveness of antivenin (Crotalidae) polyvalent following injection of Crotalus venom. *Toxicon*, **11**: 461-464.

Sakurai, Y., Fujimura, Y., Kokubo, T., Imamura, K., Kawasaki, T., Handa, M., Suzuki, M., Matsui, T., Titani, K. and Yoshioka, A. (1998). The cDNA cloning and molecular characterization of a snake venom platelet glycoprotein Ib-binding protein, mamushigin, from *Akistrodon halys blomhoffii* venom. *Thrombosis and Haemostasis*, **79**: 1199-1207.

Samsa, G.P., Matchar, D.B., Williams, G.R. and Levy, D.E. (2002). Cost-effectiveness of ancrod treatment of acute ischaemic stroke: results from the Stroke Treatment with Ancrod Trial (STAT). *Journal of Evaluation of Clinical Practice*, **8**: 61-70.

Sanchez, E.F., Santos, C.I., Magalhaes, A., Diniz, C.R., Figueiredo, S., Gilroy, J. and Richardson, M. (2000). Isolation of a proteinase with plasminogen-activating activity from *Lachesis muta muta* (bushmaster) snake venom. *Archives of Biochemistry and Biophysics*, **378**: 131-141.

Scarborough, R.M., Rose, J.W., Hsu, M.A., Phillips, D.R., Fried, V.A., Campbell, A.M., Nannizzi, L. and Charo, I.F. (1991). Barbourin. A GPIIb-IIIa-specific integrin antagonist from the venom of *Sistrurus m. barbouri*. *Journal of Biological Chemistry*, **266**: 9359-9362.

Schlondorff, J. and Blobel, C.P. (1999). Metalloprotease-disintegrins: Modular proteins capable of promoting cell-cell interactions and triggering signals by protein-ectodomain shedding. *Journal of Cell Science*, **112**: 3603-3617.

Sekiya, F., Atoda, H. and Morita, T. (1993). Isolation and characterization of an anticoagulant protein homologous to botrocetin from the venom of *Bothrops jararaca*. *Biochemistry*, **32**: 6892-6897.

Serrano, S.M.T., Mentele, R., Sampio, C.A.M. and Fink, E. (1995). Purification, characterization and amino acid sequence of a serine proteinase, PA-BJ, with platelet-aggregating activity from the venom of *Bothrops jararaca*. *Biochemistry*, **34**: 7186-7193.

Serrano, S.M. and Maroun, R.C. (2005). Snake venom serine proteinases: sequence homology *vs.* substrate specificity, a paradox to be solved. *Toxicon*, **45**: 1115-1132.

Sherman, D.G., Atkinson, R.P., Chippendale, T., Levin, K.A., Ng, K., Futrell, N., Hsu, C.Y. and Levy, D.E. (2000). Intravenous ancrod for treatment of acute ischemic stroke: The STAT study: a randomized controlled trial. Stroke Treatment with Ancrod Trial. *JAMA*, **283**: 2395-2403.

Shieh, T.C., Kawabata, S.I., Kihara, H., Ohno, M. and Iwanaga, S. (1988). Amino acid sequence of a coagulant enzyme, flavoxobin, from *Trimeresurus flavoviridis* venom. *Journal of Biochemistry*, **103**: 596-605.

Singhamatr, P. and Rojnuckarin, P. (2007). Molecular cloning of albolatin, a novel snake venom metalloprotease from green pit viper (*Trimeresurus albolabris*), and expression of its disintegrin domain. *Toxicon*, **50**: 1192-1200.

Soszka, T., Knudsen, K.A., Beviglia, L, Rossi, C., Poggi, A. and Niewiarowski, S. (1991). Inhibition of murine melanoma cell- matrix adhesion and experimental metastasis by albolabrin, an RGD-containing peptide isolated from the venom of *Trimeresurus albolabris*. *Experimental Cell Research*, **196**: 6-12.

Suehiro, K., Smith, J.W. and Plow, E.F. (1996). The ligand recognition specificity of beta3 integrins. *Journal of Biological Chemistry*, **271**: 10365-10371.

Swenson, S., Costa, F., Minea, R., Sherwin, R.P., Ernst, W., Fujii, G., Yang, D. and Markland, F.S. Jr. (2004). Intravenous liposomal delivery of the snake venom disintegrin contortrostatin limits breast cancer progression. *Molecular Cancer Therapy*, **3**: 499-511.

Swenson, S. and Markland, F.S. Jr. (2005). Snake venom fibrin(ogen)olytic enzymes. *Toxicon*, **45**: 1021-1039.

Swenson, S., Ramu, S. and Markland, F.S. (2007). Anti-angiogenesis and RGD-containing snake venom disintegrins. *Current Pharmaceutical Design*, **13**: 2860-2871.

Takeda, S., Igarashi, T., Mori, H. and Araki, S. (2006). Crystal structures of VAP1 reveal ADAMs' MDC domain architecture and its unique C-shaped scaffold. *EMBO Journal*, **25**: 2388-2396.

Takeda, S., Igarashi, T. and Mori, H. (2007). Crystal structure of RVV-X: An example of evolutionary gain of specificity by ADAM proteinases. *FEBS Letters*, **581**: 5859-5864.

Tokunaga, F., Nagasawa, K., Tamura, S., Miyata, T., Iwanaga, S. and Kisiel, W. (1988). The factor V-activating enzyme (RVV-V) from Russell's viper venom: Identification of isoproteins RVV-V alpha, -V beta, -V gamma and their complete amino acid sequence. *Journal of Biological Chemistry*, **263**: 17471-17481.

Toombs, C.F. (2001). Alfimeprase: pharmacology of a novel fibrinolytic metalloproteinase for thrombolysis. *Haemostasis*, **31**: 141-147.

Trummal, K., Tõnismägi, K., Siigur, E., Aaspõllu, A., Lopp, A., Sillat, T., Saat, R., Kasak, L., Tammiste, I., Kogerman, P., Kalkkinen, N. and Siigur, J. (2005). A novel metalloprotease from *Vipera lebetina* venom induces human endothelial cell apoptosis. *Toxicon*, **46**: 46-61.

Tseng, Y.L., Lee, C.J. and Huang, T.F. (2004). Effects of a snake venom metalloproteinase, triflamp, on platelet aggregation, platelet-neutrophil and neutrophil-neutrophil interactions: involvement of platelet GPIbalpha and neutrophil PSGL-1. *Thrombosis and Haemostasis*, **91**: 315-324.

Wang, W.J., Ling, Q.D., Liau, M.Y. and Huang, T.F. (2003). A tetrameric glycoprotein Ib-binding protein, agglucetin, from Formosan pit viper: structure and interaction with human platelets. *Thrombosis and Haemostasis*, **90**: 465-475.

Wang, W.J. (2008). Agglucetin, a tetrameric C-type lectin-like venom protein, regulates endothelial cell survival and promotes angiogenesis by activating integrin alphavbeta3 signaling. *Biochemical Biophysical Research Communications*, **369**: 753-760.

Weis, W.I., Kahn, R., Fourme, R., Drickamer, K. and Hendrickson, W.A. (1991). Structure of the calcium-dependent lectin domain from a rat mannose-binding protein determined by MAD phasing. *Science*, **254**: 1608-1615.

Wu, W.B., Peng, H.C. and Huang, T.F. (2001). Crotalin, a vWF and GP Ib cleaving metalloproteinase from venom of *Crotalus atrox*. *Thrombosis and Haemostasis*, **86**: 1501-1511.

Xu, G., Liu, X., Zhu, W., Yin, Q., Zhang, R. and Fan, X. (2007). Feasibility of treating hyperfibrinogenemia with intermittently administered batroxobin in patients with ischemic stroke/transient ischemic attack for secondary prevention. *Blood Coagulation and Fibrinolysis*, **18**: 193-197.

Yang, R.S., Tang, C.H., Chuang, W.J., Huang, T.H, Peng, H.C., Huang, T.F. and Fu, W.M. (2005). Inhibition of tumor formation by snake venom disintegrin. *Toxicon*, **45**: 661-669.

You, W.K., Seo, H.J., Chung, K.H. and Kim, D.S. (2003). A novel metalloprotease from *Gloydius halys* venom induces endothelial cell apoptosis through its protease and disintegrin-like domains. *Journal of Biochemistry*, **134**: 739-749.

Zhang, Y., Wisner, A., Xiong, Y. and Bon, C. (1995). A novel plasminogen activator from snake venom: purification and characterization, and molecular cloning. *Journal of Biological Chemistry*, **270**: 10246-10255.

Zhang, Y., Wisner, A., Maroun, R.C., Choumet, V., Xiong, Y. and Bon, C. (1997). *Trimeresurus stejnegeri* snake venom plasminogen activator: site-directed mutagenesis and molecular modeling. *Journal of Biological Chemistry*, **272**: 20531-20537.

Zhang, Y., Gao, R., Lee, W.H., Zhu, S.W., Xiong, Y.L. and Wang, W.Y. (1998). Characterization of a fibrinogen-clotting enzyme from *Trimeresurus stejnegeri* venom, and comparative study with other venom proteases. *Toxicon*, **36**: 131-142.

Zhou, Q., Dangelmaier, C. and Smith, J.B. (1996). The hemorrhagin catrocollastatin inhibits collagen-induced platelet aggregation by binding to collagen via its disintegrin-like domain. *Biochemical Biophysical Research Communications*, **219**: 720-726.

Zhou, Q., Nakada, M.T., Arnold, C., Shieh, K.Y. and Markland, F.S. Jr. (1999). Contortrostatin, a dimeric disintegrin from *Agkistrodon contortrix contortrix*, inhibits angiogenesis. *Angiogenesis*, **3**: 259-269.

Zingali, R.B., Ferreira, M.S., Assafim, M., Frattani, F.S. and Monteiro, R.Q. (2005). Bothrojaracin, a *Bothrops jararaca* snake venom-derived (pro) thrombin inhibitor, as an anti-thrombotic molecule. *Pathophysiology of Haemostasis and Thrombosis*, **34**: 160-163.

11

Bee-Pollen Therapeutical Value

MARIA GRAÇA R. CAMPOS*, CHRISTIAN FRIGERIO AND FRANCISCO FERREIRA

ABSTRACT

Around the world many things have been related to the health benefits of bee-pollen and different therapeutic bioactivities. Few researches have been done in a way to prove the importance to explore the potential of bee-pollen as a source of new drugs. In the 90ths the interest for this product increased and relevant research has been undertaken. The major lack on the most of the results published is the relationship between the pharmacological activity and the compounds involved. And in some cases it can only be verified through the pharmacodynamic activity because the assay with a well correlated dose effect and the quantification of the molecules and the pharmacokinetic of them is very hard to attribute. Different therapeutic bioactivities attributed to bee-pollen have been reviewed in this paper presenting the more relevant research published so far. From the data published here it's obvious that pollen itself and bee-pollen have a good potential of bioactivity that can be explored with different approaches. However, research is still needed to confirm many of these preliminary findings. Nevertheless it still remains a promising remedy to cure various human ailments.

Key words : Antiartherosclerotic, anti-inflammatory, antioxidant, bee-pollen, osteoporosis, prostatitis

1. Centre of Pharmaceutical Studies, Laborary of Pharmacognosy and Phytotherapy, Faculty of Pharmacy University of Coimbra, Pólo III-Health Sciences Azinhaga de Santa Comba, Celas 3000-548, Coimbra, Portugal.

* *Corresponding author* : E-mail : mgcampos@ff.uc.pt

INTRODUCTION

Bee products, including bee-pollen, were used as Gods food many centuries ago and the diet was linked to the Medicine and therapeutics. About 487-380 B.C., Hérodicos de Sélymbrie, master of gymnastics, inaugurated his diet method, the "Grand Arte", *i.e.* the Medicine, Hippocrate de Cos (460-377 A.C.) establish the roles in his Diet Treaty, The Food and the Man Nature (Debry, 1991). Even then, empirically or not, they knew that the frequent use of a correct diet could have a prophylactic effect avoiding the precocity or total appearance of some illnesses.

In respect to bee products many papers have been published mainly in honey. Royal jelly and bee pollen still need research to be done and results to be published.

A problem frequently found in the majority of the papers published concerning the nutrients of bee-pollen is the lack of floral species identification used on the samples analysed. It is known that the major *taxon* on the mix of bee collected pollen makes a difference in the average in the compounds referred.

Nowadays, it is known that bee pollen content in macronutrients make this product a good complement to the daily diet mainly due to the well balanced proportions of proteins, fats and carbohydrates, especially these last two. The caloric value of bee-pollen can be assessed with a standard 100 g supply that contains an average of 381.70 $\pm$ 14.69 kcal (1595.51Kj) (Orzaez-Villanueva *et al.*, 2002).

According to Peris (1984), bee-pollen covers the minimum amount in amino acids that the human body needs with a daily dose of 15 g (more or less a soup spoon). Further, 40% of the content is made up by various forms of sugars. Anyway they depend of what scientists can include in this group. Huidobro *et al.* (1986, 1987) had obtained a value of 61%, but they consider in this fraction the carbohydrates like reducing sugars (fructose, glucose), maltose, sucrose and polysaccharides. They have done a special group with the starch and other polysaccharides that can't be absorbed including cellulose, hemicelluloses, lignin, esporopolenin, etc. We believe that it is important, because we also verified that about 50% of the weight of bee-pollen corresponds to a material that can't be extracted by organic or aqueous solvents, were the fibber might be included (Campos, 1997).

Lipids and minerals (carriers of calcium, phosphorus, magnesium, iron, copper and manganese, etc.) represent 5 and 3% respectively. The major part (60%) of the fatty acid is in the free form. Bound fatty acids, with rather reflect the compositional profile of pollen, were characterised by a high content of α-linolenic acid (70%) followed by small amounts of linoleic and oleic acid. Palmitic acid is the most abundant saturated fatty acid (Seppänen *et al.*, 1989).

Several vitamins can be found in bee-pollen like B complex vitamins, ascorbic acid, and also A, D and E vitamins.

The values variation between the pollen species can be explained as the average results of the analysis presented in bibliography (Hagedorn & Burger, 1967; Herbert *et al.,* 1987) and research is still needed to be done in a way to standardise bee pollen samples if they will be used for therapeutic purposes (Campos, 1997).

THERAPEUTIC BIOACTIVITY

Anaemia and the Hemopoietic System

Anaemia is characterized by a low number of red blood cells. This could be caused by a lot of reasons like haemolysis, a low iron diet or the production of some kinds of haemoglobin, resulting in the different kinds of anaemias, like Haemolytic anaemia, Nutritional ferropenic Anaemia, Sickle cell anaemia, among others.

Some research has been carried-out with bee-pollen, and other bee products, related to this disease. Wang *et al.* (1993), verified the effects of 10 g/kg/day of oral bee-pollen on haemolytic anaemia animals. The hemopoietic system was studied in mice and rats. The results showed that bee-pollen markedly antagonized the inhibition of the hemopoietic system and reduced white blood cells in these animals. Haro *et al.* (2000), made a similar studied in healthy rats and rats with nutritional ferropenic anaemia. They studied the effect of the addition of 10 g/kg/day of multifloral bee-pollen on a standard diet. The bee-pollen group showed a better weight gain, an increase in the haemoglobin levels and a decrease in platelets. Platelet concentration constitute a hematic parameter that reflects the state of the iron within an organism. It was concluded that bee-pollen increase the digestive utilization of iron, showing it to be useful in this kind of diseases.

Other studies with quercetin, one of the main flavonoids in bee-pollen, showed that this compound have an antioxidant activity in the red blood cells. Cesquini *et al.* (2003) studied the effect of quercetin and rutin in sickle red blood cells. The results showed a better activity of quercetin as an antioxidant. The level of oxyhemoglobin (oxyHb) increase and the levels of metahemoglobin (metHb), hemichrome, lipid peroxidation and the binding of hemoglobin (Hb) to the sickle red blood cell membrane decrease. The results of Pawlikowska-Pawlega *et al.* (2003) about the effect of quercetin in human red blood cell membrane showed a protective effect of quercetin against hypotonic haemolysis, and it was demonstrated that it could decrease the cell diameter and alter it shape. Hypotonic haemolysis protective effect is dependent on the concentration at 90 μg/mL quercetin that can prevent 50% of the haemolysis. The possible explanations for this are its competitive

quenching of singlet oxygen, an increase in the surface area/volume ratio of the cell or a change of permeability of the membrane.

Bee-pollen could have a lot of beneficial effects in the hemopoietic system and the related diseases but more studies are needed to understand the mechanism involved and their contribution to the therapeutic activity.

Activity in Disorders of the Prostate

One of the therapeutic activities more claimed for pollen in general is the prophylactic and curative activity in prostate disorders.

The prostate is usually healthy in younger men. As a man grows older, however, the prostate gland frequently becomes a source of troubles. The three most common prostate problems are inflammation (prostatitis), prostate enlargement (benign prostatic hyperplasia/BPH), and prostate cancer. Neither prostatitis nor prostate enlargement is known to cause cancer. However, it is possible for men who have one or both of these conditions to develop prostate cancer as well (http://www.cancer.gov). Prostatitis, or prostate inflammation, can cause difficult or painful urination that is often accompanied by a burning sensation, by a strong and frequent urge to urinate, that often results in only small amounts of urine, and by pain in the lower back or abdomen. The causes of prostatitis remain unclear. Sometimes it results from a bacterial infection. Occasionally, prostatitis is accompanied by chills and a high fever. When prostatitis is the result of a bacterial infection, it usually can be treated with antibiotics.

Benign prostatic hyperplasia (BPH) is an enlarged prostate. Benign means non-cancerous and hyperplasia means excessive growth of tissue. BPH is the result of small non-cancerous growths inside the prostate. It is not known what causes these growths, but they may be related to hormone changes that occur with aging. By age 60, more than half of all American men have microscopic signs of BPH, and by age 70, more than 40% will have enlargement that can be felt on physical examination.

By age 50, one-third of American men have microscopic signs of prostate cancer, and by age 75, half to three-quarters of men's prostates will have cancerous changes. Most of these cancers either remain latent, producing no signs or symptoms, or they are so slow-growing, or indolent, that they never become a serious threat to health (http://www.cancer.gov).

Chronic prostatitis is a very common and poorly understood condition with significant impact on quality of life for men. Given the lack of proven efficacy of conventional therapies such as antibiotics, it is not surprising that patients have turned with increasing frequency to phytotherapy and other complementary treatments. Although some of these last therapies could be plentiful, and only few have been subjected to scientific scrutiny and prospective controlled clinical trials maybe some can be helpful.

These treatments include zinc, cernitin pollen extract (bee-pollen extract) (Becker & Ebeling, 1988; Buck *et al.*, 1990; Macdonald *et al.,* 1999; Wilt *et al.,* 2000), quercetin (flavonoid world wild spread), saw palmetto (*Serenoa repens*), and acupuncture. Complementary therapies may indeed have much to offer to the patients, particularly those with chronic degenerative conditions in which allopathic therapies have proven less successful. However, they require the same scientific criteria for validation and acceptance as do conventional medical therapies (Shokes, 2002). Although many of them appear promising in small preliminary studies, sometimes the final results in humans did not show a good relationship drug-activity (Shoskes *et al.*, 2002).

Lin *et al.* (1990) studied the morphological changes in aged canine prostatic hyperplasia treated with bee-pollen. Bee-pollen 5-10 g/kg was administered in oral doses for 2 months to aged dogs with prostatic hyperplasia. Prostate size was reduced both at one month and at 2 months. Microscopic examination showed marked diminution in gland diameter, epithelial cell heights and less papillary infolding of the epithelia compared to untreated controls. No effect on plasma estradiol or testosterone levels was observed. No toxicities were reported.

Bruneton (1999) published in a "Compendium of Pharmacognosy" that in certain countries an extract of pollen from a selected flora in the South of Suede was commercialized for prostatitis. The extract includes two fractions that support the activity. One is water-soluble and the other soluble in acetone rich in sterols. The hydrosoluble fraction from AB Cernelle, Vegeholm, Sweden, was analysed by Zhang *et al.* (1995) and inhibits *in vitro* tumoral and normal prostatic cells to grow. The bioactivity described was attributed to the 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA), a cyclic hydroxamic acid.

This hydroxamic acid is an active compound in the pollen extract that which might be responsible for the symtomatic relief in patients with benign prostate hyperplasia. Seventy-nine patients, ages ranged from 62 to 89 years, with this disease were treated in this essay with pollen extract that showed a mild beneficial effect on prostate volume and urination (Yasumoto *et al.,* 1995).

The total extract decreases the prostate hypertrophy in rats, but given to humans no change was verified in blood levels of LH, FSH, testosterone or dihydro-testosterone. In patients with prostatic adenoma the improvement was in nycturie, important decreases in the residue post-urinate and in long term treatment, decrease the diameter antero-posterior of prostate. The debit urinary did not suffer any changes. The effect on the other symptoms usual in the hyperthrophie benign of prostate was not of statistical significance (Bruneton, 1999).

DIBOA activity, and of others structural analogues, was also studied by Roberts *et al.* (2000). DIBOA shows the capacity of inhibit the hormone independent prostate cancer cells DU-145. In contrast to the results reported from others studies, it doesn't inhibit only prostate cancer cells, but exhibit more potent effects on MCF-7 cells (human breast cancer cells line derived from a patient with metastatic mammary carcinoma) and COS-7 cells.

The pollen extract Prostat/Poltit (produced by Allergon) show, in a double blind placebo controlled study, to improve symptomatic relief in man with chronic nonbacterial prostatitis/chronic pelvic pain syndrome (CNBP/CPPS) (Elist, 2005).

After 6 months the patients treated with Prostat/Poltit (3 tablets/day eq. 222 mg of pollen extract/day) shown a significant lower of pain score, less of voiding symptoms, less of urine storage symptoms and better sexual function than the patients who had received placebo. No adverse effects were reported.

Quercetin is a flavonoid that we can be easily found in bee-pollen (Campos *et al.*, 1997). That compound show, *in vitro*, a permanent inhibition of androgen-independents cancer cells PC-3 at the dose of 100 μm. This activity, in prostate cancer cells, in due to the quercetin ability of block the cell cycle in various phases through an inhibition of the expression of several G_1-, S-, and G_2-specific genes. Quercetin also up-regulate expression of various tumor suppressor genes while down-regulate some oncogenes expression.

Kaempferol, another flavonoid comune in bee-pollen, show a reversible inhibition of PC-3 cells grow, blocking the cells cycle progression from G_2 to M phase (Nair *et al.*, 2004). That progression is modulating by $p34^{cdc2}$ kinase (Nurse, 1990), and is known that others flavonoids (like apigenin) are able to depress the kinase activation (Lepley *et al.*, 1996).

In a prospective, double blind, placebo-controlled trial made by Shoskes *et al.* (1999), the patients who had been taking quercetin (500 mg, 2 time/day for 4 weeks) showed a mean improvement in NIH chronic prostatitis symptom score from 21 to 13.1 ($p=0.003$), while those taking placebo had a improvement from 20.2 to 18.8. The 67% of patients taking quercetin had a significant improvement of symptoms. A third group was treated with Prosta-Q (Farry Labs El Segundo, Calif.) that include quercetin with bromelain and papain, substances that increase quercetin absorption. In this group the NIH symptoms score improve from 25.1 to 14.6 (44%), and the 82% of patients had a significant improvement of symptoms.

The beneficial effects of quercetin on CPPS can be due to it's antioxidant and anti-inflammatory activity. In fact CPPS is associated to a high oxidative stress, and also to inflammatory processes. Various studies

have shown the anti-oxidant property of quercetin (Potapovich & Kostyuk, 2003), and also it has been demonstrated that the therapy with quercetin reduce inflammation with lessening of PGE_2 in EPS and consequently increase the prostatic levels of β-endorfines (Shahed & Shokes, 2001).

An overview of one promising pharmacologic agents in complementary medicine for their use in benign prostatic hyperplasia and prostate cancer was presented by Thompson (2001), including agents such as *Glycine max* (soy), PC-SPES (a mixture of 8 herbs), *Prunus africana (Pygeum africanum;* Tadenan), and Cernilton (the previous cited bee-pollen extract) (Thompson, 2001). Using this last extract Dutkiewicz (1996) carried out a clinical essay with a total of 89 patients with benign prostatic hyperplasia (BPH) that were treated pharmacologically for 4 months: 51 received Cernilton and 38 Tadenan (controls). Significant subjective improvement was found in 78% of the patients in the Cernilton group compared to only 55% of the Tadenan-treated patients. The obstructive and irritative symptoms responded best to the therapy. In the Cernilton-treated patients a significant improvement in the uroflow rate, decrease in residual urine and in prostate volume were found. This study shows that Cernilton can be an effective therapy for patients with BPH.

Antioxidant Activity

The lipidic oxidation in presence of oxygen, carry out to form the rancidity, has been recognised since the antiquity like the problem to preserve oils and fats. The oxidation of the vegetal oils and the animal fats, carry to organoleptic changes (colour, smell and taste), as well change the density, viscosity and up to the solubility. Anyway, only at 1950 the scientists started to give emphasis to the lipidic oxidation associated with the biology and medicine.

One of the most important events that cause cellular death is related to the oxidation of the phospholipids that built the cellular wall. This compounds need to be preserved to keep the cells "healthy". When they started to be oxidised many intruders can go inside the cell, as xenobiots, bacteria, virus, etc. and cause diseases.

This process starts with the first breath when we are born but the endogenous antioxidants can balance the equilibrium. This oxidation is directly involved in the degradation of our body conducting us to death. Many are the theories in literature presented to explain the ageing process:

- *Genetic Theory* - the ageing and the long life are genetic programmed ("Programmed Ageing"). These theory was presented at the beginning by Hayflick (1965), that it was the first to show that the human diploid cells in culture, were mortal (could die), all this have a new impulse with the development of the molecular genetic. More recent results from this theory was presented by

Warner *et al.* (1987), and for Holliday *et al.* (1985), that give the hypothesis that some oncogenes, in the inactive state, can be the ageing genes, named gerontogenes.

- *Stochastic Theory* - this theory say that the ageing is the result of a series of alienable events that affect all the level of cellular organisation ("Random ageing"). These events can be:

  Transferring mistakes in the genetic information, that induces the abnormal proteins, *Error catastrophe theory* (Orgel, 1963).

  The decrease of disequilibrium between the producing systems and the radical protected species (Harman, 1956) conducting to a disorganisation of the membranes and abnormal proteins and nucleic acids*: Free radical theory.*

All these events accumulated and amplified induced to the ageing and cellular death. In fact both theory, stochastic and genetic don't exclude one to the other, because the genetic program only result from it's "products" and the mistakes can result from a programme that we haven't the key.

Therefore, all this is presented in literature under the form of theory were the discussion is contradictory, and this is special true for the free-*radical theory of the ageing*. Remember that a free radical is a neutral or charged structure that possess a celibate proton, represented by the symbol $R^{\bullet}$. Our body produce, all the time, active forms of oxygen and free oxygen radicals by different pathways.

These radical species are very reactive, they increase important molecular disorders: the producing of secondary free radicals, bridges intra and intermolecular, oxidation, halogenations, fragmentation.

A part of the structure, the polyinsaturation of the fatty acids and this particular organisation that can pomote the "propagation", the membrane lipids are a privileged place for free radicals. This "peroxidation" give many derivatives that are instant or cumulative witness of the intensity of the phenomena of cellular oxidation: lipid hidroperoxids, aldeids (malondyaldeid - MDA; 4-OH nonenal), conjugated dialdeids, hydrocarbons, fluorescence conjugates (lipofuscins).

Some of these derivatives have biological activities as quimiotatic action, cellular division effect. Other deleterious effects of free radicals result from the action on the polysaccharides (hialuronic acid despolimerisation), proteins (chemical modification of the crucial aminoacids for the enzymatic functions, fragmentation of the peptide chain), and nucleic acids (chromosome bridges).

Further evidence that the maintenance and repair pathways are the main determinants of longevity comes from experiments performed to retard ageing and to increase the lifespan of organisms. For example, anti-ageing and life-prolonging effects of calorie restriction are seen to be accompanied by the stimulation of various maintenance mechanisms. These include increased efficiency of DNA repair, increased fidelity of genetic information transfer, more efficient protein synthesis, more efficient protein degradation, more effective cell replacement and regeneration, improved cellular responsiveness, fortification of the immune system, and enhanced protection from free-radical- and oxidation-induced damage (Bardhan *et al.*, 1985; Xiao *et al.*, 1993; Masoro, 1995; Weindruch, 1996; Masoro & Austad, 1996; Yu, 1999).

Throughout human history, including the present times, search for means to prevent or retard ageing has followed three main lines: (i) cleansing from impurities and wastes; (ii) nutritional supplements, including the use of medicinal plants; and (iii) replacement therapy. The immense popularity of various spas and water therapies even today, is an example of the first type of anti-ageing approach. The claims made for various herbal and other medicinal plant products, such as bee-pollen, ginseng, *Ginkgo biloba* and garlic, as nutritional supplements and anti-ageing drugs have received support, however, from preliminary from laboratory and/or clinical tests (Bardhan *et al.*, 1985; Lu & Dice, 1985; Petkov *et al.*, 1993; Xiao *et al.*, 1993; Svendsen *et al.*, 1994).

There are other conflicting data regarding the anti-ageing effects of antioxidants and very little is known about the efficacy and bioavailability of the externally supplied antioxidants as compared with the body's own antioxidant levels in normal healthy circumstance (Le Bourg, 2001).

The free radical theory have more adepts after the discovery that the active free radicals are involved in almost all the cellular degradation process, like cardiovascular diseases, arthritis, cancer, diabetes, etc. (Various Communications, 1993), and can also be responsible for the Parkinson's and the Alzheimer's disease (Féher *et al.*, 1987).

Bee-pollen was used during centuries to protect the body from the diseases and more especially to reduce the process of ageing.

Following this line Dudov and Starodub (1994) had feeding rats with bee-pollen during a month and studied the state of erythrocyte redox system. It was established that the content of glutathione, total SH-groups as well as activities of glutathione peroxidase and glutathione reductase in these animals in comparison with the control group were increased. Simultaneously a decrease of malondialdehyde and dienic conjugates in erythrocytes was shown. The activity of catalase and superoxide dismutase were increased but was not statically reliable. A conclusion was made that

the antioxidative system is non-specifically active and oxidative processes are blocked in erythrocytes of rats fed on bee-pollen load.

Primary and secondary humoral immune response (the level of specific IgM and IgG) as well as the intensity of delayed-type hypersensitivity to sheep erythrocytes were investigated in the rabbits fed with bee-pollen load for a month. It showed that bee-pollen is an immunomodulator. It stimulated humoral immune response and changed the reaction of delayed-type hypersensibility (Dudov *et al.,* 1994).

Maybe all this can be explained with the anti-oxidant theory. The physical exercise with aerobic characteristics impose to the organism a supplementary consumption of oxygen that in humans go to ten times the amount for the basal consumption. The concept of "oxidative stress" supposes beforehand the disruption in the precarious equilibrium between the production and remission of the free radical of oxygen. With the physique exercise the "stress" is installed by an excess of production of the radical cited above. However, it would be paradoxical, to know that the people doing sports with regularity live in a situation of "permanent oxidative stress" at the biological cost in the situation. Today the scientists know that the resistance training, like an adaptive mechanism, increase the anti-oxidant defences making available more consumption of oxygen without negative consequences.

Liu and Li (1990) had shown the morphological observation of effect of bee-pollen on intercellular lipofuscin in NIH mice. The results of mouse studies demonstrate a reduction of lipofuscin in cardiac muscle, liver, brain and adrenal glands following administration of bee-pollen.

Trying to find a relationship between the antioxidant activity and the constituents of pollen we have published a few papers in this field. In fact, it was not possible to establish an absolute link between them but it was verified that the major involvement could be attributed to phenolic compounds (Campos *et al.,* 1994; Campos, 1997; Campos *et al.,* 2000; Campos *et al.,* 2003; Almaraz-Abarca *et al.,* 2004; Leja *et al.,* 2007). It was also found that the bee-pollen antioxidant activity is species-specific, and independent of its geographical origin. This free radical scavenging capability decreases with the aging of bee-pollen, mainly if the storage conditions are not ideal (Campos *et al.,* 2003).

Bee-pollen antioxidant activity was also studied for linoleic acid peroxidation and hydroxyl radical-scavenging activity showing good results (Liu & Li, 1990).

Bevzo and Grygor'eva (1997) established that small x-irradiation doses activate the lipid peroxidation and antioxidant system enzymes in mice liver. The introduction of bee-pollen extract to the diet of the animals normalized the activity glutathione system enzymes in mice liver.

Chronic combined exposure to ionizing radiation with dose of 0.25 Gy and cadmium chloride or atrazine in drinking water at five-fold Limited Permissible Concentration (LPC) values led to the additively reduced intercellular K^+ level in rat brain, that was at first choice caused by the active ion transport disorders in case of irradiation and by the changes in membrane permeability in the case of toxic loading. Applying beta-carotene oil and bee-pollen both abolished radiation effects but no chemical toxicant ones. Authors believed that the selective action of the observed drugs is connected with the antioxidant activity of them (Anan'eva & Dvoretskii, 1999).

Uzbekova *et al.* (2003) studied the effects of bee-pollen on liver functions in old rats. After one month they had a diminution of malondyaldehyde levels and the sulphydryl groups (SH-G) content was normalized. Also serum urea and protein contents were significantly improved at the end of the experiments.

In traditional Chinese medicine a mixture of bee-pollen, radix polygoni multiflore, semen ziziphi spinosae, radix salviae multiorhizae, fructus schisandrae and fructus ligustris lucidae, known as "NaO Li Su", has a reputation as a remedy against declining memory functions. In the present study the effect of the preparation on failing memory was assessed in 100 elderly Danish volunteers who complained of a deteriorating memory. The study published by Iversen *et al.* (1997) was a double-blind placebo controlled cross-over trial. The effect was evaluated after treatment periods of 3 months duration by a battery of psychological and biochemical tests. No desirable effects on memory functions were achieved by the active treatment. Increases in the number of red blood cells and in the serum creatinine levels were seen after active treatment. In the subgroup initially showing a number of red blood cells below the median, a significant positive correlation was found between changes in the number of red blood cells and changes in the Wechsler Memory Scale scores.

As could be seen, several methods can be used to establish the antioxidant activity of a product. This happen because different damages can be attributed to the oxidant activity of certain compounds in our body and also because there are several ways to check the occurrence of metabolites produced in the metabolic pathways involved.

Antiartherosclerotic Activity

Atherosclerosis is an important health problem nowadays. Atherosclerosis is a type of arteriosclerosis. It comes from the Greek words athero (meaning gruel or paste) and sclerosis (hardness). It's the term for the process of fatty substances, cholesterol, cellular waste products, calcium and fibrin (a clotting material in the blood) building up in the inner lining of an artery. The buildup that results is called plaque.

Arteriosclerosis is a general term for the thickening and hardening of arteries. Some hardening of arteries normally occurs when people grow older. Plaque may partially or totally block the blood's flow through an artery. Two things that can happen where plaque occurs are:

- Bleeding (hemorrhage) into the plaque
- Formation of a blood clot (thrombus) on the plaque's surface

If either of these events occur and blocks the entire artery, a heart attack or stroke may result. Atherosclerosis affects large and medium-sized arteries. The type of artery and where the plaque develops varies with each person.

Some groups have revealed that the pollen extract has beneficial properties, lowering serum lipid levels (Samochowiec & Wójcicki, 1981; Wójcicki & Samochowiec, 1984), reducing atherosclerosis plaque intensity (Wójcicki *et al.,* 1986) and decreasing platelet aggregation both *in vitro* (Kosmider *et al.,* 1983) and *in vivo* (Wójcicki & Samochowiec, 1984). These essays have been confirmed in humans (Wójcicki *et al.*, 1983).

Studies in humans suggest that a diet supplemented with polyunsaturated fatty acids decreases whole blood viscosity and reduces triglyceride and cholesterol levels in patients with cardiovascular disease. Seppänen *et al.* (1989) have analysed (by gas chromatography) the fatty acid composition of the fat-soluble pollen extract (Cernitin GBX) with regard to its proven anti-atherosclerotic activity. The analyses of the fat-soluble pollen extract revealed that the major part (more than 60%) of the fatty acid was in the free form, characterised by a high content of linolenic acid (18:3 n-3, α-ALA) around 70%. α-linolenic acid, is an essential fatty acid, which means that it is essential to human health but cannot be manufactured by the body. For this reason, ALA must be obtained from food. ALA, as well as the fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), belongs to a group of fatty acids called omega-3 fatty acids. EPA and DHA are found primarily in fish while ALA is highly concentrated in certain plant oils such as flaxseed oil and to a lesser extent, canola, soy, perilla, and walnut oils. ALA is also found in wild plants such as purslane. Once ingested, the body converts ALA to EPA and DHA, the two types of omega-3 fatty acids more readily used by the body.

If fatty acids are involved in the beneficial effects refer, the role of α-linolenic acid as a precursor of eicosapentaenoic acid (20:5 n-3, EPA) is significant, since EPA is considered to be responsible for reduced platelet aggregation. EPA *in vivo* is incorporated into platelet phospholipids, to some extent replacing arachidonic acid and exerting an antithrombotic effect either by competing with remaining arachidonic acid for cyclo-oxygenase and lipoxygenase or by being converted to less proagreggatory PGH_3 and TXA_3 (Moncada & Vane, 1984).

Activity on Osteoporosis

Osteoporosis is defined as a reduction in bone mass and disruption of bone architecture resulting in reduced bone strength and increased fracture risk. Fragility fractures are the hallmark of osteoporosis and are particularly common in the spine, hip and forearm. They show a steep age-related increase and have a major impact on the health of elderly populations in the Western world, causing significant morbidity and mortality and imposing huge financial burdens on health services throughout the European Union. Demographic changes and increasing life expectancy will lead to a dramatic increase in the number of people suffering from fractures over the next few decades (Blanchard *et al.*, 1998).

Bone consists of an extracellular collagenous matrix, composed predominantly of type I collagen, in which bone mineral is deposited in the form of calcium salts.

The skeleton contains 99% of the total body calcium which homeostasis is closely linked with bone metabolism. Serum calcium concentration is regulated mainly by parathyroid hormone and calcitriol, the biologically active metabolite of vitamin D, formed in the kidney from calcidiol. Parathyroid hormone affects calcium homeostasis via effects on bone, kidney and vitamins D metabolism.

During childhood and adolescence bone mass continue to increase until a peak bone mass (3^{rd} decade of life) is achieved. The regulation of peak bone mass is not fully understood but a number of factors have been identified. Of these the most important are genetic influences (Morrison *et al.*, 1994; Houston *et al.*, 1996; Grant *et al.*, 1996) other determinants which are potentially modifiable, include physical activity (Slemenda *et al.*, 1994; Valimaki *et al.*, 1994), nutritional factors and hormonal status (Villareal, 1991; Mazess & Barden, 1991; Khaw & Sneyd, 1992; Hodges *et al.*, 1993; Welten *et al.*, 1995; Bonjour *et al.*, 1996; Karkkainen & Lamberg, 1996).

After peak bone mass has been attained, there is a period of consolidation of bones and integrity of the skeleton is maintained by the process of bone remodelling, in which old bone is removed by osteoclasts and subsequently replaced by new bone formed by osteoblasts.

The age at which bone loss starts is uncertain but is believed to be around the age of 40 years, both in men and women. In men, bone loss averages between 0.5 and 1% per year. In women, there is an acceleration in the rate of bone loss around the time of the menopause to about 2% per year (with wide variation from 1% to 6%).

After menopause there is an increase in bone turnover and a decrease in bone formation within individual remodelling units leading to rapid bone loss (Compston, 1994). It's estimate that in women approximately 35% and

50% of cortical and cancellous bone are lost over the course of lifetime (Riggs *et al.*, 1981; Mazess, 1982).

Primary osteoporosis has traditionally been classified into Type-1 or postmenopausal osteoporosis and Type-II, or senile osteoporosis (Riggs & Melton, 1983). Oestrogen deficiency is believed to be the major pathogenetic factor responsible for Type I osteoporosis, characterised by predominantly cancellous bone loss. Type II osteoporosis, which occurs in elderly men and women, results from loss of both cortical and cancellous bone. It is postulated that vitamin D deficiency and secondary hyperparathyroidism are largely responsible for bone loss in Type-II osteoporosis.

Many risk factors for osteoporosis have been identified: endogens factors like female gender, age, slight body build, Asian or Caucasian race, and exogenous factors like premature menopause, primary or secondary amenorrhoea, primary or secondary hypogonadism in man, low dietary calcium intake, vitamins D deficiency, glucocorticoid therapy.

Current pharmacological interventions for prevention of fractures in patients with osteoporosis aim mainly at reducing bone resorption and bone turnover. The treatment of the disease is doing with oestrogens, bisphosphonates, calcium, calcitonin, vitamin D metabolites and fluoride. Calcium and vitamin D are also used in primary/secondary prevention.

In a recent study Hamamoto *et al.* (2006) showed that bee-pollen water-solubilized extract from *Cistus ladaniferus* have inhibitory effects on bone resorption in rats femoral tissues and osteoclastic cell formation in bone marrow cell culture *in vitro*. Thus bee-pollen extract has stimulatory effects on bone formation *in vitro*.

Also Yamaguchi *et al.* (2006) showed that water-soluble extract from *Cistus ladaniferus* cause a significant increase in alkaline phosphatase, enzyme that participate in bone mineralization (Majeska & Wuthier, 1975), and DNA content in the rats femoral-diaphyseal and –metaphyseal tissues *in vitro*. That's increases are completely inhibited in the presence of cycloheximide, an inhibitor of proteine synthesis. These facts suggest that the activity of bee-pollen on bone formation can results from newly synthetized protein components *in vitro*. The oral administration of the water-solubilized extract from *Cistus ladaniferus* to rats caused a significant increase in calcium content, alkaline phosphatase activity and DNA content in the femoral-diaphyseal and metaphyseal tissues, indicating that the extract exerts anabolic effects on bone components *in vivo*.

Antidiarrhoeal Activity

Other therapeutic activity of bee-pollen is as an antidiarrhoeal agent. There are few works on this field, but the existing ones corroborate this therapeutic activity.

Campos (1997) studied the effect of bee-pollen extracts from *Eucalyptus globulus* Labill. and *Salix atrocinerea* Brot. in Swiss OFFI mice. The results showed that both bee-pollen species have antidiarrhoeal activity. However, they have some differences, *Eucalyptus globulus* Labill. bee-pollen extract was more effective on retarding the diarrhoea, where as *Salix atrocinerea* Brot. had a better effect in reducing the percentage of diarrhoeal excrements, but both floral types reduced the diarrhoeal excrements by 30%.

This study concluded that the antidiarrhoeal activity, of the studied bee-pollen, may be due to polyphenolic compounds, especially quercetin, although some others compounds could have a role on this activity and may be responsible for the differences on the results.

Antimicrobial Activity

Although there are few scientific studies corroborating the antimicrobial activity of bee-pollen, there are several ones showing that pollen had this activity against some bacterial species, specially the plant pathogens species.

Campos *et al.* (1998) made preliminary assays using different flavonoids isolated from *Eucalyptus globulus*, *Ranunculus sardous* and *Ulex Europeans* bee-pollens and concluded that the herbacetin derivates from *Ranunculus sardous* and *Ulex Europeans* had a marked antibiotic activity against *Pseudomonas aeruginosa*. On the other hand, *Eucalyptus globulus*, rich in mainly quercetin derivates, did not show any activity.

Basin *et al.* (2006) studied the antibacterial activity of Turkish pollen in 13 different bacterial species pathogens for plants (*Agrobacterium tumefaciens, A. vitis, Clavibacter michiganensis subsp. michiganensis, Erwinia amylovora, E. carotovora* pv. *carotovora, Pseudomonas corrugata, P. savastanoi* pv. *savastanoi, P. syringae* pv. *phaseolicola, P. syringae* pv. *syringae, P. syringae* pv. *tomato, Ralstonia solanacearum, Xanthomonas campestris* pv. *campestris and X. axonopodis* pv. *vesicatoria*). The results showed that the bee-pollen extract of Turkish pollen have an inhibitory effect in all pathogens, although there were differences on the effect according with the pathogen in study. The conclusion of the study shows that this bee-pollen extract have a potential to became a seed protectans because some of the bacterial pathogens are transmitted through the seeds.

Anti-inflammatory Activity

Inflammation is a physiological response to the damage, due to physical or biological agents, of tissues or cells that involve a conjunction of reactions destined to remove the cause and repair the damage. The process is characterized by redness, oedema, fever, pain, and loss of function. We can distinguish two kinds of inflammation:

- Acute inflammation, that it's relatively short, it's characterized by oedema and immunity cells movement from the vascular system to extra vascular tissues with the objective of eliminate the damage cause and repair the tissues.
- Chronic inflammation, that's a long term process were inflammation, tissues destruction, and recover proceeding at the same time.

For the treatment of acute inflammatory process are currently used non-steroidal anti-inflammatory drugs (NSAID) and steroidal anti-inflammatory drugs (SAID) but these conventional drugs have not been successful to cure chronic inflammatory disorders.

For that reason there is a need for new and safe class of anti-inflammatory drugs.

Choi (2007) evaluated the antinociceptive and anti-inflammatory activity of pine (*Pinus densiflora*) pollen extracts (100 and 200 mg/kg) in mice. They used six different tests to obtain preliminary information on the mechanism of action. The positive results at the tests of acetic acid induced writhing, formalin induced paw licking (both phases) and hot plate test suggest that the analgesic effect may be related to its anti-inflammatory, neurogenic and narcotic proprieties. Positive results in carragenan-induced paw oedema and arachidonic acid-induced ear oedema suggest that *Pinus densiflora* pollen extract acts on cycloxygenase and lypoxygenase. The author suggests that this activity can be done from the flavonoidic fraction of the extract according to the extended bibliography existing about their anti-inflammatory activity (Kim *et al.,* 2004).

Allergic Reactions and Toxicity

Bee-pollen is normally well tolerated, but the presence of allergenic pollens and substances can't be excluded. Puente *et al.* (1997) have reported a case of a 34-year-old Spanish woman with a lifelong history of seasonal rhinoconjunctivitis and honey intolerance which developed eosinophilic gastroenteritis after ingestion of bee-pollen.

Greenberger and Flais (2001) studied the allergic reaction in a patient who experienced a non-life-threatening anaphylactic reaction after bee-pollen ingestion. The patient had a 7 mm/28 mm wheal/erythema reaction to bee-pollen at 1 mg/mL concentration.

CONCLUSIONS

From this results it's obvious that pollen itself and bee-pollen as a good potential of bioactivity that can be explored with different approaches, however more research is still needed to confirm many of these preliminary assays, nevertheless it remains a promising drug.

REFERENCES

Almaraz-Abarca, N., Campos, M.G., Ávila-Reyes, J.A., Naranjo-Jiménez, N., Herrera-Corral, J. and Gonzales-Valdez, L.S. (2004). Variability of antioxidant activity among Honeybee-collected pollen of different botanical origin. *Interciencia,* **29**: 574-578.

Anan'eva, T.V. and Dvoretskii, A.I. (1999). Effect of beta-carotene oil and bee pollen on ion transport in rat brain slices following radiation-chemical exposure. *Radiats Biol Radioecol,* **39(2-3)**: 341-4.

Bardhan, P., Sharma S.K. and Garg, N.K. (1985). Effect of geriforte *in vivo* and *in vitro* on age-related enzyme changes in liver and brain of rats. *Ind. J. Exp. Biol.*, **23**: 323-326.

Becker, H. and Ebeling, L. (1988). Conservative treatment of benign prostatic hyperplasia (BPH) with Cernilton® N – Results of a placebo-controlled double-blind study. *Urologe,* **28**: 301-306.

Bevzo, V.V. and Grygor'eva, N.P. (1997). Effect of bee pollen extract on glutathione system activity in mice liver under X-ray irradiation. *Ukr Biokhim Zh.*, **69(4)**: 115-7.

Blanchard, F., Papapoulos, S. and Compston, J. (1998). Report on Osteoporosis in the European Community - Action for Prevention. Office for Official Publications of the European Communities. Luxembourg.

Bonjour, J.P., Schurch, M.A. and Rizzoli, R. (1996). Nutritional aspects of hip fractures. *Bone,* **18(suppl 3)**: S139-44.

Bruneton, J. (1999). Pharmacognosie – Phytochimie & Plantes Medicinales. *Ed.* Tec & Doc. – Lavoisier 3ªed. Revise.

Buck, A.C., Cox, R., Rees, R.W., Ebeling, L. and John, A. (1990). Treatment of outflow tract obstruction due to benign prostatic hyperplasia with pollen extract, Cernilton. A double-blind, placebo-controlled study. *Brit. J. Urology,* **66**: 398-404.

Campos, M.G., Cunha, A.P., Navarro, M.C. and Utrilla, M.P. (1994). Free radical sacavenger activity of Bee-pollen. *Bull. Groupe Polyphenols*, **17**: 415-417.

Campos, M.G. (1997). "Caracterização do pólen apícola pelo seu perfil em compostos fenólicos e pesquisa de algumas actividades biológicas", PhD Thesis. Faculty of Pharmacy, University of Coimbra.

Campos, M.G., Markham, K.R., Mitchell, K.A. and Cunha, A.P. (1997). An approach to the characterization of bee pollens via their Flavonoid/Phenolic profiles. *Phytochem. Anal.,* **8**: 181-185.

Campos, M.G., Webby, R., Kevin, A., Coleta, M., Markham, K. and Cunha, A.P. (2000). Free-radical scavenging properties of bee-pollens – the non-involvement of flavonoids? *Bull. Groupe Polyphenols,* **20(2)**: 347-348.

Campos, M.G., Cunha, A.P. and Markham, K.R. (1998). Inibition of virulence of *Pseudomonas aurugınosa* cultures, by flavonoids isolated from bee-pollen: possible structure-activity relantionships. *Polyphenol communications 98, XIXth ınternational conference on polyphenols, Lille.*

Campos, M.G., Webby, R.F., Markham, K.R., Mitchell, K.A. and Cunha, A.P. (2003). Age-induced diminution of free radical scavenging capacity in bee-pollens and the contribution of constituent flavonoids. *J. of Agrı. Food Chem.,* **51**: 742-745.

Cesquini, M., Torsoni, M.A., Stoppa, G.R. and Ogo, S.H. (2003). t-BOOH-induced oxidative damage in sickle red blood cells and the role of flavonoids. *Biomed Pharmacother.* **57(3-4)**: 124-129.

Choi, E. (2007). Antinociceptive and anti-inflammatory activities of pine (*Pinus densiflora*) pollen extract. *Phytother Res.*, **21**: 471-475.

Compston, J.E. (1994). Hormone replacement therapy for osteoporosis: clinical and pathophysiological aspects. *Repro. Med. Rev.*, **3**: 209-24.

Daniel, A., Shoskes, S.I., Zeitlin, A.S. and Rajfer, J. (1999). Quercetine in men with category III chronic prostatitis: a preliminary prospective, double-blind, placebo-controlled trial. *Urology,* **54**: 960-963.

Debry, G. (1991). Evolution des concepts en nutrition humain. *Cah. Nutr. Diét.,* **26(6)**: 435-442.

Dudov, I.A., Morenets, A.A., Artiukh, V.P. and Starodub, N.F. (1994). Immunomodulatory effect of honeybee flower pollen load. Ukr. *Biokhim. Zh.*, **66(6)**: 91-93.

Dudov, I.A. and Starodub, N.F. (1994). Antioxidant system of rat erythrocytes under conditions of prolonged intake honeybee flower pollen load. *Ukr. Biokhim. Zh.*, **66(6)**: 94-96.

Dutkiewicz, S. (1996). Usefulness of Cernilton in the treatment of benign prostatic hyperplasia. *Int Urol Nephrol.*, **28**: 49-53.

Elist, J. (2005). Effects of pollen extract preparation Prostat/Polit on lower urinary tract symptoms in patient with Chronic Nonbacterial Prostatitis/Chronic Pelvic Pain Syndrome: a randomized, double blind, placebo-controlled study. *Urology*, **6(1)**: 60-63.

Esin, B., Hüseyin, B. and Musa, Ö. (2006). Antibacterial activities of Turkish pollen and propolis extracts against plant bacterial pathogens. *J. Food Eng.*, **77**: 992-996.

Eun-Mi, C. (2007). Antinociceptive and antiinflammatory activities of Pine (*Pinus densiflora*) pollen extract. *Phytother. Res.*, **21**: 471-475.

Féher, J. and Csmós, Vereckei A. (1987). Clinical importance of free radical reactions and their role in the pathogenesis of various human diseases in *Free Radical Reactions in Medicine* 48-147. Springer-Verlag New York, Inc.

Grant, S.F.A., Reid, D.M. and Blake, G. (1996). Reduced bone density and osteoporosis associated with a polymorphic Sp1 binding site in the collagen Ia1 gene. *Nature Genet.*, **14**: 1-6.

Greenberger, P.A. and Flais, M.J. (2001). Bee pollen-induced anaphylactic reaction in an unknowingly sensitized subject. *Ann Allergy Asthma Immunol.*, **86(2)**: 239-242.

Hagedorn, H.H. and Burger, M. (1967). Effect of the age of pollen used in pollen supplements on their nutritive value for the honeybee. II. Effect of vitamin content on pollens. *J. Apic. Res.*, **7**: 97-101.

Hamamoto, R., Ishiyama, K. and Yamaguchi, M. (2006). Inhibitory effects of bee pollen *cistus ladanifer* extract on bone resorption in femoral tissues and osteoclast-like cell formation in bone marrow cells *in vitro*. *J Health Sci.*, **52(3)**: 268-275.

Harman, D. (1956). The free radical theory of ageing. in "*Free Radicals in Biology*, A. Pryor (*Ed.*) Academic Press, 1984, **5**:, 255-275.

Haro, A., López-Aliaga, I., Lisbona, F., Barrionuevo, M., Alférez, M.J. and Campos, M.S. (2000). Beneficial effect of pollen and/or propolis on the metabolism of iron, calcium, phosphorus, and magnesium in rats with nutritional ferropenic anemia. *J. Agric. Food Chem.*, **48**: 5715-5722.

Hayflick, (1965). The limit *in vitro* lifetime of human diploid strains. *Exp. Cell Res.*, **37**: 614-636.

Herbert, E.W. Jr., Vanderslice, J.T., Meis-Hsia, H. and Higgs, D.J. (1987). Levels of thiamine and its esters in bee collected pollen using liquid chromatography and robotics. *Apidologie.*, **18(2)**: 129-136.

Hodges, S.J., Akesson, K. and Vergnaud, P. (1993). Circulating levels of vitamins K1 and K2 decreased in elderly women with hip fracture. *J Bone Miner Res.*, **8**: 1241-5.

Holliday, R., Kirkwood, T.B.L. and Cuzin, F. (1985). EMBO Workshop on "*Oncogenes, immortalization and cellular Ageing*" Grignon, 3-7 September.

Houston, L.A., Grant S.F.A., Reid, D.M. and Ralston, S.H. (1996). Vitamin D receptor polymorphisms, bone mineral density and osteoporotic vertebral fracture: studies in a UK population. *Bone*, **11**: 249-52.

http://www.cancer.gov/cancerinfo/understanding-prostate-changes/page3.

Huidobro, J.F., Simal, J. and Muniategui, S. (1986). El polen: Determinación del contenido en água. *Offarm.*, **5(3)**: 73-77.

Huidobro, J.F., Simal, J. and Muniategui, S. (1987). El polen apícola: Determinación del contenido em glucidos. *Offarm.*, **6(5)**: 57-71.

Kim, H.P., Son, K.H., Chang, H.W. and Kang, S.S. (2004). Anti-inflammatory plant flavonoids and cellular action mechanisms. *J Pharmacol Sci.*, **96**: 229-245.

Iversen, T., Fiirgaard, K.M., Schriver, P., Rasmussen, O. and Andreasen, F. (1997). The effect of NaO Li Su on memory functions and blood chemistry in elderly people. *J Ethnopharm.*, **56(2)**: 109-16.

Kärkkäinen, M. and Lamberg-Allardt, C. (1996). An acute intake of phosphate increases parathyroid hormone secretion and inhibits bone formation in young women. *J Bone Miner Res.*, **11**: 1905-12.

Khaw, K.T., Sneyd, M.J. and Compston, J. (1992). Bone density, parathyroid hormone and 25-hydroxyvitamin D concentrations in middle-aged women. *BMJ.,* **305**: 273-7.

Knowles, L.M., Zigrossi, D.A., Tauber, R.A., Hightower, C. and Milner, J.A. (2000). Flavonoids suppress androgen-independent human prostate tumor proliferation. *Nutr Cancer,* **38**: 116-122.

Kosmider, K., Wójcicki, J., Samochowiec, L., Woyke, M. and Górnik, W. (1983). Effect of Cerniton on platelet aggregation *in vivo*. *Herba Polon.*, **29**: 237.

Le Bourg, E. (2001). Oxidative stress, aging and longevity in *drosophila melanogaster*. *FEBS Lett.,* **498**: 183-186. http://www.imsb.au.dk/~rattan/biogerontology.html.

Leja, M., Mareczek, A., Wyzgolik, G. Klepacz-Baniak, J. and Czekonska, K. (2007). Antioxidative properties of bee pollen in selected plant species. *Food Chem.,* **100**: 237-240.

Lepley, D.M., Li, B., Birt, D.F. and Pelling, J.C. (1996). The chemopreventive flavonoid apigenin induces G_2/M arrest in keratinocytes. *Carcinogene,* **17**: 2367-2375.

Lin, X.L., Zhu, L.Q., Yuan, Y.Y. and Li, L.M. (1990). Morphological changes in aged canine prostatic hyperplasia treated with bee pollen. *Chin Tradit Herb Drugs,* **21**: 164-166.

Liu, X.L. and Li, L.M. (1990). Morphological observation of effect of bee pollen on intercellular lipofuscin in NIH mice. *J Chin Mater Med Zhongguo Zhongyao Zazhi.,* **15**: 561-563.

Lu, Z.Q. and Dice, J.F. (1985). Ginseng extract inhibits protein degradation and stimulates protein synthesis in human fibroblasts. *Biochem. Biophys. Res. Commun.*, **126**: 636-640.

Macdonald, R., Ishani, A., Rutks, I. and Wilt, T. (1999). A systematic review of Cenilton for the treatment of benign prostatic hyperplasia. *BJU Int.,* **85**: 836-841.

Majeska R.J. and Wuthier, R.E. (1975). Studies on matrix vesicles isolated from chick epiphyseal cartilage. Association of pyrophosphatase and ATPase activities with alkaline phosphatase. *Biochem. Biophys. Acta*, **391**: 51-60.

Masoro, E.J. (1995). Dietary restriction. *Exp. Gerontol.,* **30**: 291-298.

Masoro, E.J. and Austad, S.N. (1996). The evolution of the antiaging action of dietary restriction: a hypothesis. *J. Gerontol. Biol. Sci.*, **51A**: B387-B391.

Mazess, R.B. (1982). On aging bone loss. *Clin Orthop.,* **165**: 239-52.

Mazess, R.B. and Barden, H.S. (1991). Bone density in premenopausal women: effects of age, dietary intake, physical activity and birth-control pills. *Am J Clin Nutr.,* **53**: 132-142.

Moncada, S. and Vane, J.R. (1984). Prostacyclin and its clinical applications. *An. Clin. Res.,* **16**: 241.

Morrison, N.A., Qi, J.C., Tokita, A. *et al.* (1994). Prediction of bone density from vitamin D receptor alleles. *Nature,* **367**: 284-7.

Nair, H.K., Kesava, K.R., Ravikumar, A., Supriya, M., Ram, C. and Stanley A.S. (2004). Inhibition of prostate cancer cell colony formation by the flavonoid quercetin correlates with modulation of specific regulatory genes. *Clinical and Diagnostic Laboratory Immunology,* **11(1)** 63-69.

Nurse, P1. (1990). Universal control mechanism regulating onset of M-phase. *Nature,* **334**: 503-508.

Orgel, L.E. (1963). *The maintenance of the accuracy of protein synthesis and its relevance's to ageing. Prod. Natl. Acad. Sci.*, **49**: 517-521.

Orzaez-Villanueva, M.T., Diaz-Marquina, A., Bravo-Serrano, R. and Blazquez-Abellan, G. (2002). The importance of bee-collected pollen in the diet: a study of its composition *Int. J. Food. Sci. Nutr.,* **53(3)**: 217-224.

Pawlikowska-Pawlega, B., Gawron, A., Gruszecki, W.I. and Misiak, L.E. (2003). The study of the quercetin action on human erythrocyte membranes. *Biochem Pharmacol.,* **66(4)**: 605-12.

Pekkarinen, S.S., Heinonen I.M. and Hopia, A.I. (1999). Flavonoids quercetin, myricetin, kaemferol and (+)-catechin as antioxidants in methyl linoleate. *J Sci Food Agric.,* **79(4)**: 499-506.

Peris, J. (1984). Producción y comércio de los produtos apícolas en España. El Campo del Banco de Bilbao. *Apicultura,* **93**: (Bilbao).

Petkov, V.D., Kehayou, R., Belcheva, S., Konstantinova, E., Petkov, V.V., Getova, D. and Markovska, V. (1993). Memory effects of standardized extracts of *Panax ginseng* (G115), *Ginkgo biloba* (GK501) and their combination Gincosan (PHL-00701) *Planta Med.*, **59**: 106-114.

Pietta, P.G. (2000). Flavonoids as antioxidants. *Journal of Natural Products,* **63(7)**: 1035-1042.

Potapovich, A.I. and Kostyuk, V.A. (2003). Comparative study of antioxidant properties and cytoprotective activity of flavonoids. *Biochem Moscow,* **68(5)**: 514-519.

Puente, S., Iniguez, A., Subirats, M., Alonso, M.J., Polo, F. and Moneo, I. (1997). Eosinophilic gastroenteritis caused by bee pollen sensitization. *Med Clin (Barc).,* **108(18)**: 698-700.

Riggs, B.L. and Melton, L.J. (1983). Evidence for two distinct syndromes of involutional osteoporosis. *Am J Med.*, **75**: 899-901.

Riggs, B.L., Wahner, H.W., Dunn, W.L. *et al.* (1981). Differential changes in bone mineral density of the appendicular and axial skeleton with aging: relationship to spinal osteoporosis. *J Clin Invest.,* **67**: 328-35.

Roberts, K.P., Liu, L.T., Lind, R.E., Iyer, R.A., Prasad, G. and Hanna, P.E. (2000). Cyclic hydroxamic acid inhibitors of prostate cancer cell growth: selectivity and structure activity relationships. *Prostate,* **34(2)**: 92-99.

Samochowiec, L. and Wójcicki, J. (1981). Effect of pollen on serum and liver lipids in rats fed on a high-lipid diet. *Herba Polon.*, **27**: 33.

Seppänen, T., Laakso, I., Wójcicki, J. and Samochowiec, L. (1989). An Analytical study on fatty acids in pollen extract. *Phytother Res.,* **3(3)**: 115-116.

Shahed, A.R. and Shoskes, D.A. (2001). Correlation of β-endorphin and prostaglandin E2 levels in prostatic fluid of patients with chronic prostatitis with diagnosis and treatment response. *The Journal of Urology,* **166**: 1738-1741.

Shoskes, D.A. (2002). Phytotherapy and other alternative forms of care for the patient with prostatitis. *Curr Urol Rep.,* **3(4)**: 330-334.

Shoskes, D.A., Fowler, Jr. J.E. and Nickel, J.C. (2002). Phytotherapy in chronic prostatitis. *Urology*, **60(6)**: supp A 35-37.

Slemenda, C.W., Reister, T.K. and Hui, S.L. *et al.* (1994). Influences on skeletal mineralisation in children and adolescents: evidence for varying effects of sexual maturation and physical activity. *J Pediat.,* **125**: 201-207.

Svendsen, L., Rattan, S.I.S. and Clark, B.F.C. (1994). Testing garlic for possible anti-ageing effects on long-term growth characteristics, morphology and macromolecular synthesis of human fibroblasts in culture. *J. Ethnopharm.,* **43**: 125-133.

Thompson, I. (2001). Pharmacologic agents in complementary medicine in prostatic disease. *Drugs Today,* **37**: 427-433.

Uzbekova, D.G., Makarova, V.G., Khvoynitskaya, L.G. and Slepnev, A.A. (2003). Evaluation of Bee-collected pollen influence in lipid peroxidation, antioxidant system and liver function in old animals. *J Hepatol.,* **38(Suppl. 2)**: 703.

Välimäki, M.J., Kärkkäinen, M., Lamberg-Allardt. C. *et al.* (1994). Exercise, smoking and calcium intake during adolescence and early adulthood as determinants of peak bone mass. *BMJ.,* **309**: 230-5.

Various Comunications - 1° Congresso de Radicais Livres em Química, Biologia e Medicina. Instituto Superior Técnico, Lisboa. Portugal 21-23 de Junho de 1993.

Villareal, D.T., Civitelli, R., Chines, A. and Avioli, L.V. (1991). Subclinical vitamin D deficiency in postmenopausal women with low vertebral bone mass. *J. Clin. Endocrinol. Metab.,* **72**: 628-634.

Wang, M.S., Fan, H.F. and Xu, H.J. (1993). Effects of bee pollen on blood and hemopoietic system in mice and rats. *Chin Tradit Herb Drugs.*, **24**: 588-591, 601.

Warner, H.T., Butler, R.N., Sprott, R.L. and Schneider E.L. (1987). Modern theories of ageing. *Ageing,* **31**: Raven Press N.Y.

Weindruch, R. (1996). Calorie restriction and aging. *Sci. Amer.*, **274**: 32-38.

Welten, D., Kemper, H.C.G., Post, G.B. and Van Staveren, W. (1995). A meta-analysis of the effect of calcium and bone mass in young and middle aged females and males. *J. Nutr.*, **125**: 2802-2813.

Wilt, T., Macdonald, R., Ishani, A., Rutks, I. and Stark. G. (2000). Cernilton for benign prostatic hyperplasia. *Cochrane Database Systematic Reviews,* **2**: CD001042.

Wójcicki, J., Kósmider, K., Samochowiec, L. and Woyke, M. (1983). Clinical evaluation of Cerniton as lipid-lowering agent. *Herba Polon.*, **29**: 55.

Wójcicki, J. and Samochowiec, L. (1984). Further studies in Cernitins: screening of the hypolipidemic activity in rats. *Herba Polon.,* **30**: 115.

Wójcicki, J., Samochowiec. L., Bartlomowicz, B., Hinek, A., Jaworska, M. and Gawronska-Szklarz, B. (1986). Effect of atherosclerosis in rabbits. *Atheroscler,* **62**: 39.

Xiao, P.G., Xing, S.T. and Wang, L.W. (1993). Immunological aspects of Chinese medicinal plants as antiageing drugs. *J. Ethnopharmacol.*, **38**: 167-175.

Yamaguchi, M., Hamamoto, R., Uchiyama, S., Ishiyama, K. and Hashimoto, K. (2006). Anabolic effects of bee pollen *Cistus ladaniferus* extract on bone components in the femoral-diaphyseal and -metaphyseal tissues of rats *in vitro* and *in vivo. J Health Sci.,* **52(1)**: 43-49.

Yasumoto, R., Kawanishi, H., Tsujino, T., Tsujita, M., Nishisaka, N., Horii, A. and Kishimoto, T. (1995). Clinical evaluation of long term treatment using cernitin pollen extract in patients with benign prostatic hyperplasia. *Clin. Ther.*, **17(1)**: 82-87.

Yu, B.P. (1999). Approaches to anti-aging intervention: the promises and the uncertainities. *Mech. Ageing Dev.,* **111**: 73-87.

Zhang, X., Habib, F.K., Ross, M., Burger, U., Lewenstein, A., Rose, K. and Jaton, J.C. (1995). Isolation and characterisation of a cyclic hydroxamic acid from a pollen extract, which inhibits cancerous cell growth *in vitro. J. Med. Chem.*, **38(4)**: 735-738.

"This page is Intentionally Left Blank"

12

Herbal Products in Healthcare: Challenges and Potential

MOHAMMED MOSIHUZZAMAN[1*] AND LEIV K SYDNES[2]

ABSTRACT

It is understandable that the modern pharmaceuticals will remain out of reach of many and the dream of 'health for all' may only be realized by modernizing the use of herbal products. Although the use of herbal medicine is increasing worldwide, there are concerns about the safety, efficacy and standard of much of the herbal products. These issues have been reviewed and the salient features are included in this article. The historical background and the philosophy of herbal medical practice along with its status at the present time science have been outlined. Although herbal products are generally considered safe due to their age-old usage, but serious adverse effects have been reported for many herbal products including herbal medicines. These are primarily due to accidental contamination or intentional adulteration. Methods of identifying contaminants and assuring safety by proper toxicity and risk assessments have been outlined. Due to the holistic approach of herbal medicine, assessment of claimed efficacy is difficult. Practical ways of assessing efficacy of herbal medicine by adapting the methodologies used for modern pharmaceutical are described. The maintenance of appropriate dose format and standard of herbal medicine have been discussed in details and, pragmatic approaches of assuring reproducible standard of herbal medicine by using modern tools of fingerprinting the chemical profile of herbal medicine have been discussed. As much of the traditional herbal medical knowledge is scattered around the world at the family and community levels and in tribal areas, there is

1. H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi - 75270, Pakistan.
2. Department of Chemistry, University of Bergen, NO - 5007 Bergen, Norway.
* *Corresponding author* : E-mail : mmosihuzzaman@yahoo.com

the obvious danger of losing the knowledge unless they are quickly documented. Difficulties in documenting of herbal medical knowledge specially due to concerns of Intellectual Property Rights (IPR), have been highlighted. Formats of documenting single medicinal plant profile and that of combined herbal drug have been outlined.

Key words : Herbal Products, herbal medicine, philosophy of herbal medicine, safety, contamination, adulteration, adverse effects, efficacy, standardization, fingerprinting, documentation, intellectual property rights

INTRODUCTION

Herbal products have been used for human healthcare throughout the human civilization. The consumption of plant-based medicines and other botanicals has increased manifold in recent time especially in Europe and the USA. For example, between 1990 to 2000, an increase of over 380% in sales of such drugs has been recorded in the USA. A global sale of herbal products, including herbal medicines, is already over USD 10^{11} and is expected to exceed USD 10^{12} in the next 20 years at the present growth rate.

All around the world there is talk about 'health for all' but it has been realized that modern pharmaceuticals are and will remain out of reach of a large proportion of the human population for the foreseeable future. This necessitates the use of other sources of human knowledge to provide common health benefits. Thus, herbal medicine is now regarded as important but underutilized tool against disease. The World Health Organization (WHO) recognized this fact in the early 1970s and encouraged governments to effectively utilize local knowledge of herbal medicines for disease prevention and health promotion. Herbal medicines, however, suffer from a range of shortcomings mainly arising from insufficient and unacceptable evidences of safety, efficacy and inconsistent production practices. In this article we try to summarize the challenges of modernizing or 'scientificating' of herbal medicine for universal use and how to overcome them. As the isolation and analytical techniques have become more powerful, and the capability to apply them in countries rich in medical herbs has increased, there is a growing interest in herbal medicine.

From the point of view of countries rich in herbal plants with certain medicinal potential or even proven activity this is indeed a great opportunity. If a fraction of this opportunity is to be realized, there is a strong need to develop more reliable and comprehensive chemical analyses, and to improve the knowledge of the active ingredients in the herbal medicines with respect to structure, function, and toxicity as well as efficacy. For this purpose a global perspective is required and cooperation between the stakeholders

has to be increased well beyond the present level because there are a number of obstacles that have to be diminished or removed.

HISTORICAL BACKGROUND

The first generally accepted use of plants as healing agents was depicted in the cave paintings discovered in the Lascaux caves in France, which have been radiocarbon-dated to 13,000-25,000 BCE. Medicinal herbs were found in the personal effects of an "Ice man" whose body was frozen in the Swiss alps for more than 5,300 years, which appear to have been used to treat the parasites found in his intestines (Capasso, 1998). It is said that animals seek out bitter plants rich in secondary metabolites, such as tannins and alkaloids (Hutchings *et al.*, 2003), which they normally would not eat (Huffman, 2003). The use of herbs and spices in cuisine developed in part as a response to the threat of food-born pathogens. Studies show that in tropical climes, where pathogens are the most abundant, recipes are most highly spiced. Further, the spices with the most potent antimicrobial activity tend to be selected (Sherman & Billing, 1998). In all cultures vegetables are spiced less than meat, presumably because they are more resistant to spoilage (Sherman & Hash, 2001). In light of the growing resistance of parasites and pathogens to synthetic drugs, the study of animal self-medication and ethno-medicine offers a novel line of investigation to provide ecologically-sound methods for the treatment of parasites using plant-based medicines in populations and their livestock living in the tropics (Huffman, 2003).

Herbal Medicine Documents

The early records of medicinal use of plants date back 5,000 years to the Sumerians (Anonymous, 2008a). About the same time Rishi (Sages) Srila Vyasadeva wrote down the Vedas including a part called Ayurveda or "Science of Life". The written text of that part as Rig Veda came just about 2000 years ago (Anonymous, 2008b). Current practice of Ayurveda is based on the Ayurvedic Pharmacopoeia of India (API, 1989) and other recent publications (Kapoor, 2001). Around 1500 BCE, Ayurveda grew into a respectable system of medicine which has continued to the present time.

Traditional Chinese Medicine (TCM) can be traced back to about 2700 BCE when Nejing Suwen was composed by the Yellow Emperor (2698-2596 BCE). The standardized format of TCM was created during the Mao Zedong era in the 1960s (Anonymous, 2008c). Other relevant sources are the Encyclopedia of Chinese Materia Medica (Zhong, 1977) and the Pharmacopoeia of the Peoples' Republic of China (Gi, 1992).

The Unani system of medicine originated in Greece by Galen. Later, the Arab and Persian scholars enriched the system and the earlier 'Galenic' system became known as Unani (Arabic name for Greek) system of medicine. The Unani system was introduced in India around 10th century

AD and is now has become an important system of medicine after Ayurveda in the Indian subcontinent (Anonymous, 2008d) While Rishis in north India founded Ayurveda, Siddhars (saints) practiced, what is known as Siddha system of medicine (Anonymous, 2008e), in the south of India. Both the systems use herbs, minerals and metals but differ in philosophy in that the prescriptions came from Gods and nowadays, it is limited only to the followers of hereditary and traditional people of Siddha medicine.

Africa and the Americas

In the Americas, the history of herbal medicine is very different as there are no age-old systems like those in China and India. Recorded pharmacopoeias only have come by in recent times with the resurgence of herbal medicine worldwide. The plant knowledge of the Native peoples of North, Central and South America, refined over millennia of practice, has integrated itself throughout human cultures. There is no exhaustive source of Native North American plant knowledge, but Daniel Moerman's magnificent Native American Ethnobotany (Moerman, 1998) comes the closest. This rich *materia medica*, refined by talented healers over generations, includes scores, or even hundreds, of plants which have entered general human use through Native contact with European, African and Asian peoples (Stephen, 2007).

In Africa, herbal medicines are often used as primary treatment for HIV/AIDS and for HIV-related problems. In general, traditional medicines are not well researched, and are poorly regulated (Mills *et al.*, 2005), although a very high percentage of people in Africa depend on herbal treatment for their primary healthcare. In recent times a group of researchers is assembling a pan-African pharmacopoeia, a database of African plants with medicinal properties (Anonymous, 2006).

Besides the major systems of use of herbal medicine, there are numerous pharmacopoeia, material medica and other methods usually on a country or region basis, like those in Iran, Germany, and other EU countries. Moreover there are large number of books and monographs on herbal medicine all across the world.

PHILOSOPHY OF HERBAL MEDICINE

Practically all systems of herbal medicine take a holistic approach where the physical, mental and social well-being of an individual are considered collectively for the treatment of a particular medical condition. Traditional medicine, including herbal medicine, implies knowledge and practice of herbal healing for the prevention, diagnosis, and elimination of physical, mental, or social imbalance (Akerele, 1993). Herbal medicine practitioners or herbalists, view their field as a study of a web of relationships rather than a quest for a single condition as most modern medical practitioners do. Herbalists also view their goal as prevention as well as cure. They

argue that different phytochemicals present in many herbs will interact with synergy and multifunctionality to enhance the therapeutic effects of the herb and dilute toxicity. Although in specific cases, the claim of synergy (Williamson, 2001) and multifunctionality (Izhaki, 2002) have been supported by scientific evaluation, generalization is difficult. The argument is that, plants under selection pressure develop resistance to threats such as radiation, reactive oxygen species and microbial attack and herbs may simultaneously address several of these factors. Synergistic interactions are of vital importance in phytomedicines, to explain difficulties in always isolating a single active ingredient, and explain the efficacy of apparently low doses of active constituents in a herbal product. This concept, that a whole or partially purified extract of a plant offers advantages over a single isolated ingredient, also underpins the philosophy of herbal medicine. However, most herbalists concede that pharmaceuticals are more effective in emergency situations, for example when a patient has elevated blood pressure threatening imminent danger. But they claim that over the long term, herbs can help the patient resist disease and in addition provide nutritional and immunological support that pharmaceuticals lack.

CHEMISTRY OF HERBAL MEDICINE

All types of herbs and plants produce various chemical compounds as part of their normal metabolic activities as well as for protection against disease and predators. Primary metabolites like sugars and fats are found in all plants. On the other hand many of the secondary metabolites, found in most plants, are useful as therapeutic agents. Some secondary metabolites are toxins used to deter predation, and others are pheromones used to attract insects for pollination. Phytoalexines protect against bacterial and fungal attacks. Allelochemicals inhibit rival plants that are competing for soil and light. The chemical profile of a plant may vary over time as it reacts to changing conditions. Major classes of phytochemicals useful for therapy, produced by plants include alkaloids, phenolics, terpenoids and glycosides. The word 'drug' itself comes from the Swedish word "druug", which means 'dried plant'. Some examples of drugs from plants are inulin from the roots of dahlias, quinine from the cinchona, morphine and codeine from the poppy, digoxin from the foxglove, and parent aspirin from willow bark. Reports of isolation of hundreds of compounds from different medicinal plants are abundant in scientific literature, but hardly any new drug from plant sources is coming up in recent times.

The exact composition of a herbal product depends on the method of extraction. Alcohol and water are extensively used for herbal medicine preparation. Tinctures are prepared by extraction with ethanol usually containing certain percentage (10-40%) of water whereas herbal wine and elixirs macerated products of herbs in wine and spirits containing 12-38% of alcohol (Annoymous, 2008a).

PRESENT STATUS OF HERBAL MEDICINE

During the past few decades public interest in traditional, complementary and alternative (TCAM) medicine and use of herbal medicines has increased dramatically in industrialized countries (Bodeker & Mumford, 2007). This has increased the international trade in herbal medicine enormously. WHO said in 2003 (WHO, 2003) that the global market for herbal medicines stood at US $ 60 billion and was growing steadily. Global sales of herbal products including herbal medicine has already crossed 100 billion in the last five years and is expected to exceed one trillion in the next 20 years at the present growth rate. Many pharmaceutical companies are showing interest in the production and marketing of herbal medicines.

Historically, about two centuries ago, our medicinal practices were largely dominated by plant-based medicines. However, the medicinal use of herbs went into decline in the West when more predictable synthetic drugs were made commonly available. In contrast, many developing nations continued to benefit from the rich knowledge of medical herbalism. For example Ayurvedic medicine in India, Kampo medicine in Japan, Traditional Chinese Medicine and Unani Medicine in the Middle East and South Asia are still used by a large majority of people.

In the recent era of herbal renaissance, the demand of herbal medicines and other botanicals by Western communities has been increasing steadily, particularly over the past two decades. Figures from the Consumers Association in the UK suggest an increase in utilisation of herbal therapies by their members from one in seven in 1985 to one in four in 1991 (BMA, 1993). In 1997, Figures from the United States suggested that as high as 67.6% of the population had used complementary and alternative therapy at least once in their lifetime (Kessler *et al.,* 2001). In Europe, North America and other industrialized regions, over 50% of the population have used complimentary or alternative medicine at least once (WHO, 2003). A study in Australia indicated that 57.2% of herbal users did not discuss herbal use with their physician. Estimates of the national cost of both herbal medicines and practitioner visits have been placed at approximately 2.3 billion Australian dollars annually (MacLennan, 2002) In parallel with this trend, Traditional Chinese Medicine (TCM), represented by acupuncture and Chinese herbal medicine, is currently being used more widely than ever before in Western countries (Cassidy, 1998). Along with herbal medicines, other herbal products such as cosmetics, fragrances, teas, health foods and nutraceuticals are equally popular and constitute a large proportion of global herbal business.

PRESENT CHALLENGES

The fact remains that 80% of the population of the developing world rely on herbal medicine for their primary healthcare, (WHO, 2002a) and a growing

number of people in the industrialized countries are using herbal therapy. For example, in the USA about 20,000 herbal products are available, although of the ten most commonly used herbs only four are likely to be effective (Bent & Ko, 2004). WHO recognized this fact more than 35 years ago and encouraged governments to effectively utilize local knowledge of herbal medicines for disease prevention and health promotion. Herbal medicines, however, suffer from a range of shortcomings. These include insufficient and unacceptable evidences of safety, efficacy, standardization and inconsistent production practices. The shortcomings are played well by the promoters of modern medicine and are also responsible for a lower confidence level among the elite in the developing as well as developed countries. The increased use of herbal medicine may be considered as an increase in the confidence level among the people and may be related to two factors. First, the extraction, isolation and analytical techniques have become more powerful and reliable and made the isolation more reproducible and characterization more accurate; and secondly, the capability to apply these techniques in countries rich in medicinal herbs has increased. There is therefore a growing interest in expanding the activity in this field, and from the point of view of the countries rich in medicinal plants this is very good news. If this potential is to be realized, there is a strong need to develop more reliable and comprehensive chemical analyzes, and to improve the knowledge of the active ingredients in the herbal medicines with respect to structure, function and toxicity as well as efficacy.

Consideration of the growing importance of herbal medicine and other herbal preparations, concerns about the safety and claimed efficacy of many herbal products, and lack of proper scientific evaluation resulted in International Union of Pure and Applied Chemistry (IUPAC) supporting a project with the objective of preparing protocols on safety, efficacy, standardization and documentation of herbal medicine. These protocols has been published in *Pure and Applied Chemistry* as an IUPAC Technical Report (Mosihuzzaman & Choudhary, 2008). In this document various aspects of the science of herbal medicine in modern times have been compiled in one place and it is expected to significantly advance the scope of scientific assessment of herbal products, enhance confidence in herbal medicine and ensure safe and efficacious products prepared following certain standards. With the target of utilizing the protocols in developing herbal medicine for better healthcare specially in the developing countries, establishment of an International Centre for Natural Product Research has been initiated in Dhaka, Bangladesh.

SAFETY ISSUES

There is a general perception among many consumers that herbal products including herbal medicines are safe as they are being used through the ages. True, considering the adverse effects of pharmaceuticals, herbal

medicine may offer an inexpensive and safe alternative. In the US, which has only 4% of world's population, 106,000 patients died from and 2.2 million were seriously injured by adverse effects of pharmaceuticals in the year 1994 (Lazarou *et al.*, 1998). Although no such exhaustive study is available for herbal medicine, unregulated or inappropriate use of herbal medicine may have serious consequences. There are numerous case reports of serious adverse events after administration of herbal products. In a lot of cases the toxicity has been traced to contaminants and adulteration. However, some of the plants used in herbal medicines can also be highly toxic. As a whole, herbal medicines can have a risk of adverse effects and drug-drug and drug-food interactions if not properly assessed.

Contamination

Considering the serious risks involved, assessment of the safety of herbal products is a priority in herbal research. There are various approaches towards the evaluation of safety of herbal medicines. The toxic effects of herbal preparation may be attributed mainly to a) inherent toxicity of plant constituents and ingredients, and b) manufacturing malpractices and contamination. Evaluation of the toxic effects of plant constituents of herbal formulation requires detailed phytochemical, and pharmacological studies. It is, however, safe to assume that, based on human experiences in various cultures, the use of toxic plant ingredients has already been largely eliminated and recent reports of toxicity could largely be due to misidentification and accidental or intentional contamination.

Unintentional contaminants of herbal medicines may include microorganisms, microbial toxins, pesticides, fumigation agents, radioactivity and presence of toxic compounds of toxic metals (Cassidy, 1998; Ernst, 2001). Intentional contaminants are usually synthetic drugs (Ernst, 2002). Contamination other than toxic metals in Chinese herbal preparations has not been reported in the literature; other contaminants have not been the focus of any extensive investigation so far. Reports of toxic metal contamination include lead, arsenic, mercury, thallium and cadmium poisoning (Chan *et al.*, 1993; Brown & Ede, 1996; Mitchell-Heggs, 1990).

Substitution and Adverse Effects

Inadvertent substitution due to similarity in appearance or difficulty in nomenclature has been reported (Chan *et al.*, 1993). But intentional substitution for reducing cost or due to poor practice is also known. Adverse effects associated with herbal medicine products were first reported for medicinal plants of the Asteraceae family, *Hypericin* and *Aristolochia* genus, and Kava-Kava. A number of cases of inadvertent or deliberate substitution of the constituents of Chinese herbal preparations are cited in the literature. For example Siberian ginseng (*Eleutherococcus senticosus),* American

ginseng (*Panax quinquefolium*), and Japanese ginseng (*Panax pseudo-ginseng*) have been substituted for Korean or Chinese ginseng (*Panax ginseng*). Sometimes the substitute has a much greater toxicity than the original material. Examples of substitution resulting in an adverse effect include reported cases of hepatitis with Jin Bu Huan (Woolf *et al.*, 1994), renal fibrosis due to *Aristolochia fangchi* (Vanherweghem *et al.*, 1994) and podophyllin poisoning due to *Podophyllum emodi* (Chan & Critchley, 1996). Acute hepatitis by a Chinese herb Shou-Wu-Pian, mainly containing *Polygonum multriflorum,* has also been reported (Kaptchuk, 1995).

Cases of hepatitis related to consumption of Jin Bu Huan (*Lycopodium serratum*) were reported in seven patients in the USA. Jin Bu Huan is used for pain relief and insomnia (Woolf *et al.,* 1994). The reaction developed after an average of 20 weeks of therapy and was resolved in most patients in a mean of 8 weeks. The information enclosed with the preparation indicated that the product contained 30% levo-alkaloid from *Polygala chinensis,* but analysis suggested a more toxic substitute (Park *et al.,* 2001), an alkaloid from *Stephania* and *Corydalis* genera and not from *Polygala*. In Belgium 70 people required renal transplant or dialysis for interstitial fibrosis of the kidney after taking a herbal preparation from the wrong species of plant as slimming treatment (Vanherwegham, 1998; WHO, 2003). The preparation was known to contain *Stephania tetranda* and *Magnolia officinalis*. Extensive investigation ultimately found the presence of aristolochic acid from *Aristolochis fangchi* which may have been incorporated due to possible mistakes in nomenclature. *Aristolochia* spp. contain aristolochic acid, which is nephrotoxic in animals and humans (De Smet *et al.,* 1992).

There are numerous reports of inadvertent substitution of "Gui Jiu" (*Podophyllum emodi* Wall) for "Long Dan Cao" (*Gentiana* spp.). This resulted in severe neurological, gastrointestinal, renal and hepatic manifestations to the consumers (Chan & Critchley, 1996).

Regulatory Approaches

Herbal medicines are largely unregulated as drugs, and the legal situation regarding herbal preparations varies from country to country. Herbal products range from phytomedicines to food and dietary supplements where therapeutic claims are not allowed. There may be a strong connection with traditionally-used herbal medicines and folkloric knowledge which in some countries justifies less stringent regulations. Generally, there are no universal legislative criteria for use in regulating herbal products but different countries have developed their own regulations and many others follow the WHO guidelines.

Based on the recommendations of the WHO-sponsored 6th International Conference on Drug Regulatory Authorities (ICDRA), WHO prepared guidelines for basic criteria for quality, safety and efficacy of

herbal medicines (ICDRA, 1991). This was followed by development of monographs on herbal medicines on the basis of the guidelines, in the following ICDRA meeting in 1996 (ICDRA, 1996).

Herbal products can be marketed in the USA only as food supplements. Specific health claims need FDA approval. The European Guidelines for the Assessment of Herbal Medicines state that a substance's historical use is valid to document safety and efficacy, in the absence of scientific evidence to the contrary. However, the legislative framework for herbal medicine is pretty complex in the European Community. Directive 91/507/EEC gives details of quality, safety and efficacy. Further directives followed *e.g.* European Directive 2004/24/EC of 31 March 2004. Similarly United Kingdom has adopted a Statutory Instrument No. 2750 entitled "The Medicines (Traditional Herbal Medicinal Products for Human Use) Regulations 2005". The German Federal Health Agency Commission E has developed monographs of herbal medicine containing pharmacological, toxicological and clinical documentation. Developments in other countries have been summarized (Shia *et al.,* 2007).

The risks associated with the use of herbal substances are related to problems associated with the failure of good handling and manufacturing of certain products. Lack of standardization, contamination with toxic metals, inadvertent and deliberate substitution of other herbs and adulteration with Western pharmaceuticals also contribute in associated risks.

Monitoring and Surveillance

There is hardly any monitoring or surveillance system in place in most of the developing countries. In many countries herbal medicinal products are considered as a drug without a therapeutic claim and therefore are not subject to drug regulations. In some countries microbiological tests are used to assess safety. Thus, regulations and proper legislation must be introduced to ensure that required standards of safety and efficacy are met. Serious herb-drug interactions are rare and trials are unlikely to detect most cases. Quality assurance is, therefore of paramount importance. What is needed is a balance between industry and regulation in order to protect public health. The practice developed for herbal medicines through the Joint Agency Model – Joint Tasman Project (TGA, 2004) used a risk-based approach by substance and by claim.

For the evaluation of herbal products several steps are to be followed (ICDRA, 1991; TGA, 2004) such as (a) identity of the ingredients, (b) history of use, (c) any reported adverse reaction, (d) toxicity (if any) and (e) any clinical trial data. The presence of impurities is either intended addition, or accidental contamination *via* processing. The substitution of plants arises because of similar plants/wrong identification, or use of cheaper alternatives.

Toxicity Assessment

Analysis of herbal products may be best approached by analysis of one or more hypothesized active ingredients, analysis of a chemical constituent that constitutes a sizeable percentage of total ingredients, and chemical fingerprinting of ingredients (TDR, 2005). Toxicity investigation will also be required because the analysis alone is unlikely to reveal the contributions to toxicity itself. In assessing toxicity of a herbal medicinal product, the dose chosen is very important. Toxicity assessment involves one or more of the following techniques:

1. *In vivo* techniques
2. *In vitro* techniques
3. *Cell line* techniques
4. Micro-array and other modern techniques

The issues of mixtures and deviation from conventional pharmacological approaches remain a major problem, along with the consequences of using the wrong source plants or ingredients, variable content of active constituents and a narrow therapeutic window with herbal medicines. Concurrent contamination with any one of the toxic metals, bacteria, viruses, or pesticides may also occur.

Risk Assessment

The risk assessment procedure considers the following steps in risk characterization:

- Hazard identification
- Dose response assessment
- Exposure assessment

Risk characterization enables the estimation of any adverse effect and provides a means of devising risk management, if appropriate. Risk management can then be applied on the basis of the assessments. In the risk assessment process, studies need to take into account formulation of the herbal products and their bio-availabilities. There are two groups at risk (Sim & McNeil, 1999), namely (i) workers handling herbal products, and (ii) people using herbal products. Currently accepted procedures in Australia (EnHealth, 2002) and by the USEPA (USEPA, 1998) enable the formalized approach of risk assessment to be applied when required. Calculation of dose enables recommendations to be made regarding safety criteria for public health. The understanding of risk assessment and implementation and management are two sequential steps where assessment is first undertaken followed by development of a management tool based on identified risks. The outcome of the risk assessment process is, therefore, to determine whether risk characterization identifies any

significant aspect of the toxicant(s) in any particular herbal product. A key challenge is to objectively assess conflicting toxicological, epidemiological and other data and the verification of herbal materials used. This requires use of the audit process to identify potential contaminants in herbal products.

EFFICACY OF HERBAL MEDICINE

One of the central issue of modern debate about herbal medicine is its ability to improve health and well being. The use of herbal remedies is often justified by their long usage but, age-old wisdom does not necessarily guarantee that the product in question is efficacious with reasonable specificity. Modern pharmaceuticals takes a direct approach onto a certain disease condition, but herbal medicine usually takes an overall view of well-being of an individual. One way to bridge the philosophical gap between these two systems (and thus to adopt more of an integrated approach) is the WHO concept of health which emphasizes the best part of both systems. Through this "One System of Medicine" we may be able to design appropriate indicators of efficacy and the practical methodology to test them.

Assessment of Efficacy

Although herbal medicines are inherently different from conventional pharmaceuticals but presently there is no way to assess their efficacy other than by currently used clinical, laboratory or diagnostic methodologies. Clinical outcomes include parameters such as improved morbidity and mortality, reduced pain or discomfort, improved appetite and weight gain, reduction of blood pressure, reduction of tumor size or extent, and improved quality of life. Laboratory or diagnostic outcomes include parameters such as reduction of blood glucose, improvement of hemoglobin status, improvement in the findings of radiological, electrical, imaging or diagnostic techniques.

One of the crucial questions is whether these two groups (clinical and laboratory) of efficacy measures are sufficient for assessing the efficacy of herbal medicines. In particular, it is argued that socioeconomic, cultural and psychological variables should be integrated with the clinical and diagnostic parameters in assessing the efficacy of herbal medicine. Given the fact that most of the users of herbal medicine in developing countries have limited accessibility to and affordability for modern medicine, it would be natural to agree in favor of a greater emphasis on socioeconomic variables in this system of medicine. While this approach is attractive from a social and political point of view, it is still difficult to incorporate it into the practical assessment of efficacy of a particular herbal medicine. There is, however, scope to utilize the flexibility inherent in the modern scientific method for conducting studies on herbal medicines.

Methods for Assessment of Efficacy

The various methods used for testing the efficacy of a conventional drug may not be applicable as such to herbal medicine. The design of a study of efficacy will have to be sound regarding evidence, subject selection, randomization, mode of treatment, controlling, confounding variables and reporting of results (Hennekens & Buring, 1987). Usually, a) anecdotal reports, b) case reports, c) case series, and d) randomized clinical trials are applied for testing the efficacy of drugs (Plantadosis, 2005).

(i) *Anecdotal Reports* are usually not taken into account in conventional medicine but they are important components of the efficacy assessment in herbal medicine. This is due to the fact that knowledge about their usefulness is often limited to individual practitioners or tribes, sometimes, living in isolated communities or peripheral locations. Organization of these anecdotal reports into well designed case series may provide useful data on the efficacy of a large group of herbal agents.

(ii) *Case Reports* are the starting point for efficacy (or toxicity) tests of many drugs, as evident from the contents of many reputable clinical journals (Fletcher & Fletcher, 1979). These can represent the first clues in the identification of new diseases, new interventions or previously unknown adverse effects. Case Reports can be retrospective (data collected from previous experiences or records) or prospective (follow-up from a baseline) in nature. The latter may again be divided into observational (collection of data by passive observation) or interventional (adjusting the system by standardized design) in nature.

(iii) *Case Series* are collections of individual case reports, organized to explore a particular association. Case Series may be retrospective or prospective (observational or interventional) in nature, as explained in case of Case Reports.

(iv) *Randomized Clinical Trials* (with double-blind ones being the gold standard) are the ultimate measure of efficacy in conventional medicine. Substantial efforts and resources are necessary for such trials.

Randomized Clinical Trial

Although randomized clinical trials should be the ideal way for evaluating the efficacy of herbal drugs, it cannot always be implemented (Plantadosis, 2005). These trials are inherently interventional in nature and it may be sometimes ethically questionable whether such studies can be designed without sufficient existing evidence about its efficacy and safety in animals. For edible plants or for plants used as herbal medicines since early times by human beings and recorded in some pharmacopoeia, there may be an

argument for conducting trials without animal experimentation but there still remain unsolved ethical issues in this area.

Numerous variables (including socio-cultural factors, beliefs and placebo effects) influencing the effectiveness and quality of herbal drugs also make it difficult to design trials on these drugs. Use of conventional protocols for chemical analysis, animal experimentation and toxicity studies blur the demarcation between traditional and modern pharmaceutical approaches. Moreover, there is a risk that the economic consequences of such an approach could offset the low-cost healthcare benefit currently enjoyed by the majority of the population who are most dependent on herbal drugs.

Case Reports and Case Series

In the context of difficulties with randomized clinical trials, an increased scoring can be given to Case Report and Case Series approaches in assessing the efficacy of herbal medicines. Retrospective observational data would be difficult to be compiled due to the lack of proper record systems in the case of individual or institutional traditional practitioners. It may, however, be possible to conduct qualitative studies on age-long practices and the inferences may be used to strengthen the evidence for efficacy. Prospective observational design will be the easiest to implement as a substantial amount of data from a large number of subjects, prescribed with herbal drugs under the existing legislations of a particular country, can be brought into a proper record system and analyzed scientifically. Data from Case Series studies may provide sufficient scientific and ethical validity to conduct randomized clinical trials of herbal medicines, but acceptance of this protocol needs a paradigm change in the methodology of drug evaluation as understood in conventional medicine. Even with all the above-cited limitations, a significant number of randomized clinical trials on herbal medicines have been successfully conducted. For example, garlic (*Allium sativum* L.) for lipid-lowering properties and gingko (*Gingko biloba* L.) for pain relief.

WHO Guidelines

In order to proceed with the validation of the efficacy of medicinal plants, WHO believes that several levels of evidence including ethnobotanical claims, anecdotal information, pharmacological studies and observational studies should be taken into account (WHO, 1999; WHO, 2002b).

STANDARDIZATION OF HERBAL MEDICINE

It is a common observation that people diagnosed with incurable chronic disease states such as diabetes, arthritis and AIDS turn to herbal therapies for a sense of control and mental comfort from taking action (Brown & Marcy, 1991). Herbs are considered to be dietary supplements in the United

States and therefore are subjected to a very limited form of regulation and oversight (Lee, 1999). Herbal product studies cannot be considered scientifically valid if the product tested has not been authenticated and characterized in order to ensure reproducibility in the manufacturing of the product in question. Several studies have indicated quantitative variations in marker constituents in herbal preparations. Moreover, many dangerous and lethal side effects have recently been reported, including direct toxic effects, allergic reactions, effects from contaminants, and interactions with drugs and other herbs. Therefore, there is a need and scientific requirement to accelerate research in phytomedicine (Ulrich-Merzenich *et al.*, 2007).

For consistent quality and well-defined constituents, standardization of herbal products is absolutely necessary. Pharmacological properties of a herbal formulation depend on phytochemical constituents present therein. Development of authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analyses of marker/bioactive compounds and other major constituents, is a major challenge to scientists. Resurgence of interest and the growing market of herbal medicinal products necessitate strong commitment by the stakeholders to safeguard the consumer and the industry. Standardization is the first step for the establishment of a consistent therapeutic activity and a consistent chemical profile. In other words a quality assurance program for production and manufacturing needs to be in place. In this context, the European Union has defined herbal products (a) containing definite chemical compound(s) with known efficacy, (b) those containing chemically defined constituents possessing relevant pharmacological properties which are likely to contribute to the clinical efficacy and (c) those in which no constituents have been identified as being responsible for the therapeutic activity.

Standardization of herbal medicines may be made by analyzing the constituents with known therapeutic activity or by following marker compounds. The European Medicines Agency (EMEA) defines marker compounds as chemically defined constituents of a herbal drug which are of interest for control purposes, independent of whether they have any therapeutic activity or not (EMEA, 2006).

Current Status

Herbal medicine may be prepared from leaves, stems, flowers, roots or seeds (Winslow & Kroll, 1998) of a single plant or combinations of several different plant materials that are believed to have complementary effects. Some herbal products, including those of traditional Chinese medicine formulations, also include animal products and minerals (Rotblatt & Ziment, 2002). Herbal products are sold as either raw plants or extracts of portions of the plant. The extraction involves boiling, percolating or macerating the

herb in water, ethanol, or other solvents to release biologically active constituents from the cell matrices of the plant into the solvents. The herb to be extracted may be in its dried or fresh forms.

Regulatory requirements for the quality of herbal products vary depending on the country and the regulatory category. The same herbal product can be marketed as a drug in Europe and as a dietary supplement in the USA. In Europe, most herbal products are produced according to quality standards typical for pharmaceutical products. Individual governments, the WHO and panels of academic experts and clinicians often provide guidelines for manufacturing and quality control. Many of these guidelines are compiled in pharmacopoeial monographs. These documents publish traditional and standardized therapeutic uses of herbs and provide a foundation for clinical practice. Monographs consist of a description of the herb, including botanical information, laboratory analysis, therapeutic indications, and drug interactions (if relevant). Although the monographs may be tedious to read, they include specific information that may not be available in other references. Some examples are (a) United States Pharmacopoeia, (b) German Commission E Monographs, (c) ESCOP Monographs, (d) British Herbal Pharmacopoeia and (e) WHO Monographs (Tyler, 1997). According to the draft guidelines stated by the United States Food and Drug Administration (USFDA) (USFDA, 2000) and The European Agency for the Evaluation of Medicinal Products (EMEA, 2001), various aspects of analysis as recommended by their respective Pharmacopoeias must be performed for the purpose of certification of botanical drugs and herbal preparations.

Extraction

For any form of quality control or standardization of herbal medicine, the identity of the plant material has to be ascertained first. Next comes the question of extraction and chemical analysis. The most important step in the analysis of botanicals and herbal preparations is the sample preparation. In most cases, air-dried and powdered plant materials are used for sonication, heating under reflux, Soxhlet extraction, and more recent times cold extraction are commonly used (USP, 2002; PRC, 2000). To reduce the time and the use of organic solvent, and improve the extraction processes, newer sample preparation methods (Huie, 2002), such as microwave assisted extraction (MAE) (Kaufmann *et al.*, 2007), subcritical water extraction (SFE) (Jimenez-Carmona *et al.*, 1998) and accelerated solvent extraction (ASE) (Brachet *et al.*, 2001) have been introduced.

Separation of Constituents

In whatever way the plant material is extracted, the extract will be a complex mixture of many compounds. Various forms of chromatographic methods are utilized to separate individual compounds for bioactivity evaluation. TLC, HPLC and NMR are often used to record some kind of fingerprints of

chemical constituents specially marker compounds. HPLC in combination with UV using photodiode array detector, multistage mass and nuclear magnetic spectrometers allow identification and characterization of individual compounds (Hostettmann & Wolfender, 1999; Guliyeva *et al.*, 2004). NMR Metabonomics in combination with chemometrics, especially Principal Component Analysis (PCA) and Simulated Independent Modeling of Class Analogy (SIMCA) algorithms, has been recognized as a very powerful tool to classify samples according to their total chemical composition. The resolution of high field NMR can provide information in the orders of magnitude higher than of other fingerprinting technologies as usual NMR spectrometry or HPLC (Belton *et al.,* 1998; Frederich *et al.*, 2004; Choi *et al.*, 2004). The presence of heavy metals is also one of the criteria included in pharmacopoeias. The tool primarily used to detect and quantitate the metalic elements in most analyses is based on atomic absorption spectrometry (AAS). Currently there have been a number of instruments developed based on the same principle such as inductively coupled plasma – optical emission spectrometry (ICP-OES). Detection and quantitation based on mass spectrometry has also been available using inductively coupled plasma – optical mass spectrometry (ICP-MS).

Challenges

Plant materials and extracts from them contain complicated mixtures of organic chemicals, which may include fatty acids, sterols, alkaloids, flavonoids, glycosides, saponins, tannins, lignans, terpenes as well as peptides and oligosaccharides. It is often difficult to determine which component, if any, of the herb has biological activity in humans. Processing and environmental factors may affect the levels of components in any given batch of a herb. Other factors, including infections, insects, planting density, competition with other plant species, seeding time, and genetic factors, play an important role in producing uniform herbal products (Wijesekera, 1991). Moreover, pharmacological experiments with single isolated compounds versus the original extract or extract fractions have confirmed that many plant constituents, among them primarily phenolic compounds and terpenoids, exert "polyvalent pharmacological" effects (Wagner, 2001). This might explain some of the pharmacological synergetic effects and the phenomenon that very often an extract possesses a much better therapeutic effect than single isolated constituents. Synergistic interactions are of vital importance in phytomedicines, to explain difficulties in always isolating a single active ingredient, and explain the efficacy of apparently low doses of active constituents in a herbal product. This concept, that a whole or partially purified extract of a plant offers advantages over a single isolated ingredient, also underpins the philosophy of herbal medicine. Evidence to support the occurrence of synergy in within phytomedicines is now accumulating and is reviewed (Williamson, 2001).

As there are evidences of substantial difference in the mode of action of a combination drug from the mode of action of single chemical component, the question arises about the relevance of determining marker constituents in herbal medicines. A new challenge is then towards understanding the effects of complex mixtures on molecular and biochemical processes in health and disease. Considering all the facts, emphasis may be given on the need for botanicals and herbal preparation to approach scientific proof and clinical validation with chemical standardization, biological assays, animal models and clinical trials. The constraint is that the existing technologies are not adequate for complete analyses of constituents. In most developing countries, the costs of analyses and standardization are still too high, and instruments and infrastructure, expertise and human resources are impediments in standardization technology. A new paradigm on the concept of standardization and therapeutic validation of herbal medicines may be required to address the issues, putting a major challenge to the scientific community.

DOCUMENTATION OF HERBAL MEDICAL KNOWLEDGE AND IPR

Plant materials have been used for alleviating human suffering from the very beginning of human civilization, and records of the use of plants are available since about 5000 years ago. Well documented books, monographs and pharmacopoeias are available in China (Anonymous, 2008c; Zhong, 1977; Gi, 1992) and India (Anonymous, 2008b; API, 1989; Kapoor, 2001; Anonymous, 2008d). However, situation in Americas (Stephen, 2007; UCLA, 2008) and Africa (Mills *et al.*, 2005) are different as there is no such established system of medicine as in Ayurveda, Unani and TCM. Thus, a lot more knowledge of herbal medicine is scattered around in communities, families, tribes and with local herbal medicine practitioners. Given the effects of modernization, much of the knowledge in this sector will be lost to the world unless documented early and made accessible to all the people.

Documentation of herbal medicine should involve documentation on the cultivation, harvesting and technologies involved, including plantation and processing methods; the prior validation of products used in herbal medicine; documentation of the properties of synthetic products identical with or related to the active constituent(s) of the medicine; the chemistry of herbs believed to be responsible for the activity; the results of any clinical trials carried out on the product and aspects of marketing and trading, and legal issues including intellectual property rights. This is an unenviable task as only a fraction of the hundreds of thousands of plant species has been fully investigated in the laboratory. In the West, the demand for herbal drugs, often derived from plants exported from developing countries, has been expanding in an unprecedented manner in recent years. The global

craving for herbal products has serious implications on the survival of many plant species and a large scale program of cultivation should be initiated to replace herbal products which are unsustainably collected at the behest of manufacturers and exporters of herbal medicine. Conservation and cultivation methods must be developed and the information documented.

Current Status

Although a considerable amount of documentation is available for herbal products in the formal sector, there is very little in the non-formal sector. Even in the formal sector, where pharmacopoeias are followed, the drug dispensed by an indigenous medical practitioner may often not follow the protocol specified in the available documentation. Variations in raw materials used are inevitable, given that the chemical contents of plants quite often vary in different cultivars, location, age, the part used, storage conditions, period of storage and drying. The efficacy of formulations depends on the manufacturing procedure, the concentration of the chemical constituents and the solvent/method for extraction. It is clear that chemical standardization is the way forward, if herbal remedies are to be widely used.

Chemistry and basic bioassay of medicinal plants used in herbal products, which is important for quality control, standardization and the development of new Western drugs, are available in scientific journals but are hardly compiled for easy reference. There is much less information on herbal dietary supplements which are now being accessed by large sections of the populations of developed countries. Claims, often with no real basis, are made for their efficacy. Manufacturers of dietary supplements should be encouraged to publish a pharmacopoeia-like "nutracopoeia" giving details on preparation, active constituents, efficacy studies, chemical and clinical data (if any) on nutraceuticals.

Databases

The present day position on documentation is that a number of databases on herbal medicines are available. But many of them are not freely accessible and some are not publicly available due to intellectual property concerns. Many databases are proprietary and provide information on indications, actions and active constituents to herbal medicine practitioners in the West, *e.g.* the Phytotherapies database (www.phytotherapies.org) and the Natural Medicines Comprehensive Database (www.naturaldatabase.com). Some of these databases claim to provide impartial evidence-based information, like the nonprofit HerbMed website (www.herbmed.org/HerbMed).

Some public and private databases are accessible and contain much information about individual plant materials and in some cases composite herbal drug as such. A commercial database (www.cintcm.com/index.htm) contains about 550,000 records of TCM. The Indian government is setting

up a Traditional Knowledge Digital Library, which would be a knowledge repository of Indian traditional knowledge in Ayurvedic, Unani and Siddha medicine and yoga (Van Dogen *et al.*, 2003). Some general databases contain information about herbs and herbal medicines. They include AGRICOLA of the US Department of Agriculture (www.agricola.nal.usda.gov), MICROMEDEX (www.micromedex.com/products/altmeddex) and CRISP (www.crisp.cit.nih.gov). The EMBASE database maintained by Elsevier (www.embase.com) maintains 18 million records covering MEDLINE records as well, collates information from over 5000 biomedical journals on medical and drug-related subjects. NAPRALERT, maintained by the College of Pharmacy, University of Illinois at Chicago, contains information on ethnomedicine and Natural Products published in journals. It has about 180,000 records which contain information on academic research carried out in pharmacology, biological activity, and chemistry of natural products. Information on NAPRALERT is available at its website (http://info.cas.org/ONLINE/DBBS/napralertss.html). ESCOP database (www.escop.com/phytonet.htm) maintained by the European Scientific Co-operative on Phytotherapy (ESCOP) also includes adverse effects on the use of herbal medicine. The International Bibliographic Information on Dietary Supplements (IBIDS) database (http://ods.od.nih.gov/Health_Information/IBIDS.aspx) specializes in dietary supplements.

Intellectual Property Rights (IPR)

One of the main problems of developing herbal medicine into therapeutic agents acceptable to modern scientific communities and societies is the secretiveness of the traditional medical knowledge held by individuals, families, tribes and communities. They fear that their knowledge will be stolen and used for financial gains. Modern techniques including biotechnology can be used to enhance yields of plant products from existing sources, to develop new sources for them and to design new products with better biological properties, but these require access to both biological resources and the traditional knowledge of indigenous communities. Biological resources are unevaluated and their habitats, usually in the developing countries, are at risk, leading to extinction and loss of valuable species. Traditional knowledge of local communities is also being rapidly lost, both through the loss of traditionally used biological resources by overexploitation and changes in the life styles of indigenous peoples.

During the past twenty-five years, the prominence given to intellectual property has convinced the owners of biological resources that these resources are valuable, as they are part of a national or community heritage and no longer the common heritage of the people of the world, and that the national or community heritage has to be protected from undue exploitation. The Convention on Biological Diversity (CBD) in 1992 provided an incentive to developing countries for the sustainable use of biodiversity. The CBD also created an environment which could facilitate

research on the biodiversity of developing countries by providing for access to genetic resources and the sharing of any benefits derived from their exploitation. Countries and communities owning biological resources were expected to obtain an equitable share of the profits and the transfer of the technology and know-how in return for permitting the exploitation of their genetic resources and associated traditional knowledge by other countries on mutually agreed terms. One hundred and eighty-eight states and the European Union have ratified the Convention so far.

Although most countries are members of the CBD, very few have introduced legislation in conformity with the Convention and there are few successful working models at present that ensure an equitable distribution of benefits accruing from exploitation of traditional knowledge. Part of the problem is that there are two powerful international fora other than the CBD dealing with these issues, the World Trade Organization (WTO) and the World Intellectual Property Organization (WIPO).

One of the problems facing developing countries in protecting their biodiversity from unethical exploitation is the absence of a mechanism to protect herbal medicines in the intellectual property rights regime which most developing countries have put or are putting into place to conform to WTO's Trade Related Intellectual Property Rights (TRIPS) Agreement. TRIPS does not permit the patenting of herbal medicines, only plants or compounds with new biological activities or the biological activity itself being considered patentable. The herbal medicine and its preparation cannot be patented as much of the knowledge involved is not novel and in the public domain. Although there is a general understanding that there should be a satisfactory mechanism for the protection of traditional knowledge and many suggestions have been made, none have achieved acceptance. It is generally held that successful protection will require changes to TRIPS.

Challenges

The available databases hardly provide comprehensive information on herbal medicine. Knowledge available in indigenous knowledge systems and in the non-formal sector regarding herbal medicine is often inaccessible and there is no straightforward mechanism to collect and record data on the plants and the processing and formulation methods used in herbal medicines. Although there is some documentation of herbal medicine in the formal sector; herbal medicines produced by individual medical practitioners or commercial organizations do not often conform to recipes recorded in national pharmacopoeia. The herbs used in the formulation may differ, the content of the active constituent varies widely and the processing methods are rarely uniform. Indigenous communities and family practitioners should be assured that their Intellectual Property Rights (IPR) will not be unfairly exploited. Existing IPR laws should be modified to permit the patenting of

new drugs developed from traditional herbal medicines in order to attract multinational pharmaceutical companies to the field and thereby increase research and development in herbal medicine. Although herbal medicines, prepared according to established pharmacopoeia like the Auyrveda, Unani or TCM cannot be patented, new composition and processes of herbal drugs have been patented (Hakky, 1989; Lee, 2004; Glaser, 2007).

Finally, it is desirable to establish a document database, containing information on each approved medicinal herb or herbal medicine. A central digital document database which is regularly updated containing this information with linkages to references in other databases should be established for easy access by all beneficiaries, producers and stakeholders. The knowledge base for a herb or herbal medicine, promoted for wider use, should be strengthened and expanded so that there is a sound scientific basis for each use. This would require presentation of information on individual single plants as well as for composite herbal medicine in a detailed format easily understood by all the stakeholders, the consumers, traditional medicine practitioners and herbal product manufacturers.

UTILIZATION OF PLANT MATERIAL

The biological resources are not evenly distributed around the world, and their utilization has therefore become possible due to work carried out by international teams of researchers. Such cooperation has in fact been a necessity to achieve utilization of bio-resources because some regions, which are rich in such resources, have not yet been in a position to extract the maximum benefit from those assets. An essential element of such cooperation is a just sharing of the benefits between the partners (properly) involved. This issue has been discussed internationally on several occasions and has subsequently been incorporated in several international declarations dealing with biological resources and biodiversity, for instance the Manila Declaration (February 1992), the Bukit Tinggi Declaration (October 1992), the Melaka Accord (June 1994), and the Phuket Declaration of November 1997 (IUPAC, 1998) The topic was also addressed at a broader international level at the United Nations Conference on Environment and Development held in Rio de Janeiro, June 1992, and has subsequently been incorporated in international law through the Convention on Biological Diversity.

Innovative and sustainable utilization of biological resources requires close co-operation between the main stakeholders in such an endeavour, *viz.*, governments, academia, and industry, in both the preservation and the harvest of the biological resources. Such co-operation can not be limited to the production processes; any activity that adds value to a biological resource should help to support its conservation by making its natural resource and its sustainable use better known. In order to achieve this, it is quite obvious that an increasing public awareness should be encouraged.

Analyses show that most significant value chains in modern societies are based on molecules and molecular systems with defined structures. Therefore, the chemical community has an important role to play in discussions of scientific, economic, and ethical issues that are associated with international cooperation involving molecules and molecular systems. IUPAC has met this expectation and in 2002 the Union published a so-called IUPAC Technical Report entitled *"Molecular Basis of Biodiversity, Conservation, and Sustained Innovative Utilization"* with a set of recommendations for global cooperation on sustainable prospecting for molecular systems and information at the molecular level derived from natural resources (Fischli *et al.,* 2002).

The recommendations detailed in the report were arrived at through an extensive and international process involving representatives of all the main groups of stakeholders. The discussions were based on the principles summarized in Box 1 and the outcome was a set of recommendations related to authorization of cooperation and consideration of the interests and obligations of cooperating partners.

Box 1. Excerpts from the preamble (Fischli *et al.*, 2002)

Recognizing the important ecological roles played by local and regional biodiversity.

Recognizing the contributions which the molecular diversity of natural products has made to the health and welfare of humankind.

Affirming their commitment to cooperate fairly and equitably with stakeholders for the benefit of humankind and the sustainable use of diversity at both the molecular and organism level.

Recognizing the sovereign rights of states over their own natural resources and the authority of national governments to determine access to biological and genetic resources, subject to national legislation.

Acknowledging the interests of other stakeholders from the country or from abroad, including indigenous and local communities and farmers, in natural resources and existing knowledge.

Determined to honour the spirit of international, regional, national, and sub national laws and policies concerning biological and molecular diversity as well as intellectual property rights.

Committed to ensure fair and equitable sharing of benefits arising from the sustainable utilization of natural resources.

Dedicated to the enhancement of the scientific and technological expertise and resources of developing countries.

The recommended guidelines for international cooperation on sustainable utilization of bio-resources are quite rigorous and it may be

asked if the recommendations will work in practice. Based on experience, in particular with projects involving National Cancer Institute (NCI) in Bethesda, Maryland, USA, it is quite reasonable to believe that the answer is yes (Baker *et al.*, 1995; Kashman *et al.*, 1992). Since the beginning of the 1990's NCI has carried out many sampling and screening projects based on principles similar to those put forward in the IUPAC technical report. Some examples of projects involving co-operation with partners in Asia are listed in Box 2, and it is promising to learn that the results from these and similar projects have been rather encouraging (Cragg *et al.*, 2000).

Box 2. Some examples of Asian research institutions involved in screening projects

Bangladesh: Bangladesh National Herbarium, Dhaka (1994).

Cambodia: Forest and Wildlife Research Institute, Department of Forestry and Wildlife, Phnom Penh (2000).

Laos: Research Institute of Medicinal Plants, Vientiane (1998).

Papua New Guinea: University of Papua New Guinea, Port Moresby (2001).

Philippines: Philippines National Museum, Manila (1992).

Sarawak, Malaysia: State Government of Sarawak: State Department of Forests (1994).

State Government of Sarawak: Sarawak Biodiversity Center (2002).

Vietnam: Institute of Ecology and Biological Resources, Hanoi (1997).

REFERENCES

Akerele, O. (1993). Summary of WHO guidelines for the assessment of herbal medicine. *Herbalgram*, **28**: 13-16.

Anonymous, (2006). *The Economist*, (25 October 2006). A catalogue of African Herbal Medicines. <http://www.economist.com/science/displaystory.cfm?story_id=7995196>.

Anonymous, (2008a). *Herbalism*. <http://en.wikipedia.org/wiki/Herbalism>.

Anonymous, (2008b). History of Ayurveda, *ayurvediccure.com* <http://www.ayurvedicure.com/ayurveda_ayurvedic_herbs/ayurveda_history.htm>.

Anonymous, (2008c). Traditional Chinese Medicine <http://en.wikipedia.org/wiki/Traditional_Chinese_medicine>.

Anonymous, (2008d). Medical Systems Unani.<http://www.webindia123.com/medicine/systems/unaniindex.htm>.

Anonymous, (2008e). Siddha system of medicine <http://www.sysindia.com/medical/aboutsiddha.html>.

API, (1989). *Ayurvedic Pharmacopoeia of India* (API), Department of Health, Ministry of Health and Family Welfare, Government of India. New Delhi.

Baker, J.T., Borris, R.P., Cordell, G.A., Soejarto, D.D., Cragg, G.M., Gupta, M.P., Iwu, M.M., Madulid, D.R. and Tyler, V.E. (1995). Natural product drug discovery and development: new perspectives on international collaboration. *J. Nat. Prod.*, **58**: 1325-1357.

Belton, P.S., Colquhoun, I.J., Kemsley, E.K., Delgadillo, I., Roma., P., Dennis, M.J., Sharman, M., Holmes, E., Nicholson, J.K. and Spraul, M. (1998). Application of chemometrics to the ^{1}H-NMR spectra of apple juices: discrimination between apple varieties. *Food Chemistry*, **61**: 207-213.

Bent, S. and Ko, R. (2004). Commonly used herbal medicine in the United States: a review. *Am J Medicine*, **116**: 478-485.

Bodeker, G. and Mumford, G. (*Eds.*) (2007). *Traditional, Complementary and Alternative Medicine: Policy and Public Health Perspectives*. Imperial College Press, London, U.K.

Brachet, A., Rudaz, S., Mateus, L., Christen, P. and Veuthey, J.-L. (2001). Optimisation of accelerated solvent extraction of cocaine and benzoylecgonine from coca leaves. *Journal of Separation Science*, **24**: 865-873.

British Medical Association, (1993). *Complimentary Medicine New Approaches to Good Practice*, Oxford, UK, Oxford University Press.

Brown, J.S. and Marcy, S.A. (1991). The use of botanicals for health purposes by members of a prepaid health plan. *Res. Nurs Health*, **14**: 339-350.

Brown, S. and Ede, R. (1996). Occult lead poisoning. *British J Hospit Med.*, **53**: 469-476.

Capasso, L. (1998). *'5300 years ago, the Ice man used natural laxatives and antibiotics'. Lancet*, **352**: 1424. PMID: 9851424.

Cassidy, C.M. (1998). Chinese medicine users in the United States. Part 1: Utilization, satisfaction, medical pleurality. *J. Altern. Complement. Med,* **4**: 17-28.

Chan, T.Y.K. and Critchley, J.A.J.H. (1996). Usage and adverse effects of Chinese herbal medicine. *Hum Exp Toxicol.*, **15**: 5-12.

Chan, T.Y.K., Chan, J.C.N., Tomlinson, B. and Critchley, J.A.J.H. (1993). Chinese herbal medicines revisited: a Hong Kong perspective. *Lancet*, **342**: 1532-1534.

Choi, H.K., Choi, Y.K., Verberne, M., Lefeber, A.W.M., Erkelens, C. and Verpoorte, R. (2004). Metabolic fingerprinting of wild type and transgenic tobacco plants by 1H NMR and multivariate analysis technique. *Phytochemistry,* **65**: 857-864.

Cragg, G.M., Boyd, M.R., Hallock, Y.F., Newman, D.J., Sausville, E.A. and Wolpert, M.K. (2000). Natural Product Drug Discovery at the National Cancer Institute. Past Achievements and New Directions for the New Millenium. *In*: Wrigley, S.K., Hayes, M.A., Thomas, R., Chrystal, E.J.T. and Nicholson, N. (*Eds.*), *Biodiversity, New Leads for the Pharmaceutical and Agrochemical Industries*, Royal Society of Chemistry, London, UK.

De Smet, P.A.G.M., De Smet, P.A.G.M., Keller, K., Hansel, R. and Chandler, R.F. (*Editors*) (1992). *Adverse effects of herbal drugs*, Berlin: Springer-Verlag; *Aristolochia* Species, pp. 79-89.

EMEA, (2001). *Note for guidance on specifications: test procedures and acceptance criteria for herbal drugs, herbal drug preparations and herbal medicinal products, In*: CPMP/QWP/2820/00 (EMEA/CVMP/815/00).

EMEA, (2006). *Guideline on specifications: test procedures and acceptance criteria for herbal drugs, herbal drug preparations and herbal medicinal products/traditional herbal medicinal products*, EMEA, London, UK, EMEA/CPMP/QWP/2820/00 Rev 1 and (EMEA/CVMP/815/00), Rev 1.

EnHealth, (2002). Environmental Health Risk Assessment: Guidelines for assessing human health risks from environmental hazards. *Department of Health and Aged Care and Health Council*: Canberra.

Ernst, E. (2001). Heavy metals in traditional Chinese medicines: a systematic review. *Clin. Pharmacol. Ther.*, **70**: 497-504.

Ernst, E. (2002). Adulteration of Chinese herbal medicines with synthetic drugs. *J Intern. Med.*, **252**: 107-113.

Fischli, A.E., Pandit, U.K. and Black, D.StC. (2002). Molecular basis of biodiversity. conservation, and sustained innovative utilization. *Pure Appl Chem.*, **74**: 697-702.

Fletcher, R.H. and Fletcher, S.W. (1979). Clinical research in general medical journals: a 30-year perspective. *New Engl J Med.*, **301**: 180-183.

Frederich, M., Choi, Y.H., Angenot, L., Harnischfeger, G., Lefeber, A.W.M. and Verpoorte, R. (2004). Metabolomic analysis of *Strychnos nux-vomica*, *Strychnos icaja* and *Strychnos ignatii* extracts by ^{1}H nuclear magnetic resonance spectrometry and multivariate analysis techniques. *Phytochemistry,* **65**: 1993-2001.

Gi, T. (*Ed.*) (1992). *Pharmacopoeia of the Peoples' Republic of China*, (English Edition), Guangdon Science and Technology Press, Beijing,.

Glaser, R. (2007). US patent 20070059384. <http://www.freepatentsonline.com/20070059384.html>.

Guliyeva, V.B., Gulb, M. and Yildirima, A. (2004). *Hippophae rhamnoides* L.: chromatographic methods to determine chemical composition, use in traditional medicine and pharmacological effects. *J. Chromtogr.* **B, 812**: 291-307.

Hakky, S.I. (1989). US patent 4826684. <http://www.freepatents.com/4826684.html>.

Hennekens, C.H. and Buring, J.E. (1987). Design Strategies in Epidemiologic Research, *In*: Mayrent, S.L. (*Editor*): *Epidemiology in Medicine*. Lippincot Williams & Wilkins, Philadelphia, PA.

Hostettmann, K. and Wolfender, J.L. (1999). *In*: *Advances in Plant Glycosides, Chemistry and Biology*. *Edited* by Yang, C.R. and Tanaka, O.E., Amsterdam: Elsevier; pp. 233.

Huffman, M.A. (2003). Animal self-medication and ethno-medicine: exploration and exploitation of the medicinal properties of plants. *Proc Nutr Soc.*, **62**: 371-381, PMID: 14506884.

Huie, C.W. (2002). A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Analytical and Bioanalytical Chemistry*, **373**: 23-30.

Hutchings, M.R., Athanasiadou, S., Kyriazakis, I. and Gordon I.J. (2003). Can animals use foraging behavior to combat parasites. *Proc Nutr Soc.*, **62**: 361-370, PMID: 14506883.

ICDRA, (1996). 8th *International Conference on Drug Regulatory Authorities*. WHO, Bahrain, pp. 10-13.

ICDRA, (1991). 6th *International Conference on Drug Regulatory Authorities*. WHO.

IUPAC, (1998). The Phuket Declaration concerning the conservation and sustainable utilization of biological diversity. *Pure Appl. Chem.*, pp. 2065-2145.

Izhaki, I. (2002). Emodin - a secondary metabolite with multiple ecological functions in higher plants. *New Phytologist*, **155**: 205-217.

Jimenez-Carmona, M.M., Ubera, J.L. and Luque de Castro, M.D. (1998). Comparison of continuous subcritical water extraction and hydrodistillation of marjoram essential oil. *Journal of Chromatography, A,* **855**: 625-632.

Kapoor, L.D. (2001). *CRC Handbook of Ayurvedic Medicinal Plants*, Herbal Reference Library, Boca Raton, FL, USA, CRC Press.

Kaptchuk, T.J. (1995). Acute hepatitis associated with Jin Bu Huan. *Ann Intern Med.*, **122**: 636.

Kashman, Y. Gustafson, K.R., Fuller, R.W., Cardellina, J.H., McMahon, H.J.B., Currens, M.J., Buckheit, R.W., Hughes, S.H., Cragg, G.M. and Boyd, M.R. (1992). *J Med. Chem.,* **35**: 2735-2743.

Kaufmann, B., Rudaz, S., Cherkaoui, S., Veuthey, J.L. and Christen, P. (2007). Influence of plant matrix on microwave-assisted extraction process. The case of diosgenin extracted from fenugreek (*Trigonella foenum-graecum* L.). *Phytochemical Analysis,* **18**: 70-76.

Kessler, R.C., Davis, R.V., Foster, D.A., Van Rompay, M.I., Walters, E.E., Wilkey, S.A., Kaptchuk, T.J. and Eisenberg, D.M. (2001). Long-term trends in the use of complementary and alternative medical therapies in the United States. *Ann Intern Med.*, **135**: 262-268.

Lazarou, J., Pomeranz, B.H. and Corey, P.N. (1998). Incidence of adverse drug reactions in hospitalized patients, A meta-analysis of prospective studies. *J Am Med Assn.*, **279**: 1200-1205.

Lee, L. (1999). Introducing herbal medicine into conventional healthcare settings. *Journal of Nurse-Midwifery*, **44**: 253-266.

Lee, S.Y. (2004). US patent 66927777. <http://www.patentstorm.us/patents/66927777.html>.

MacLennan, A.H., Wilson, D.H. and Taylor, A.W. (2002). The escalating cost and prevalence of alternative medicine. *Prev Med.,* **35**: 166-173.

Mills, E., Cooper, C., Seely, D. and Kanfer, I. (2005). African herbal medicines in the treatment of HIV: hypoxis and sutherlandia, an overview of evidence and pharmacology. *Nutr J.*, <javascript:AL_get(this,%20'jour',%20'Nutr%20J.');> **4**: 19. PMID: 15927053.

Mitchell-Heggs, C.A., Conway, M. and Cassar, J. (1990). Herbal medicine as a cause of combined lead and arsenic poisoning. *Hum Exp Toxicol.*, **9**: 195-196.

Moerman, D.E. (1998). North American Ethnobotany, Timber Press, Portland, Oregon, USA. <http://www.timberpress.com/books/isbn.cfm/9780881924534/>.

Mosihuzzaman, M. and Choudhary, M.I. (2008). Protocols on safety, efficacy, standardization and documentation of herbal medicine: IUPAC Technical Report. *Pure and Applied Chemistry*, **80**: 2195-2230.

Park, G.J., Mann, S.P. and Ngu, M.C. (2001). Acute hepatitis induced by Shou-Wu-Pian, a herbal product derived from *Polygonum multiforum. J. Gastroenterol Hepatol.*, **16**: 115-117.

Plantadosis, S. (2005). *In*: *Clinical Trials-A Methodologic Perspective. Editor*: Plantadosis, S, Wiley-Interscience, New Jersey.

PRC, (2000). *Pharmacopoeia of the People's Republic of China, English ed.* Beijing: The Pharmacopeia Commission of PRC.

Rotblatt, M. and Ziment, I. (2002). *Evidence-based herbal medicine*. Henley and Belfus Inc., Philadelphia, PA.

Sherman, P.W. and Billing, J. (1998). Antimicrobial functions of spices: Why some like it hot. *Q. Rev. Biol.*, **73**: 3-49. PMID: 9586227.

Sherman, P.W. and Hash, G.A. (2001). Why vegetable recipes are not so spicy. *Evol. Hum. Behav.*, **22**: 147-163. PMID: 11384883.

Shia, G., Noller, B. and Burford, G. (2007). *Safety Issues and policy. In*: *Traditional, Complementary and Alternative Medicine: Policy and Public Health Perspectives. (Eds.* Bodeker, G. & Burford, G.). Imperial College Press, London, Chap. 4, pp. 83-98.

Sim, M.R. and McNeil, J.J. (1999). *Med. J. Austral.*, **170**: 372-374.

Stephen, H.B. (2007). Sacred Plants: Native American Herbal Medicine. *Native Peoples Magazine.* <http://www.nativepeoples.com/article/articles/243/1/Sacred-Plants:-Native-American-Herbal-Medicine>

TDR, (2005). *Operational guidance*: Information needed to support clinical trials of herbal products. UNICEF/UNDP/World Bank/WHO Special Programme for research and training in Tropical Diseases (TDR).

TGA, (2004). *Therapeutic Goods Administration* (Australia), www/health.gov.au/-tga/complementarymedicines.

Tyler, V.E. (1997). The Complete German Commission E Monographs. *American Botanical Council*, Boston.

UCLA, (2008). University of California, Los Angeles, Online Archive of American Folkmedicine. <http://folkmed.ucla.edu>.

Ulrich-Merzenich, G., Zeitler, H., Jobst, D., Panek, D., Vetter, H. and Wagner, H. (2007). Application of the "-Omic-" technologies in phytomedicine. *Phytomedicine*, **14**: 70-82.

USEPA, (1998). *Guidelines for ecological risk assessment*; U.S. Environmental Protection Agency, Washington, DC.

USFDA, (2000). *Guidance for industry, botanical drug products, draft guidance*, CDER/USFDA.

USP, (2002). *United States Pharmacopeia and National Formulary*, USP 25, NF 19, Rockville: United States Pharmacopeial Convention Inc.

Van Dongen, M., Van Rossum, E., Kessels, A., Seilhorst, H. and Knipschild, P. (2003). Ginkgo for elderly people with dementia and age-associated memory impairment: a randomized clinical trial. *Journal of Clinical Epidemiology*, **56**: 367-376.

Vanherwegham, L.J. (1998). Misuse of herbal remedies: the case of an outbreak of terminal renal failure in Belgium (Chinese herbs nephropathy). *J Altern Complement Med.*, **4**: 9-13.

Vanherweghem, J.L., Depierreux, M., Tielemans, C., Abramowicz, D., Dratwa, M., Jadoul, M., Richard, C., Vandervelde, D., Verbeelen, D. and Vanhaelen-Fastre, R. (1993). Rapidly progressive interstitial renal fibrosis in young women: association with slimming regimen including Chinese herbs. *Lancet*, **341**: 387-391.

Wagner, H. (2001). Trends and challenges in phytomedicine: research in the new millennium. *In*: *Handbook of Medicinal Plants. Edited* by Yaniv, Z. and Bachrach, U.E., Bindhamton (UK): Haworth Medical Press, Inc. pp. 3-28 (chapter 21).

WHO, (1999). *WHO Monographs of Selected Medicinal Plants. Vol. 1*, WHO, Geneva, Switzerland.

WHO, (2002a). Traditional Medicine Strategy 2002-2005. *In*: <http://www.who.int/medicines/library/trm/trm_stat_eng.pdfS>.

WHO, (2002b). *WHO Monographs of Selected Medicinal Plants. Vol. 2*, WHO, Geneva, Switzerland.

WHO, (2003). *World Health Organization Fact Sheet # 134*, revised May, 2003. <http://www.who.int/mediacentre/factsheets/fs134/en/print.html>.

Wijiesekera, R.O.B. (*Ed.*) (1991). *The medicinal Plant Industry*, CRC Press, Inc., Boca Raton, Florida.

Williamson, E.M. (2001). Synergy and other interactions in phytomedicines. *Phytomedicine*, **8**: 401-409.

Winslow, L.C. and Kroll, D.J. (1998). Herbs as medicines. *Arch Intern Med.*, **158**: 2192-2199.

Woolf, G.M., Petrovich, L.M., Rojter, S.E., Wainwright, S., Villamil, F.G., Katkov, W.N., Michieletti, P., Wanless, I.R., Stermitz, F.R., Beck, J.J. and Vierling, J.M. (1994). Acute hepatitis associated with Chinese herbal product Jin Bu Huan. *Ann Intern Med.*, **121**: 729-735.

Zhong, Y.D.C.D. (1977). *Encyclopedia of Chinese Materia Medica, Vol. 1-3,* Shanghai Scientific Publication, Shanghai.

13

Anticancer Properties of Plant-Derived Food, Phytochemicals and Plant-Expressed Recombinant Pharmaceuticals

KUMAR V.L.[1*] AND KUMAR V.[2]

ABSTRACT

Plant derived dietary constituents are well known to prevent chronic diseases including cancer. Phytochemicals are non-nutrient bioactive compounds of plant origin that include alkaloids, flavonoids, phytosteroids, carotenoids, lycopenes and indoles. The chemopreventive potential of some of these compounds has been demonstrated both in vitro cell culture and in preclinical models of cancer. These compounds have been demonstrated to prevent initiation, proliferation and/or progression of cancer. At the molecular level these compounds have been shown to interfere with aberrant signaling cascades involved in cancerous growth by way of detoxification and enhanced excretion of carcinogens, suppression of inflammatory processes, inhibition of mitosis and/or induction of apoptosis. These phytochemicals show synergy with chemotherapy and radiotherapy and have the potential to be used as complementary and alternative medicine. Besides a rich source of phytochemicals, plants are now being used as a biofermenter for large scale production of recombinant pharmaceuticals like antibodies, interleukins and viral antigens to meet their ever growing demand in cancer prevention and therapy. This review gives an overview of pharmaceuticals

1. Department of Pharmacology, All India Institute of Medical Sciences, Ansari Nagar New Delhi - 110 029, India.
2. Virology Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi - 110 067, India.
* *Corresponding author* : E-mail : kumarvl98@hotmail.com; kumarvl98@gmail.com

both derived from plants as well as expressed in plants for their use in cancer therapeutics.

Key words : Herbal Products, herbal medicine, philosophy of herbal medicine, safety, contamination, adulteration, adverse effects, efficacy, standardization, fingerprinting, documentation, intellectual property rights

INTRODUCTION

Cancer is one of the most prevalent diseases worldwide which accounts for 13% of all deaths every year (Pujol *et al.*, 2007). It is a major health problem in developed countries and its incidence is increasing in developing countries as well due to a changing lifestyle including dietary habits. The use of plant-based medicines for cancer prevention has been mentioned in Ayurveda, a traditional medicinal system practiced in India (Gardia *et al.*, 2007). The root extract of the plant *Podophyllum peltatum* has been used by Penobscot Indians of Maine for the treatment of a condition believed to be cancer and the anticancer properties of this plant have been mentioned in the Materia Medica and Therapeutics published in 1849 (Kost, 1849). The root extract of this plant contains podophyllotoxin that has been derivatized to obtain chemotherapeutic drugs etoposide and teniposide. Similarly, alkaloids exhibiting anticancer properties, vinblastin and vincristine, have been derived from the plant *Vinca rosea* (Van Der Heijden *et al.*, 2004). A survey of literature and folklore conducted by Hartwell revealed that more than 3000 different species of plants from all over the world exhibit reputed efficacy in cancer (Hartwell, 1976). A large scale screening program was initiated by National Cancer Institute in 1960's to identify active compounds of plant origin that led to the discovery of paclitaxel from the bark of the Pacific yew tree, *Taxus brevifolia* (Zubrod, 1984).

It has been well known that populations that have relied on plant based diet have lower risk of certain cancers like that of breast, colon, lung and prostate (World Cancer Research Fund, 1997). It is important to note that 75% of colorectal cancers could be prevented by increasing the amount of plant-based food in the diet (Thomson *et al.*, 2003). According to World Cancer Research Fund (1997), consumption of fruits and green vegetables is associated with reduced breast cancer risk. Besides, several other epidemiological studies also suggest that consumption of foods of plant origin such as fruits, vegetables, whole grains, legumes, nuts, seeds and tea decrease the risk of developing various cancers (World Cancer Research Fund, 1997; Gescher *et al.*, 1998). Several herbs and plant derived food items that are either included as one of the constituents of food or consumed are known to possess anti-cancer properties (Table 1, 2). In view of the well recognized beneficial role of plants, the National Cancer Institute has

set up dietetics guidelines for the prevention of cancer that includes fruits and vegetables in the diet (Butrum *et al.*, 1988). Such food items are not only a good source of vitamins, minerals and fiber, but they also contain non-nutritional components known as phytochemicals that are biologically active. The medicinal properties of these phytochemicals are well recognized. Some of the families of phytochemicals include alkaloids, flavonoids, isoflavones, isothyiocyanates, capsaicinoids and phytosterols. Phytochemicals well known for their anticancer properties are listed in Table 3. They have been shown to inhibit carcinogenesis through multiple mechanisms that may affect the functions of immune system, levels of various hormones, pharmacokinetics of various chemotherapeutic agents and cell cycle. Structures of some of the plant derived products are shown in Fig 1 and products that have shown promising results and have been well studied are described below:

Table 1. Herbs known for their cancer-preventing properties (Craig, 1999)

Plant family	Example
Liliaceae	Garlic, Onion, Chive
Labiatae	Basil, Mint, Oregano, Rosemary, Sage, Thyme
Zingiberaceae	Turmeric, Ginger
Umbelliferae	Anise, Caraway, Celery, Chervil, Cilantro, Coriander, Cumin, Dill, Fennel, Parsley

Table 2. Plant based food item known to exhibit anticancer properties

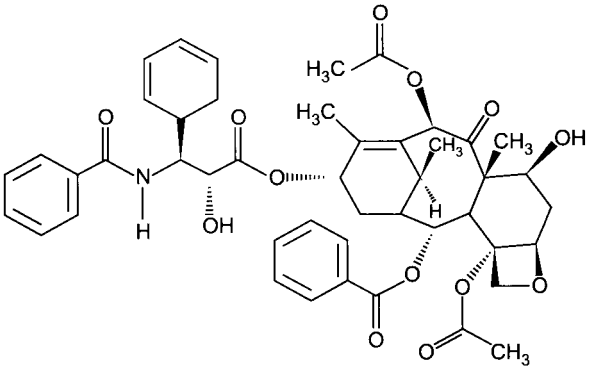
Food item	Cancer	Reference(s)
Soya	Prostate cancer	Herbert *et al.*, 1998
	Colon cancer	Billings *et al.*, 1990
	Lung metastasis of melanoma	Li *et al.*, 1999
Green tea	Esophageal cancer	Weisburger *et al.*, 1998
	Colon cancer	Wang *et al.*, 2007
	Skin cancer	Gensler *et al.*, 1996
Red grapes	Skin cancer	Chatterjee *et al.*, 1999
	Mammary cancer	Jang *et al.*, 1997
Turmeric	Colon cancer	Huang *et al.*, 1988

Table 3. Dietary phytochemicals, their sources and efficacy in cancer

Phytochemical	Source	Efficacy	Reference(s)
Curcumin	Turmeric	Colon Cancer Pancreatic cancer	Goel *et al.*, 2008
Genistein	Soy	Prostate cancer	Zhang *et al.*, 2008
Resveratrol	Grapes, berries	Prostate cancer	Wang *et al.*, 2008
Allicin	Garlic	Gastric cancer	Sun & Wang, 2003
Lycopene	Tomatoes	Prostate cancer	Dahan *et al.*, 2008
Diosgenin	Fenugreek	Breast cancer	Chiang *et al.*, 2007

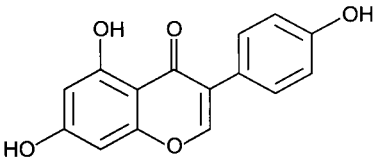
Table 3. *Contd.*

Phytochemical	Source	Efficacy	Reference(s)
[6]-Gingerol	Ginger	Skin cancer	Kim *et al.*, 2005
Ellagic acid	Berries	Pancreatic cancer	Edderkaoul *et al.*, 2008
Eugenol	Clove	Melanoma	Ghosh *et al.*, 2005
Isothiocyanates	Cruciferous	Colorectal cancer Vegetables	Moy *et al.*, 2008
Isoflavones	Soy	Colon cancer	MacDonald *et al.*, 2005
Phytosterols	Vegetable oils, nuts Legumes	Colon, breast and prostate cancer	Bradford & Awad, 2007
Folic acid	Leafy vegetables	Colorectal and Cervical cancer	Powers, 2005
Epigallocatechin gallate	Green tea	Pancreatic cancer	Shankar *et al.*, 2008



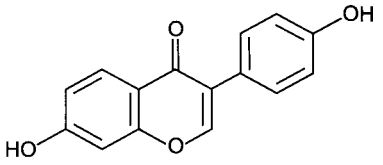
Paclitaxel

($C_{47}H_{51}NO_{14}$; MW 853.92)



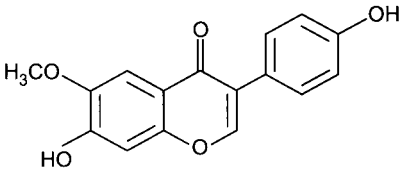
Genistein

($C_{15}H_{10}O_5$; MW 270.2)



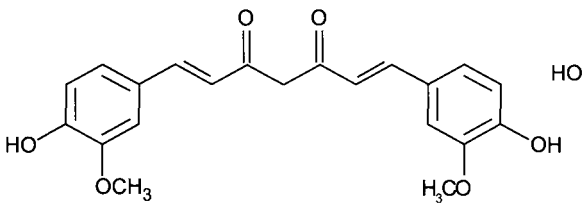
Daidzein

($C_{15}H_{10}O_4$; MW 254.24)

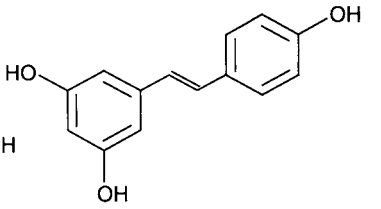


Glycitein

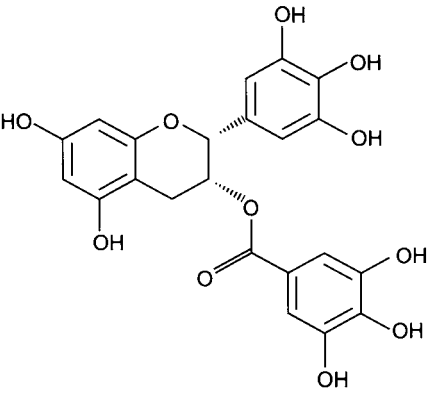
($C_{16}H_{12}O_5$; MW 284 26)



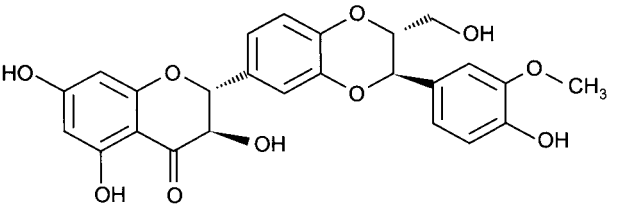
Curcumin
($C_{21}H_{20}O_6$; MW 368.39)



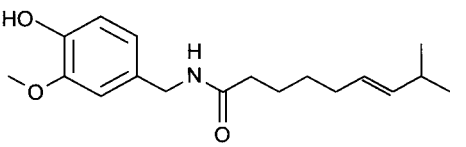
Resveratrol
($C_{14}H_{12}O_6$; MW 228.24)



Epigallocatechin gallate
($C_{22}H_{18}O_{11}$; MW 458.4)



Silybin
($C_{18}H_{22}O_{10}$; MW 482.44)



Capsaicin
($C_{18}H_{27}NO_3$; MW 305.42)

Fig 1. Structure and molecular mass of some phytochemicals with anti-cancer properties

SOY

Seeds of Soy plant, *Glycine max* are rich source of protein. They also contain lipids, glucides, vitamins, minerals, fibres, saponins and isoflavones. The non-steroidal isoflavones of soy include genistein, daidzein and glycitein and have been shown to exhibit cancer preventing activity. Soy rich diet has been shown to reduce the risk of certain cancers both in laboratory animals and in humans (Hebert *et al.*, 1998; Billings *et al.*, 1990; Li *et al.*, 1999; Aronson *et al.*, 1999). One of the phytoestrogens of soy namely, genistein has been shown to afford protection from lung metastasis of melanoma, breast cancer and to inhibit cell growth in an orthotopic human bladder tumor model (Li *et al.*, 1999; Vantyghem *et al.*, 2005; Singh *et al.*, 2006). It is known for its antioxidant properties, its ability to inhibit arachidonic acid metabolism, modulate cellular integration signal, inhibit hormonal activity, neoplastic cell growth and oncogenesis (Divisi *et al.* 2006). It has been shown to inhibit cancer cell growth through mechanisms that involve inhibition of protein tyrosine kinase, DNA topoisomerase, angiogenesis, cell differentiation and apoptosis (Watanabe *et al.*, 1989).

CURCUMIN

Curcumin, a polyphenol obtained from the rhizomes of *Curcuma longa*, is well known for its medicinal properties. It has also been shown to exhibit potent antioxidant and anti-inflammatory properties (Biswas *et al.*, 2005). Besides, several *in vitro* and *in vivo* studies have demonstrated its antitumor and chemopreventive properties where it has been shown to suppress proliferation of cancerous cells (Aggarwal *et al.*, 2007; Shishodia *et al.*, 2005). Clinical trials have revealed that curcumin is well tolerated and it may produce antitumor effects in individuals with precancerous lesions (López-Lázaro, 2008). Various mechanisms that have been proposed for these activities include down regulation of expression of cyclooxygenase, matrix metalloprotease-9, growth factors, chemokines, transcription factors and inhibition of kinases involved in signaling cascade (Deorukhkar *et al.*, 2007). At high doses curcumin enhances G2/M cell cycle arrest, induces S phase arrest and apoptosis (Chauhan, 2002).

RESVERATROL

Resveratrol, trans-3,5,4'-trihydroxystilbene, a phytoalexin present in abundance in grapes (*Vitis vinifera*), berries, peanuts and various other plants, has been shown to exhibit biological activities including anticancer properties in various animal models (Baur & Sinclair, 2006). It interferes with tumor initiation, promotion and progression. Various mechanisms that have been proposed for its anticancer properties include modulation of NF-κB, inhibition of cyclooxygenase and induction of apoptosis and cyclin

dependent kinases. It also possesses antioxidant and antiangiogenic properties and inhibits invasion and metastasis (Aggarwal *et al.*, 2004).

EPIGALLOCATECHIN GALLATE (EGCG)

Green tea polyphenols are well known for their therapeutic effects. EGCG is the most abundant catechin in the leaves of tea plant, *Camellia sinensis*. It has been shown to induce apoptosis and cell cycle arrest in many cancer cells and affect cell signaling pathways both *in vitro* and *in vivo* (Ahmad *et al.*, 1997; Khan *et al.*, 2006; Yusuf *et al.*, 2007). It brings about inhibition of mitogen activated protein kinases, growth factor related cell signaling, activation of activator protein (AP-1), NFκB, topoisomerases and metalloproteinases (Chen & Zhang, 2007). It enhances the expression of CDKI proteins, down-regulates cyclin D1, cyclin E, CDK2 and CDK4 and causes growth arrest at G1 stage of cell cycle (Gupta *et al.*, 2000; Shankar *et al.*, 2007). Clinical trials on green tea polyphenols have shown encouraging results in prostate cancer patients (Khan & Mukhtar, 2008).

SILYMARIN

Silymarin, a flavolignan comprising of silybin, silydianin and silychristine, is obtained from the plant *Silybum marianum* commonly known as milk thistle. It is known for its efficacy in treating liver and gall bladder diseases (Pradhan & Girish, 2006). It has been shown to suppress the proliferation of variety of tumor cells through cell cycle arrest at G1/S phase, induction of cyclin dependent kinase inhibitors, down-regulation of anti-apoptotic gene products, inhibition of cell survival kinases and inhibition of inflammatory transcription factors. Further, it also down-regulates gene products involved in the tumor cell proliferation, invasion, angiogenesis and metastasis (Agarwal *et al.*, 2006).

Some of the other plant derived phytochemicals that have been shown to exhibit anticancer properties include [6]-gingerol, capsaicin, flavopiridol and emodin (Kim *et al.*, 2004; Bhutani *et al.*, 2007; Takada & Aggarwal, 2004; Muto *et al.*, 2007).

SENSITIZATION TO ANTICANCER TREATMENT

Plant derived phytochemicals not only exhibit chemopreventive properties, they have been demonstrated to act as chemosensitizers and enhance the efficacy of chemotherapy (Table 4). Genistein combined with cisplatin, doxorubicin or etoposide enhances their antiproliferative effect and induces apoptosis in EGF-R expressing lung cancer cells (Lei *et al.*, 1999). Similarly pretreatment of lung cancer cells with resveratrol enhances the anti-proliferative effect of paclitaxel by inducing apoptosis (Kubota *et al.*, 2003). Further, natural products may also abrogate pro-survival signaling

pathways in cancer cells that have escaped the cytotoxic effect of chemotherapy. Such an effect has been demonstrated for curcumin and emodin (Hour *et al.*, 2002; Yi *et al.*, 2004). Besides chemosensitization, the phytochemicals have also been shown to produce radiosensitization. In this regard it has been shown that combining radiation therapy with genistein significantly inhibits the growth and colony formation in prostate cancer cell lines *in vitro* and growth of primary tumor and lymph node metastasis in orthotopic prostate carcinoma model (Hillman *et al.*, 2001; Hillman *et al.*, 2004). Such a synergy has been attributed to inhibition of NF-κB, altered expression of cell-cycle proteins and G2/M arrest (Raffoul *et al.*, 2006). Phytochemicals like curcumin, flavopiridol and resveratrol are also known for their radiosensitizing effect (Li *et al.*, 2007; Zoberi *et al.*, 2002; Jung *et al.*, 2003) (Table 4).

Table 4. Phytochemicals exhibiting anticancer properties and synergism with chemotherapeutic agents

Phytochemical	Source	Monotherapy/ combination therapy/ sensitization	Reference(s)
Genistein	Soya	Cisplatin, docetaxel	Banerjee *et al.*, 2005
		doxorubicin,	Li *et al.*, 2005
		gemcitabine CHOP	Li *et al.*, 2004
		(cyclophosphamide,	Mohammad *et al.*, 2003
		doxorubicin,	Hwang *et al.*, 2005
		vinblastine,	Satoh *et al.*, 2003
		prednisone)	
		5-FU, adriamycin	Tanos *et al.*, 2002
		tamoxifen	Lee *et al.*, 2004
			Khoshyomm *et al.*, 2000
		Bleomycin	Khoshyomm *et al.*, 2002
			Hillman *et al.*, 2001
		Cisplatin	Yashar *et al.*, 2005
		Radiotherapy	
Phenoxodiol (analog of genistein)	Soya	Monotherapy	Constantinou *et al.*, 2003
			Alvero *et al.*, 2006
		Combination therapy	Kamsteeg *et al.*, 2003
			Sapi *et al.*, 2004
Curcumin	Turmeric	Monotherapy	Pereira *et al.*, 1996
			Li *et al.*, 2002
		Cispatin, doxorubicin	Notarbartolo *et al.*, 2005
		taxol	Bava *et al.*, 2005
			Kunnumakkara *et al.*, 2007
		Gemcitabine	Li *et al.*, 2007
		Radiation therapy	

Table 4. *Contd.*

Phytochemical	Source	Monotherapy/ combination therapy/ sensitization	Reference(s)
Epigallocatechin gallate	Tea	Monotherapy	Taniguchi *et al.*, 1992 Chisholm *et al.*, 2004
		Tamoxifen Doxorubicin	Zhang *et al.*, 2004
Resveratrol	Grapes	Monotherapy	Aggarwal *et al.*, 2004 Jazirehi & Bonavida, 2004
		Paclitaxel Radiotherapy	Zoberi *et al.*, 2002
Proanthocyanidine	Grapes	Monotherapy	Martinez *et al.*, 2005 Zhang *et al.*, 2005
		Doxorubicin	Sharma *et al.*, 2004
Isothiocyanates (ITC)	Cruciferous vegetables	Cisplatin, tamoxifen	Cover *et al.*, 1999 Sarkar & Li, 2004
PhenethylITC	Cruciferous vegetables	Monotherapy	Stoner *et al.*, 1991 Hecht *et al.*, 2000
Sulforaphane	Cruciferous vegetables	Monotherapy	Chaudhuri *et al.*, 2007

PLANT MADE RECOMBINANT PHARMACEUTICALS

Plants have also been used as an alternative mean to produce large quantities of pharmaceuticals to meet the growing demand for anticancer agents. Genetic engineering has made it possible to use plants for large scale production of proteins, antibodies (plantibodies), vaccines and other therapeutic agents that include hormones, antisense oligonucleotides and immunomodulators. Not only they can perform eukaryotic post-translational modification and produce active proteins, the proteins produced by them are devoid of human pathogens and endotoxins. Plant expression system has been utilized to produce several antibodies which include anti-EGF-R antibodies Cetuximab (Erbitux) and Nimotuzumab (Thera CIM) that have been expressed in corn and *Nicotiana* respectively (Ludwig *et al.*, 2004; Rodriguez *et al.*, 2005). Other anticancer pharmaceuticals expressed in plants include human papillomavirus antigen and interleukins. In one of the studies, plant expressed human papillomavirus vaccine has been shown to afford protection from tumor development in mice (Pujol *et al.*, 2007). Further, the plant expressed protein was found to be more effective than its *E. coli* produced counterpart protein as a therapeutic agent in mice (Massa *et al.*, 2007). The efficacy of some of the plant expressed pharmaceutical in treating cancer is being evaluated in several clinical trials (Pujol *et al.*, 2007).

Thus, plants are not only good source of naturally synthesized phytochemicals, they can be engineered to serve as bioreactors for large scale production of pharmaceuticals exhibiting efficacy in various diseases including cancers. The development of standardized formulations/ preparations from plants, establishing their safety and efficacy by conducting well designed clinical trials are some of the important issues in developing drugs effective in various cancers.

REFERENCES

Agarwal, R., Agarwal, C., Ichikawa, H., Singh, R.P. and Aggarwal, B.B. (2006). Anticancer potential of silymarin: from bench to bed side. *Anticancer Research*, **26**: 4457-4498.

Aggarwal, B.B., Bhardwaj, A., Aggarwal, R.S., Seeram, N.P., Shishodia, S. and Takada, Y. (2004). Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. *Anticancer Research*, **24**: 2783-2840.

Aggarwal, B.B., Shishodia, S. and Young-Joon, S. (2007). The molecular targets and therapeutic uses of curcumin in health and disease. Springer publishers, New York, USA.

Ahmad, N., Feyes, D.K., Nieminen, A.L., Agarwal, R. and Mukhtar, H. (1997). Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *Journal of the National Cancer Institute*, **89**: 1881-1886.

Alvero, A.B., O'Malley, D., Brown, D., Kelly, G., Garg, M., Chen, W., Rutherford, T. and Mor, G. (2006). Molecular mechanism of phenoxodiol-induced apoptosis in ovarian carcinoma cells. *Cancer*, **106**: 599-608.

Aronson, W.J., Tymchuk, C.N., Elashoff, R.M., McBride, W.H., McLean, C., Wang, H. and Heber, D. (1999). Decreased growth of human prostate LNCaP tumors in SCID mice fed a low-fat, soy protein diet with isoflavones. *Nutrition and Cancer*, **35**: 130-136.

Banerjee, S., Zhang, Y., Ali, S., Bhuiyan, M., Wang, Z., Chiao, P.J., Philip, P.A., Abbruzzese, J. and Sarkar, F.H. (2005). Molecular evidence for increased antitumor activity of gemcitabine by genistein *in vitro* and *in vivo* using an orthotopic model of pancreatic cancer. *Cancer Research*, **65**: 9064-9072.

Baur, J.A. and Sinclair, D.A. (2006). Therapeutic potential of resveratrol: the *in vivo* evidence. *Nature reviews. Drug Discovery*, **5**: 493-506.

Bava, S.V., Puliappadamba, V.T., Deepti, A., Nair, A., Karunagaran, D. and Anto, R.J. (2005). Sensitization of taxol-induced apoptosis by curcumin involves down-regulation of nuclear factor-kappaB and the serine/threonine kinase Akt and is independent of tubulin polymerization. *The Journal of Biological Chemistry*, **280**: 6301-6308.

Bhutani, M., Pathak, A.K., Nair, A.S., Kunnumakkara, A.B., Guha, S., Sethi, G. and Aggarwal, B.B. (2007). Capsaicin is a novel blocker of constitutive and interleukin-6-inducible STAT3 activation. *Clinical Cancer Research*, **13**: 3024-3032.

Billings, P.C., Newborne, P.M. and Kennedy, A.R. (1990). Protease inhibitor suppression of colon and anal gland carcinogenesis induced by dimethylhydrazine. *Carcinogenesis*, **11**: 1083-1086.

Biswas, S.K., McClure, D., Jimenez, L.A., Megson, I.L. and Rahman I. (2005). Curcumin induces glutathione biosynthesis and inhibits NF-kappaB activation and interleukin-8 release in alveolar epithelial cells: mechanism of free radical scavenging activity. *Antioxidant & Redox Signaling*, **7**: 32-41.

Bradford, P.G. and Awad, A.B. (2007). Phytosterols as anticancer compounds. *Molecular Nutrition & Food Research*, **51**: 161-170.

Butrum, R.R., Clifford, C.K. and Lanza, E. (1988). **NCI** dietary guidelines: rationale. *The American Journal of Clinical Nutrition*, **48**: 888-895.

Chatterjee, L.M., Katiyar, S.K., Mohan, R.R. and Agarwal, R. (1999). A flavonoid antioxidant, silymarin, affords exceptionally high protection against tumor promotion in the SENCAR mouse skin tumorigenesis model. *Cancer Research*, **59**: 622-632.

Chaudhuri, D., Orsulic, S. and Ashok, B.T. (2007). Antiproliferative activity of sulforaphane in Akt-overexpressing ovarian cancer cells. *Molecular Cancer Therapeutics*, **6**: 334-345.

Chauhan, D.P. (2002). Chemotherapeutic potential of curcumin for colorectal cancer. *Current Pharmaceutical Design*, **8**: 1695-1706.

Chen, L. and Zhang, H.Y. (2007). Cancer preventive mechanisms of the green tea polyphenol (-)-epigallocatechin-3-gallate. *Molecules*, **12**: 946-957.

Chiang, C.T., Way, T.D., Tsai, S.J. and Lin, J.K. (2007). Diosgenin, a naturally occurring steroid, suppresses fatty acid synthase expression in HER2-overexpressing breast cancer cells through modulating Akt, mTOR and JNK phosphorylation. *FEBS Letters*, **581**: 5735-5742.

Chisholm, K., Bray, B.J. and Rosengren, R.J. (2004). Tamoxifen and epigallocatechin gallate are synergistically cytotoxic to MDA-MB-231 human breast cancer cells. *Anti-Cancer Drugs*, **15**: 889-897.

Constantinou, A.I., Mehta, R. and Husband, A. (2003). Phenoxodiol, a novel isoflavone derivative, inhibits dimethylbenz[a]anthracene (DMBA)-induced mammary carcinogenesis in female Sprague-Dawley rats. *European Journal of Cancer*, **39**: 1012-1018.

Cover, C.M., Hsieh, S.J., Cram, E.J., Hong, C., Riby, J.E., Bjeldanes, L.F. and Firestone, G.L. (1999). Indole-3-carbinol and tamoxifen cooperate to arrest the cell cycle of MCF-7 human breast cancer cells. *Cancer Research*, **59**: 1244-1251.

Craig, W.J. (1999). Health-promoting properties of common herbs. *The American Journal of Clinical Nutrition*, **70**: 491S-499S.

Dahan, K., Fennal, M. and Kumar, N.B. (2008). Lycopene in the prevention of prostate cancer. *Journal of the Society for Integrative Oncology*, **6**: 29-36.

Deorukhkar, A., Krishnan, S., Sethi, G. and Aggarwal, B.B. (2007). Back to basics: how natural products can provide the basis for new therapeutics. *Expert Opinon on Investigational Drugs*, **16**: 1753-1757.

Divisi, D., Di Tommaso, S., Salvemini, S., Garramone, M. and Crisci, R. (2006). Diet and cancer. *Acta Bio-medica*, **77**: 118-123.

Edderkaoui, M., Odinokova, I., Ohno, I., Gukovsky, I., Go, V.L., Pandol, S.J. and Gukovskaya, A.S. (2008). Ellagic acid induces apoptosis through inhibition of nuclear factor kappa B in pancreatic cancer cells. *World Journal of Gastroenterology*, **14**: 3672-3680.

Garodia, P., Ichikawa, H., Malani, N., Sethi, G. and Aggarwal, B.B. (2007). From ancient medicine to modern medicine: ayuervedic concepts of health and their role in inflammation and cancer. *Journal of the Society for Integrative Oncology*, **5**: 25-37.

Gensler, H.L., Timmermann, B.N., Valcic, S., Wächter, G.A. Dorr, R., Dvorakova, K. and Alberts, D.S. (1996). Prevention of photocarcinogenesis by topical administration of pure epigallocatechin gallate isolated from green tea. *Nutrition and Cancer*, **26**: 325-335.

Gescher, A., Pastorino, U., Plummer, S.M. and Manson, M.M. (1998). Suppression of tumour development by substances derived from the diet—mechanisms and clinical implications. *British Journal of Clinical Pharmacology*, **45**: 1-12.

Ghosh, R., Nadiminty, N., Fitzpatrick, J.E., Alworth, W.L., Slaga, T.J. and Kumar, A.P. (2005). Eugenol causes melanoma growth suppression through inhibition of E2F1 transcriptional activity. *The Journal of Biological Chemistry*, **280**: 5812-5819.

Goel, A., Kunnumakkara, A.B. and Aggarwal, B.B. (2008). Curcumin as "Curecumin": from kitchen to clinic. *Biochemical Pharmacology*, **75**: 787-809.

Gupta, S., Ahmad, N., Nieminen, A.L. and Mukhtar, H. (2000). Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by green tea constituent (-)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells. *Toxicology and Applied Pharmacology*, **164**: 82-90.

Hartwell, J.L. (1976). Types of anticancer agents isolated from plants. *Cancer Treatment Reports*, **60**: 1031-1067.

Hebert, J.R., Hurley, T.G., Olendzki, B.C., Teas, J., Ma, Y. and Hampl, J.S. (1998). Nutritional and socioeconomic factors in relation to prostate cancer mortality: a cross-national study. *Journal of the National Cancer Institute*, **90**: 1637-1647.

Hecht, S.S., Kenney, P.M., Wang, M., Trushin, N. and Upadhyaya, P. (2000). Effects of phenethyl isothiocyanate and benzyl isothiocyanate, individually and in combination, on lung tumorigenesis induced in A/J mice by benzo[a]pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Cancer Letters*, **150**: 49-56.

Hillman, G.G., Forman, J.D., Kucuk, O., Yudelev, M., Maughan, R.L., Rubio, J., Layer, A., Tekyi-Mensah, S., Abrams, J. and Sarkar, F.H. (2001). Genistein potentiates the radiation effect on prostate carcinoma cells. *Clinical Cancer Research*, **7**: 382-390.

Hillman, G.G., Wang, Y., Kucuk, O., Che, M., Doerge, D.R., Yudelev, M., Joiner, M.C., Marples, B., Forman, J.D. and Sarkar, F.H. (2004). Genistein potentiates inhibition of tumor growth by radiation in a prostate cancer orthotopic model. *Molecular Cancer Therapeutics*, **3**: 1271-1279.

Hour, T.C., Chen, J., Huang, C.Y., Guan, J.Y., Lu, S.H. and Pu, Y.S. (2002). Curcumin enhances cytotoxicity of chemotherapeutic agents in prostate cancer cells by inducing p21 (WAF1/CIP1) and C/EBPbeta expressions and suppressing NF-kappaB activation. *The Prostate*, **51**: 211-218.

Huang, M.T., Smart, R.C., Wong, C.Q. and Conney, A.H. (1988). Inhibitory effect of curcumin, chlorogenic acid, caffeic acid, and ferulic acid on tumor promotion in mouse skin by 12-*O*-Tetradecanoylphorbol-13-acetate. *Cancer Research*, **48**: 5941-5946.

Hwang, J.T., Ha, J. and Park, O.J. (2005). Combination of 5-fluorouracil and genistein induces apoptosis synergistically in chemo-resistant cancer cells through the modulation of AMPK and COX-2 signaling pathways. *Biochemical and Biophysical Research Communications*, **332**: 433-440.

Jang, M., Cai, L., Udeani, G.O., Slowing, K.V., Thomas, C.F., Beecher, C.W., Fong, H.H., Farnsworth, N.R., Kinghorn, A.D., Mehta, R.G., Moon, R.C. and Pezzuto, J.M. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*, **275**: 218-220.

Jazirehi, A.R. and Bonavida, B. (2004). Resveratrol modifies the expression of apoptotic regulatory proteins and sensitizes non-Hodgkin's lymphoma and multiple myeloma cell lines to paclitaxel-induced apoptosis. *Molecular Cancer Therapeutics*, **3**: 71-84.

Jung, C., Motwani, M., Kortmansky, J., Sirotnak, F.M., She, Y., Gonen, M., Haimovitz-Friedman, A. and Schwartz, G.K. (2003). The cyclin-dependent kinase inhibitor flavopiridol potentiates gamma-irradiation-induced apoptosis in colon and gastric cancer cells. *Clinical Cancer Research*, **9**: 6052-6061.

Kamsteeg, M., Rutherford, T., Sapi, E., Hanczaruk, B., Shahabi, S., Flick, M., Brown, D. and Mor, G. (2003). Phenoxodiol—an isoflavone analog—induces apoptosis in chemoresistant ovarian cancer cells. *Oncogene*, **22**: 2611-2620.

Khan, N. and Mukhtar, H. (2008). Multitargeted therapy of cancer by green tea polyphenols. *Cancer Letters*, **269**: 269-280.

Khan, N., Afaq, F., Saleem, M., Ahmad, N. and Mukhtar, H. (2006). Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate. *Cancer Research*, **66**: 2500-2505.

Khoshyomn, S., Manske, G.C., Lew, S.M., Wald, S.L. and Penar, P.L. (2000). Synergistic action of genistein and cisplatin on growth inhibition and cytotoxicity of human medulloblastoma cells. *Pediatric Neurosurgery*, **33**: 123-131.

Khoshyomn, S., Nathan, D., Manske, G.C., Osler, T.M. and Penar, P.L. (2002). Synergistic effect of genistein and BCNU on growth inhibition and cytotoxicity of glioblastoma cells. *Journal of Neuro-oncology*, **57**: 193-200.

Kim, S.O., Chun, K.S., Kundu, J.K. and Surh, Y.J. (2004). Inhibitory effects of [6]-gingerol on PMA-induced COX-2 expression and activation of NF-kappaB and p38 MAPK in mouse skin. *Biofactors*, **21**: 27-31.

Kim, S.O., Kundu, J.K., Shin, Y.K., Park, J.H., Cho, M.H., Kim, T.Y and Surh, Y.J. (2005). [6]-Gingerol inhibits COX-2 expression by blocking the activation of p38 MAP kinase and NF-kappaB in phorbol ester-stimulated mouse skin. *Oncogene*, **24**: 2558-2567.

Kost, J. (1849). The elements of Materia Medica and Therapeutics. *Cincinnati, Kost and Pool*, 572.

Kubota, T., Uemura, Y., Kobayashi, M. and Taguchi, H. (2003). Combined effects of resveratrol and paclitaxel on lung cancer cells. *Anticancer Research*, **23**: 4039-4046.

Kunnumakkara, A.B., Guha, S., Krishnan, S., Diagaradjane, P., Gelovani, J. and Aggarwal, B.B. (2007). Curcumin potentiates antitumor activity of gemcitabine in an orthotopic model of pancreatic cancer through suppression of proliferation, angiogenesis, and

inhibition of nuclear factor-kappaB-regulated gene products. *Cancer Research*, **67**: 3853-3861.

Lee, R., Kim, Y.J., Lee, Y.J. and Chung, H.W. (2004). The selective effect of genistein on the toxicity of bleomycin in normal lymphocytes and HL-60 cells. *Toxicology*, **195**: 87-95.

Lei, W., Mayotte, J.E. and Levitt, M.L. (1999). Enhancement of chemosensitivity and programmed cell death by tyrosine kinase inhibitors correlates with EGFR expression in non-small cell lung cancer cells. *Anticancer Research*, **19**: 221-228.

Li, D., Yee, J.A., Mc Guire, M.H., Murphy, P.A. and Yan, L. (1999). Soybean isoflavones reduce experimental metastasis in mice. *The Journal of Nutrition*, **129**: 1075-1078.

Li, M., Zhang, Z., Hill, D.L., Wang, H. and Zhang, R. (2007). Curcumin, a dietary component, has anticancer, chemosensitization, and radiosensitization effects by down-regulating the MDM2 oncogene through the PI3K/mTOR/ETS2 pathway. *Cancer Research*, **67**: 1988-1996.

Li, N., Chen, X., Liao, J., Yang, G., Wang, S., Josephson, Y., Han, C., Chen, J., Huang, M.T. and Yang, C.S. (2002). Inhibition of 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis in hamsters by tea and curcumin. *Carcinogenesis*, **23**: 1307-1313.

Li, Y., Ahmed, F., Ali, S., Philip, P.A., Kucuk, O. and Sarkar, F.H. (2005). Inactivation of nuclear factor kappaB by soy isoflavone genistein contributes to increased apoptosis induced by chemotherapeutic agents in human cancer cells. *Cancer Research*, **65**: 6934-6942.

Li, Y., Ellis, K.L., Ali, S., El-Rayes, B.F., Nedeljkovic-Kurepa, A., Kucuk, O., Philip, P.A. and Sarkar, F.H. (2004). Apoptosis-inducing effect of chemotherapeutic agents is potentiated by soy isoflavone genistein, a natural inhibitor of NF-kappaB in BxPC-3 pancreatic cancer cell line. *Pancreas*, **28**: e90-e95.

López-Lázaro, M. (2008). Anticancer and carcinogenic properties of curcumin: considerations for its clinical development as a cancer chemopreventive and chemotherapeutic agent. *Molecular Nutrition & Food Research*, **52**: S103-S127.

Ludwig, D.L., Witte, L., Hicklin, D.J., Prewett, M., Bassi, R., Burtrum, D., Pereira, D.S., Jimenez, X., Fox, F., Saxena, B., Zhou, Q., Ma, Y., Kang, X., Patel, D., Barry, M., Kussie, P., Zhu, Z., Russell, D.A., Petersen, W.L., Jury, T.P., Gaitan-Gaitan, F., Moran, D.L., Delannay, X., Storrs, B.S., Tou, J., Zupec, M.E., Gustafson, K.S., McIntyre, J., Tarnowski, S.J. and Bohlen, P. (2004). Conservation of receptor antagonist anti-tumor activity by epidermal growth factor receptor antibody expressed in transgenic corn seed. *Human Antibodies*, **13**: 81-90.

MacDonald, R.S., Guo, J., Copeland, J., Browning, J.D. Jr., Sleper, D., Rottinghaus, G.E. and Berhow, M.A. (2005). Environmental influences on isoflavones and saponins in soybeans and their role in colon cancer. *The Journal of Nutrition*, **135**: 1239-1242.

Martínez, C., Vicente, V., Yáñez, J., Alcaraz, M., Castells, M.T., Canteras, M., Benavente-García, O. and Castillo, J. (2005). The effect of the flavonoid diosmin, grape seed extract and red wine on the pulmonary metastatic B16F10 melanoma. *Histology and Histopathology*, **20**: 1121-1129.

Massa, S., Franconi, R., Brandi, R., Muller, A., Mett, V., Yusibov, V. and Venuti, A. (2007). Anti-cancer activity of plant-produced HPV16 E7 vaccine. *Vaccine*, **25**: 3018-3021.

Mohammad, R.M., Al-Katib, A., Aboukameel, A., Doerge, D.R., Sarkar, F. and Kucuk, O. (2003). Genistein sensitizes diffuse large cell lymphoma to CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy. *Molecular Cancer Therapeutics*, **2**: 1361-1368.

Moy, K.A., Yuan, J.M., Chung, F.L., Van Den Berg, D., Wang, R., Gao, Y.T. and Yu, M.C. (2008). Urinary total isothiocyanates and colorectal cancer: a prospective study of men in Shanghai, China. *Cancer Epidemiology, Biomarkers & Prevention*, **17**: 1354-1359.

Muto, A., Hori, M., Sasaki, Y., Saitoh, A., Yasuda, I., Maekawa, T., Uchida, T., Asakura, K., Nakazato, T., Kaneda, T., Kizaki, M., Ikeda, Y. and Yoshida, T. (2007). Emodin has a cytotoxic activity against human multiple myeloma as a Janus-activated kinase 2 inhibitor. *Molecular Cancer Therapeutics*, **6**: 987-994.

Notarbartolo, M., Poma, P., Perri, D., Dusonchet, L., Cervello, M. and D'Alessandro, N. (2005). Antitumor effects of curcumin, alone or in combination with cisplatin or

doxorubicin, on human hepatic cancer cells. Analysis of their possible relationship to changes in NF-κB activation levels and in IAP gene expression. *Cancer Letters*, **224**: 53-65.

Pereira, M.A., Grubbs, C.J., Barnes, L.H., Li, H., Olson, G.R., Eto, I., Juliana, M., Whitaker, L.M., Kelloff, G.J., Steele, V.E. and Lubet, R.A. (1996). Effects of the phytochemicals, curcumin and quercetin, upon azoxymethane-induced colon cancer and 7, 12-dimethylbenz[a]anthracene-induced mammary cancer in rats. *Carcinogenesis*, **17**: 1305-1311.

Powers, H.J. (2005). Interaction among folate, riboflavin, genotype, and cancer, with reference to colorectal and cervical cancer. *The Journal of Nutrition*, **135**: 2960S-2966S.

Pradhan, S.C. and Girish, C. (2006). Hepatoprotective herbal drug, silymarin from experimental pharmacology to clinical medicine. *The Indian Journal of Medical Research*, **124**: 491-504.

Pujol, M., Gavilondo, J., Ayala, M., Rodrigrez, M., Gonzalez, E.M. and Perez, L. (2007). Fighting cancer with plant-expressed pharmaceuticals. *Trends in Biotechnology*, **25**: 455-459.

Raffoul, J.J., Wang, Y., Kucuk, O., Forman, J.D., Sarkar, F.H. and Hillman, G.G. (2006). Genistein inhibits radiation-induced activation of NF-kappaB in prostate cancer cells promoting apoptosis and G2/M cell cycle arrest. *BMC Cancer*, **6**: 107.

Rodríguez, M., Ramírez, N.I., Ayala, M., Freyre, F., Pérez, L., Triguero, A., Mateo, C., Selman-Housein, G., Gavilondo, J.V. and Pujol, M. (2005). Transient expression in tobacco leaves of an aglycosylated recombinant antibody against the epidermal growth factor receptor. *Biotechnology and Bioengineering*, **89**: 188-194.

Sapi, E., Alvero, A.B., Chen, W., O'Malley, D., Hao, X.Y., Dwipoyono, B., Garg, M., Kamsteeg, M., Rutherford, T. and Mor, G. (2004). Resistance of ovarian carcinoma cells to docetaxel is XIAP dependent and reversible by phenoxodiol. *Oncology Research*, **14**: 567-578.

Sarkar, F.H. and Li, Y. (2004). Indole-3-carbinol and prostate cancer. *The Journal of Nutrition*, **134**: 3493S-3498S.,

Satoh, H., Nishikawa, K., Suzuki, K., Asano, R., Virgona, N., Ichikawa, T., Hagiwara, K. and Yano, T. (2003). Genistein, a soy isoflavone, enhances necrotic-like cell death in a breast cancer cell treated with a chemotherapeutic agent. *Research Communications in Molecular Pathology and Pharmacology*, **113-114**: 149-158.

Shankar, S., Ganapathy, S., Hingorani, S.R. and Srivastava, R.K. (2008). EGCG inhibits growth, invasion, angiogenesis and metastasis of pancreatic cancer. *Frontiers in Bioscience: A Journal and Virtual Library*, **13**: 440-452.

Shankar, S., Suthakar, G. and Srivastava, R.K. (2007). Epigallocatechin-3-gallate inhibits cell cycle and induces apoptosis in pancreatic cancer. *Frontiers in Bioscience*, **12**: 5039-5051.

Sharma, G., Tyagi, A.K., Singh, R.P., Chan, D.C. and Agarwal, R. (2004). Synergistic anti-cancer effects of grape seed extract and conventional cytotoxic agent doxorubicin against human breast carcinoma cells. *Breast Cancer Research and Treatment*, **85**: 1-12.

Shishodia, S., Sethi, G. and Aggarwal, B.B. (2005). Curcumin: getting back to the roots. *Annals of the New York Academy of Science*, **1056**: 206-217.

Singh, A.V., Franke, A.A., Blackburn, G.L. and Zhou, J.R. (2006). Soy phytochemicals prevent orthotopic growth and metastasis of bladder cancer in mice by alterations of cancer cell proliferation and apoptosis and tumor angiogenesis. *Cancer Research*, **66**: 1851-1858.

Stoner, G.D., Morrissey, D.T., Heur, Y.H., Daniel, E.M., Galati, A.J. and Wagner, S.A. (1991). Inhibitory effects of phenethyl isothiocyanate on N-nitrosobenzylmethylamine carcinogenesis in the rat esophagus. *Cancer Research*, **51**: 2063-2068.

Sun, L. and Wang, X. (2003). Effects of allicin on both telomerase activity and apoptosis in gastric cancer SGC-7901 cells. *World Journal of Gastroenterology*, **9**: 1930-1934.

Takada, Y. and Aggarwal, B.B. (2004). Flavopiridol inhibits NF-kappaB activation induced by various carcinogens and inflammatory agents through inhibition of IkappaBalpha kinase and p65 phosphorylation: abrogation of cyclin D1, cyclooxygenase-2, and matrix metalloprotease-9. *The Journal of Biological Chemistry*, **279**: 4750-4759.

Taniguchi, S., Fujiki, H., Kobayashi, H., Go, H., Miyado, K., Sadano, H. and Shimokawa, R. (1992). Effect of (-)-epigallocatechin gallate, the main constituent of green tea, on

lung metastasis with mouse B16 melanoma cell lines. *Cancer Letters*, **65**: 51-54.

Tanos, V., Brzezinski, A., Drize, O., Strauss, N. and Peretz, T. (2002). Synergistic inhibitory effects of genistein and tamoxifen on human dysplastic and malignant epithelial breast cells *in vitro*. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, **102**: 188-194.

Thomson, C.A., LeWinn, K., Newton, T.R., Alberts, D.S. and Martinez, M.E. (2003). Nutrition and diet in the development of gastrointestinal cancer. *Current Oncology Reports*, **5**: 192-200.

Van Der Heijden, R., Jacobs, D.I., Snoeijer, W., Hallard, D. and Verporate, R. (2004).The Catharanthus alkaloids: pharmacognosy and biotechnology. *Current Medicinal Chemistry*, **11**: 607-628.

Vantyghem, S.A., Wilson, S.M., Postenka, C.O., Al-Katib, W., Tuck, A.B. and Chambers, A.F. (2005). Dietary genistein reduces metastasis in a postsurgical orthotopic breast cancer model. *Cancer Research*, **65**: 3396-3403.

Wang, J.M., Xu, B., Rao, J.Y., Shen, H.B., Xue, H.C. and Jiang, Q.W. (2007). Diet habits, alcohol drinking, tobacco smoking, green tea drinking, and the risk of esophageal squamous cell carcinoma in the Chinese population. *European Journal of Gastroenterology & Hepatology*, **19**: 171-176.

Wang, T.T., Hudson, T.S., Wang, T.C., Remsberg, C.M., Davies, N.M., Takahashi, Y., Kim, Y.S., Seifried, H., Vinyard, B.T., Perkins, S.N. and Hursting, S.D. (2008). Differential effects of resveratrol on androgen-responsive LNCaP human prostate cancer cells *in vitro* and *in vivo*. *Carcinogenesis*, [In press].

Watanabe, T., Shiraishi, T., Sasaki, H. and Oishi, M. (1989). Inhibitors for protein-tyrosine kinases, ST638 and genistein: induce differentiation of mouse erythroleukemia cells in a synergistic manner. *Experimental Cell Research*, **183**: 335-342.

Weisburger, J.H., Rivenson, A., Aliaga, C., Reinhardt, J., Kelloff, G.J., Boone, C.W., Steele, V.E., Balentine, D.A., Pittman, B. and Zang, E. (1998). Effect of tea extracts, polyphenols, and epigallocatechin gallate on azoxymethane-induced colon cancer. *Proceding of the Society for Experimental Biology and Medicine at (New York, N.Y.)*, **217**: 104-108.

World Cancer Research Fund (1997). Food, Nutrition, and Prevention of Cancer: a global perspective, Washington (DC): American Institute for Cancer Research.

Yashar, C.M., Spanos, W.J., Taylor, D.D. and Gercel-Taylor, C. (2005). Potentiation of the radiation effect with genistein in cervical cancer cells. *Gynecologic Oncology*, **99**: 199-205.

Yi, J., Yang, J., He, R., Gao, F., Sang, H., Tang, X. and Ye, R.D. (2004). Emodin enhances arsenic trioxide-induced apoptosis via generation of reactive oxygen species and inhibition of survival signaling. *Cancer Research*, **64**: 108-116.

Yusuf, N., Irby, C., Katiyar, S.K. and Elmets, C.A. (2007). Photoprotective effects of green tea polyphenols. *Photodermatology, Photoimmunology & Photomedicine*, **23**: 48-56.

Zhang, L.L., Li, L., Wu, D.P., Fan, J.H., Li, X., Wu, K.J., Wang, X.Y. and He, D.L. (2008). A novel anti-cancer effect of genistein: reversal of epithelial mesenchymal transition in prostate cancer cells. *Acta Pharmacologica Sinica*, **29**: 1060-1068.

Zhang, Q., Wei, D. and Liu, J. (2004). *In vivo* reversal of doxorubicin resistance by (-)-epigallocatechin gallate in a solid human carcinoma xenograft. *Cancer Letters*, **208**: 179-186.

Zhang, X.Y., Li, W.G., Wu, Y.J., Zheng, T.Z., Li, W., Qu, S.Y. and Liu, N.F. (2005). Proanthocyanidin from grape seeds potentiates anti-tumor activity of doxorubicin *via* immunomodulatory mechanism. *International Immunopharmacology*, **5**: 1247-1257.

Zoberi, I., Bradbury, C.M., Curry, H.A., Bisht, K.S., Goswami, P.C., Roti Roti, J.L. and Gius, D. (2002). Radiosensitizing and anti-proliferative effects of resveratrol in two human cervical tumor cell lines. *Cancer Letters*, **175**: 165-173.

Zubrod, C.G. (1984).Origin and development of chemotherapy research at the National Cancer Institute. *Cancer Treatment Reports*, **68**: 9-19.

"This page is Intentionally Left Blank"

14

Global Medicinal Plants with Anti-HIV Activities

THET THET HTAR[1*] AND GABRIEL A. AKOWUAH[2]

ABSTRACT

Interest in the exploration of remedies for the treatment of Human Immunodeficiency Virus (HIV) infection, the causative of Acquired Immunodeficiency Syndrome (AIDS), was begun in 1980s and it has been carried on till to date worldwide. The anti-retroviral drugs use in the management of HIV infection today increase life expectancy but sadly, they cannot cure the infection. With the emergence of multi-drug resistant HIV strains, and rapid spread of AIDS, the search for anti-HIV agents has also been diversified to plant products. Screening of the in vitro and in vivo anti-HIV activities from traditional medicinal plants, which have been used by the world population with HIV infection as well as those plants, which have not been used by HIV patients are reviewed. It is concluded that there are many plant constituents in nature with the anti-HIV activity and synthesis of semi-derivatives of those constituents should be further encouraged to optimize the activity. A diverse mechanism of search both in medicinal plants and interventive medicines should be continued.

Key words : AIDS, anti-HIV activity, medicinal plants, protease, reverse transcriptase, traditional medicines

INTRODUCTION

The United Nations joint programme on HIV/AIDS (UNAIDS) and World Health Organization (WHO) estimated that a total of 39.5 million people

1. School of Medicine and Health Sciences, Monash University (Sunway Campus), Jalan Lagoon Selatan, 46150 Bandar Sunway, Selangor Darul Ehsan, Malaysia.
2. Faculty of Medical Sciences, School of Pharmacy, University College Sedaya International (UCSI), No. 1 Jalan Menara Gading, UCSI Heights, 56000 KualaLumpur, Malaysia.
* *Corresponding author* : E-mail : htarme@hotmail.com

would have been living with Human Immunodeficiency Virus (HIV) in 2006 including the estimated 4.3 million adults and children who were newly infected with HIV in 2006 (UNAIDS, 2006). UNAIDS and WHO also reported that a number of people with HIV in 2006 was 2.6 million more in comparison to that in 2004.

The misery of HIV/AIDS tragedy was not only the rapid growing epidemic worldwide, there is still no cure for HIV infection till to date. Although the anti-retrovirals used in the management of HIV infection today increase life expectancy, they are very expensive and unaffordable by developing countries known to have a high number of HIV infection. In addition, anti-retroviral drugs had been reported in generating serious side-effect such as lactic acidosis and developing resistance.

Currently available chemotherapeutic agents for HIV infection are targeting on the various steps of the viral life-cycle, such as viral attachment and entry, viral transcription by reverse transcriptases, viral proteases which are essential in processing of viral proteins (Mitsuya *et al.,* 1990). However, the rapid spread of AIDS, development of multi-drug resistant HIV strains and the enormous high cost of the anti-retroviral drugs, the requirement of continuous search for innovative drugs with diverse mechanisms of anti-HIV activity has been highlighted (Lipsky, 1996; Balzarini *et al.,* 1986; Tantillo *et al.,* 1994). WHO noted that 80% of the world population are relying on the traditional medicine for various diseases, therefore, *in vitro* studies of anti-HIV activity from the medicinal plants have been seriously explored worldwide (Jassim & Naji, 2003).

The aim of this paper is to review *in vitro/in vivo* screening of the medicinal plants worldwide for the anti-HIV activity. It is also noted that selection of the medicinal plants for the studies are based on the history of traditional use by AIDS patients as well as those medicinal plants which have not been used by AIDS patients. This mini-review article will be focusing on the update of recently screened medicinal plants for anti-HIV activity.

ANTI-HIV ACTIVITY OF MEDICINAL PLANTS

Many medicinal plants have been used by AIDS patients without any scientific evidence of anti-HIV activity. For instance, *Allium sativum* (garlic), *Lentinus edodes* (shiitake mushrooms), *Carica papaya* (papaya), *Panax* species (ginseng), *Aloe vera*, *Chelidonium majus* (ukrain) have been used. WHO collaborative study with Chinese and Tanzanian reported that Traditional Chinese Medicine such as *Polyporus umbellatus, Cordyceps sinensis* and *Paeonia obovata* have been used by AIDS patients although the scientific study on these plants for the anti-HIV activity is unknown.

However, current encouragement in use of traditional medicines for various diseases result in screening of anti-HIV activity from randomly

collected medicinal plants as well as medicinal plants which used to treat viral infections. Most *in vitro* and *in vivo* studies on anti-HIV activity mainly focused on the traditional targets such as reverse transcriptase (RT), protease and integrase (IN). In addition, some studies are compared to the cytopathicity of infected cells and multiplicity of infection (MOI) contributed from medicinal plants with the anti-HIV activity of standard clinically approved drugs.

Medicinal Plants Undergone for *In vitro* and *In vivo* Anti-HIV Screening

The aqueous extracts of *Ocimum gratissimum, Ficus polita, Clausena anisata, Alchornea cordifolia, Elaeophorbia drupifera* from Ghana were reported to have significant inhibition of viral cytopathicity of HIV-1 even at high multiplicity of infection (MOI) with delayed treatment for 2 h and they were active against HIV-2 (Ayisi & Nyadedzor, 2003). It was also reported that these extracts inhibited the HIV-1 reverse transcriptase activity at EC_{50} value of less than 0.01-0.03 mg/mL. In comparison, the nucleoside reverse transcriptase inhibitor, 3'-azido-2', 3'-dideoxythymidine (AZT) which is clinically approved, was only found to reduce the virus production with cytopathicity in acutely infected cells. At low multiplicity of infection (MOI), AZT was able to achieve 90% inhibition of HIV-1 but was not able to achieve such high inhibition at high MOI (Fischl *et al.,* 1987).

The anti-HIV activity of Thai medicinal plants locally named Hua-Khao-Yen, which included the following five species of *Dioscorea birmanica, Smilax corbularia, Smilax glabra, Pygmaeopremna herbacea* and *Dioscorea membranacea* had been screened for both HIV-1 protease and HIV-1 integrase inhibitory activities (Tewtrakul *et al.,* 2006). They reported that ethanolic extract of *Smilax corbularia* indicated the highest inhibitory activity against HIV-1 integrase with an IC_{50} value of 1.9 μg/mL which was two fold lower than that of the a positive control, suramin with an IC_{50} value of 3.2 μg/mL, among these five species. It was also noted that both aqueous and ethanolic extract of *Dioscorea birmanica* showed the inhibitory activity against HIV-1 integrase with IC_{50} value of 4.5 μg/mL and 4.7 μg/mL, respectively. In the case of the HIV-1 protease inhibitory activity, only the ethanol extract of *Dioscorea membranacea* showed the activity with IC_{50} value of 48 μg/mL.

Similarly, the anti-HIV activity of 69 Indian medicinal plants had been screened using virus-induced cytopathogenicity in MT-4 cells and AZT as control reference compound (Premanathan *et al.,* 2000). It was reported that only 16 of them were found to be effective against HIV-1 and the most potent activity was noted from the extract of the bark of *Cinnamomum cassia* and the shoot and fruit of *Cardiospermum helicacabum* with a selectivity index (SI) value of 6.73.

Table 1. Medicinal plants with anti-HIV activity

Plants	Parts	Target sites	Reference(s)
Boesenbergia pandurata	Rhizomes		Cheenpracha *et al.* (2006)
Ocimum gratissimum	Leaves Seeds	HIV-1 RT and taq polymerase of HIV-1 and HIV-2	Ayisi *et al.* (2002)
Ficus polita	Leaves	HIV-1 RT and taq polymerase of HIV-1 and HIV-2	Ayisi *et al.* (2002)
Clausena anisata	Leaves	Mild cytopathicity	Ayisi *et al.* (2002)
Alchornea cordifolia	Fruits Leaves	HIV-1 RT taq polymerase of HIV-1 and HIV-2	Ayisi *et al.* (2002)
Elaeophorbia drupifera	Leaves	Taq polymerase of HIV-1 and HIV-2	Ayisi *et al.* (2002)
Smilax corbularia	Rhizomes	HIV-1 IN	Tewtrakul *et al.* (2006)
Smilax glabra	Rhizomes	HIV-1 IN	Tewtrakul *et al.* (2006)
Dioscorea birmanica	Rhizomes	HIV-1 IN	Tewtrakul *et al.* (2006)
Dioscorea membranacea	Rhizomes	HIV-1 PR	Tewtrakul *et al.* (2006)
Pygmaeopremna herbacea	Root	Inactive	Tewtrakul *et al.* (2006)
Cinnamomum cassia	Rark	HIV-1	Premanathan *et al.* (2006)
Cardiospermum helicacabum	Shoot and Fruit	HIV-2	Premanathan *et al.* (2006)
Jatropha curcas	Branches	HIV-1	Matsuse *et al.* (1998)
Chamaesyce hyssopifolia	Whole plant	HIV-RT	Matsuse *et al.* (1998)
Cordia spinescens	Leaves	HIV-RT	Matsuse *et al.* (1998)
Hyptis lantanifolia	Aerial parts	HIV-RT	Matsuse *et al.* (1998)
Tetrapteris macrocarpa	Aerial parts	HIV-RT	Matsuse *et al.* (1998)
Erythroxylum citrifolium	Trunk	HIV-PR	Matsuse *et al.* (1998)
Waltheria indica	Branches	HIV-PR	Matsuse *et al.* (1998)
Xylopia frutescens	Bark	HIV-PR	Matsuse *et al.* (1998)
Sanjuisorba minor magnolia	Unknown	Inhibit cell Replication	Bedoya *et al.* (2001)
Tuberaria lignose	Unknown	Inhibit cell replication	Bedoya *et al.* (2001)
Aspilia pluriseta	Leaves	Cytotoxic	Cos *et al.* (2002)
Rumex bequaertii	Leaves	Cytotoxic	Cos *et al.* (2002)
Bridelia micrantha	Roots	HIV-RT	Bessong *et al.* (2005)
Elaendendron transvaalensis	Roots	HIV-RT	Bessong *et al.* (2005)
Mucana coriacea	Roots	HIV-RT	Bessong *et al.* (2005)
Vernonia stipulacea	Roots	HIV-RT	Bessong *et al.* (2005)

Table 1. *Contd.*

Plants	Parts	Target sites	Reference(s)
Sutherlandia frutescens	Leaves	HIV-RT	Bessong *et al.* (2005)
Ricinus communis	Roots	HIV-RT	Bessong *et al.* (2005)

Panamanian medicinal plants which were used for the treatment of viral infections and cancer had been screened for anti-HIV activity. In this study, the aqueous extracts of the whole plant of *Chamaesyce hyssopifolia* (Euphorbiaceae), the leaves of *Cordia spinescens* (Boraginaceae), the aerial parts of *Hyptis lantanifolia* (Labiatae) and the methanolic extract of the aerial parts of *Tetrapteris macrocarpa* (Malpighiaceae) were noted with the reverse transcriptase inhibition IC_{50} value of 8, 6, 7, and 8 μg/ml, respectively. Although these extracts were found to be inactive in HIV-protease inhibitory assay, the aqueous extract of the trunk of *Erythroxylum citrifolium* (Erythroxylaceae), the aqueous extract of the branches of *Waltheria indica* (Sterculiaceae) and the methanol extract of the bark of *Xylopia frutescens* (Annonaceae) were found to be moderately active against HIV-protease with IC_{50} of 43, 48, 46 μg/ml respectively (Matsuse *et al.*, 1998). This study also attempted the HIV-1 induced cytopathic effect (CPE) on MT-4 cells to determine the selectivity index (SI) and to investigate the suppressive effect of giant cell formation. It was reported that the methanolic extract of the leaves of *Jatropha curcas* (Euphorbiaceae) exhibited the most potent inhibition and a high cytotoxicity with low SI although it did not show suppression of giant cell formation at concentrations lower than 500 μg/ml.

The folk medicine of the Iberian Peninsula had been screened for the *in vitro* anti-HIV activity (Bedoya *et al.*, 2001). This study focused on the medicinal plants from the Iberian Peninsula, which had been used to treat viral infections, such as *Santolina oblongifolia, Tanacetum microphyllum, Hieracium pilosella, Sideritis phoetens, Teucrium buxifolium, Satureja obovata, Reseda lutea, Reseda suffruticosa, Sambucus ebulus, Sambucus nigra, Sanguisorba minor magnolii, Sedum album, Asphodelus ramosus, Tuberaria lignose, Cistus populifolius*. The screening of anti-viral activity of these plants had been carried out by using WHO recommended single T-cell culture assay system, human lymphocytic MT-2 cells. Among these plants, it was reported that, only the aqueous extracts of *T. lignose* and *S. minor magnolii* exhibited inhibitory effects against HIV-1 induced infections in MT-2 cells at concentrations ranging from 12.5 to 50 μg/ml and 50 μg/ml, respectively (Bedoya *et al.*, 2001).

The anti-HIV-1 activity of selected plants in a total of 38 used in Rwandan traditional medicine had been screened in Belgium and noted that 80% ethanolic extracts from the leaves of *Aspilia pluriseta* (Asteraceae) and *Rumex bequaertii* (Polygonaceae) had interesting selectivity indices (SI)

higher than 1 (Cos *et al.,* 2002). Therefore, this study concluded that the cytotoxicity of some plants might mask the antiviral properties of the active substances belonged to the plants.

In South Africa, the high prevalence of HIV and AIDS had encouraged the use of medicinal plants for AIDS related conditions (Bessong *et al.,* 2005). Like wise, the search for the anti-HIV activity from the medicinal plants had been emphasized, which reported that the methanol extracts of the roots of *Bridelia micrantha* (Euphorbiaceae) had shown the most potent reverse transcriptase inhibitory effect with an IC_{50} value of 7.3 μg/ml among the other roots of medicinal plants such as *E. transvaalensis, M. coriacea, R. communis* and *V. stipulacea* the leaves of *S. frutescens* (Bessong *et al.,* 2005). However, none of the extract of these plants was reported to have activity against HIV integrase inhibition.

The South African Medical Research Council (MRC) has launched a clinical platform for testing the safety and efficacy of medicinal plants (Morris, 2002), including some *in vivo* screening studies carried out for anti-HIV activity of Chinese herbal medicine Qi™ in 40 patients with pathogen-negative diarrhoea. Unusually, this study was aimed to see the benefit of traditional Chinese medicine in AIDS patient with chronic diarrhoea. The sustained decrease in average number of stools per day was reported for the Qi™ treatment in patients with HIV-associated, pathogen-negative diarrhoea (Cohen *et al.,* 2000).

It was also reported that glycyrrhizin isolated from *Glycyrrhiza uralensis* delayed the progression of symptoms related to HIV infection in asymptomatic HIV carriers (Ito *et al.,* 1987). The same study reported that the finding of improvement in several hematological and immunological parameters and a disappearance of HIV antigenemia if a large dose of glycyrrhizin was administered to AIDS patients.

Isolated Phytochemicals with Anti-HIV Activity

Some of the natural products isolated from the medicinal plants had been screened for the anti-HIV activity. For instance, Scutellarin (**1**) isolated from the Chinese traditional herbal plant, *Erigeron breviscapus* was found to be active against laboratory-derived HIV-1 at cell-to-cell fusion stage with the EC_{50} value of 15 μM (Zhang *et al.,* 2005).

Natural products such as quercetin (**2**) and derivative of kaempferol (**3**) isolated from *Rosa damascena* were reported to have activity against HIV by preventing gp120 and CD4 (Mahmood *et al.,* 1996). The anti-HIV activity of quercetin (**2**) was again supported as 80 % inhibition of HIV-1 reproduction at the concentration of 40 μM by (Gatto *et al.,* 2002).

Benzylisoquinoline alkaloids (+)-1(R)-Coclaurine (**4**), (-)-1-(S)-norcoclaurine (**5**) and a flavonoid glycoside, quercetin-3-*O*-β-D-glucuronide (**6**) from the leaves of *Nelumbo nucifera* were reported to have suppression

of HIV-1 replication with EC_{50} value of 0.8, less than 0.8 μg/ml and 2 μg/ml, respectively (Kashiwada *et al.*, 2005). Liensinine (**7**), neferine (**8**), isoliensinine (**9**) and nuciferine (**10**) isolated from the leaves and embryo of *Nelumbo nucifera* were also reported to have potent suppression on HIV replication with EC_{50} values of less than 0.8 μg/ml (Gatto *et al.*, 2002).

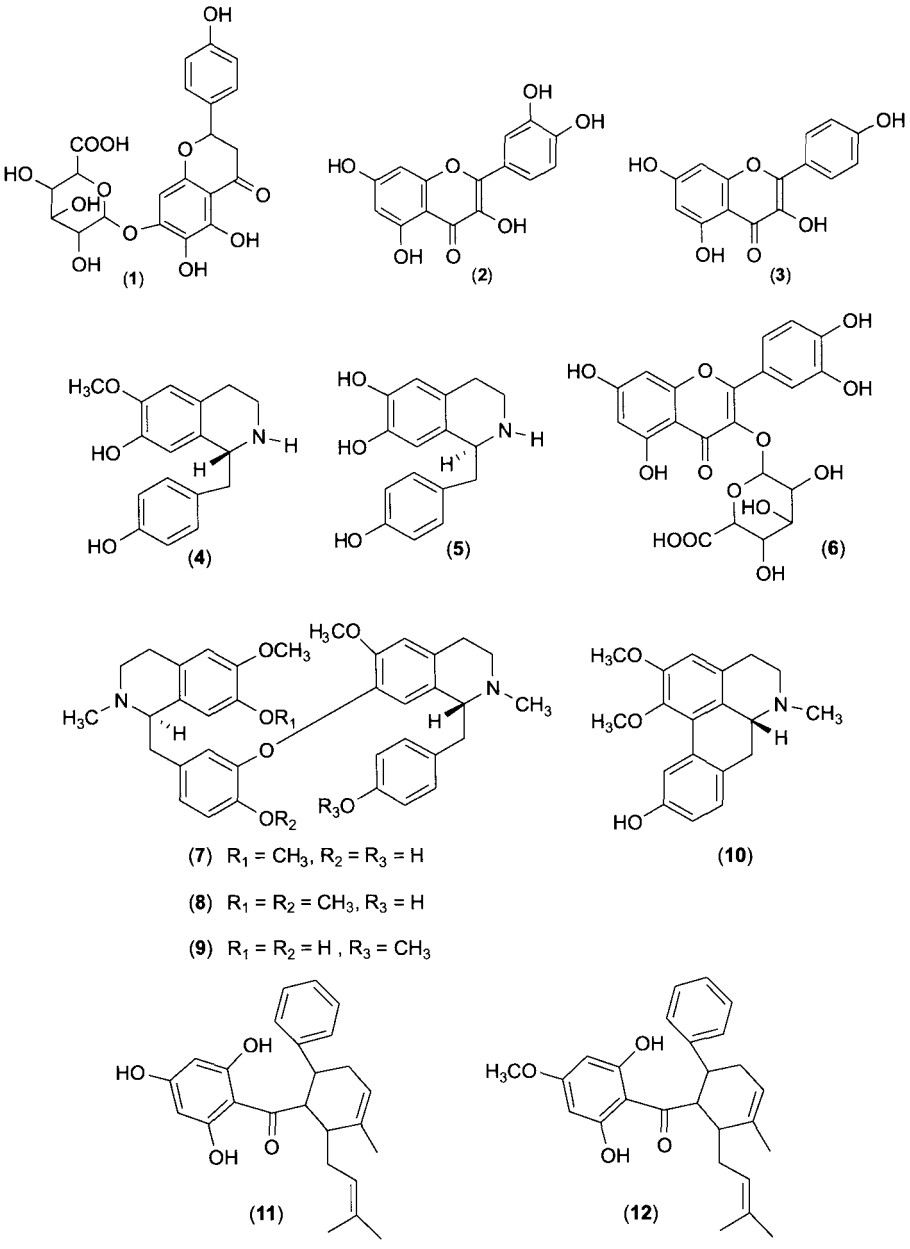


Fig 1. Natural products with anti-HIV activity

The fresh rhizome of *Boesenbergia pandurata*, Zingiberaceae family, is commonly used in Southeast Asia as a food ingredient and it is known to possess anti-bacterial (Ungsurungsie *et al.*, 1982), anti-inflammatory (Pathong *et al.*, 1989), anti-tumor (Murakami *et al.*, 1993) activities. Interestingly, it was reported that the rhizomes of *B. pandurata* were used by AIDS patients in Thailand. It was supported by a study in which compounds isolated from *B. pandurata* such as hydroxypanduratin A (**11**) and panduratin A (**12**) shown potent anti-HIV-1 protease activity with IC_{50} value of 5.6 μm and 18.7 μm respectively in comparison to the positive control, acetyl pepstatin with IC_{50} value of 3.4 μM (Cheenpracha *et al.*, 2006).

CONCLUSIONS

Traditional medicinal plants and natural products play an important role in the search of novel anti-HIV agents. It has been noted that several of these plants extracts and isolated phytochemicals exhibited multiple mechanism of actions. Although a number of *in vitro* screening of traditional medicinal plants could be documented as in this review, *in vivo* studies and clinical trials on medicinal plants are still considerably shallow. Therefore, the exploration of search on biologically active natural products from medicinal plants and synthesis of semi-derivatives bioactive products for the treatment HIV/AIDS with promotion on the safety issues of using medicinal plant products through clinical studies are required urgently.

REFERENCES

Ayisi, N.K. and Nyadedzor, C. (2003). Comparative *in vitro* effects of AZT and extracts of *Ocimum gratissimum, Ficus polita, Clausena anisata, Alchornea cordifolia,* and *Elaeophorbia drupidera* against HIV-1 and HIV-2 infections. *Antiviral Research*, **58**: 25-33.

Balzarini, J., Mitsuya, H., De Clercq, E. and Broder, S. (1986). Comparative inhibitory effects of suramin and other selected compounds on the infectivity and replication of human T-cell lymphotropic virus (HTLV-III)/lympoadenopathy-associated virus (LAV). *International Journal of Cancer*, **37**: 451-457.

Bedoya, L.M., Sanchez-Palomino, S., Abad, M.J., Bermejo, P. and Alcami, J. (2001). Anti-HIV activity of medicinal plant extracts. *Journal of Ethnopharmacology*, **77**: 113-116.

Bessong, P.O., Rojas, L.B., Obi, L.C., Tshisikawe, P.M. and lgunbor, E.O. (2005). Further screening of Venda medicinal plants for activity against HIV type 1 reverse transcriptase and integrase. *African Journal of Biotechnology*, **5**: 526-528.

Cheenpracha, S., Karalai, C., Ponglimanont, C., Subhadhirasakul, S. and Tewtrakul, S. (2006). Anti-HIV-1 protease activity of compounds from *Boesenbergia pandurata*. *Bioorganic & Medicinal Chemistry*, **14**: 1710-1714.

Cohen, M.R., Mitchell, T.F., Bacchetti, P., Child, C., Crawford, S., Gaeddert, A. and Abrams, D.I. (2000). Use of a Chinese herbal medicine for treatment of HIV-associated pathogen negative diarrhoea. *Integrative Medicine*, **2**: 79-84.

Cos, P., Hermans, N., De Bruyne, T., Apers, S., Sindambiwe, J.B., Witvrouw, M., De Clercq, E., Vanden Berghe, D., Pieters, L. and Vlietinck, A.J. (2002). Antiviral activity of Rwandan medicinal plants against human immunodeficiency virus type-1 (HIV-1). *Phytomedicine*, **9**: 62-68.

Fischl, M.A., Richman, D.D., GoGrieco, M.H., Gottlieb, M.S., Volberding, P.A., Laskin, O.L., Leedom, J.M., Groopman, J.E., Mildvan, D., Schooley, R.T., Jackson, G.G., Durack, D.T. and King, D. (1987). The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial. *New England Journal of Medicine*, **317**: 185-191.

Gatto, M.T., Falcocchio, S., Grippa, E., Mazzanti, G., Battinelli, L., Nicolosi, G., Lambusta, D. and Saso, L. (2002). Antimicrobial and anti-lipase activity of Quercetin and its C2-C16 3-O-acyl-esters. *Bioorganic & Medicinal Chemistry*, **10**: 269-272.

Ito, M., Nakashima, H., Baba, M., Pauwels, R., De Clercq, E., Shigeta, S. and Yamamoto, N. (1987). Inhibitory effect of glycyrrhizin on the *in vitro* infectivity and cytopathic activity of the human immunodeficiency virus [HIV (HTLV-III/LAV)]. *Antiviral Research*, **7**: 127-137.

Jassim, S.A.A. and Naji, M.A. (2003). A review. Novel antiviral agents: a medicinal plant perspective. *Journal of Applied Microbiology*, **95**: 412-427.

Kashiwada, Y., Aoshima, A., Ikeshiro, Y., Chen, Y-P., Furukawa, H., Itoigawa, M., Fujioka, T., Mihashi, K., Cosentino, L.M., Morris-Natchke, S.L. and Lee, K.H. (2005). Anti-HIV benzylisoquinoline alkaloids and flavonoids from the leaves of *Nelumbo nucifera*, and structure-activity correlations with related alkaloids. *Bioorganic & Medicinal Chemistry*, **13**: 443-448.

Lipsky, J.J. (1996). Antiretrovial drugs for AIDS. *Lancet*, **348**: 800-803.

Mahmood, N., Piacente, S., Pizza, C., Burke, A., Khan, A.I. and Hay, A.J. (1996). The anti-HIV activity and mechanisms of action of pure compounds isolated from Rosa damascene. *Biochemical and Biophysical Research Communications*, **229**: 73-79.

Matsuse, I.T., Lim, Y.A., Hattori, M., Correa, M. and Gupta, M.P. (1998). A search for anti-viral properties in Panamanian medicinal plants. The effects on HIV and its essential enzymes. *Journal of Ethnopharmacology*, **64**: 15-22.

Mitsuya, H., Yarchoan, R. and Broder, S. (1990). Molecular targets for AIDS therapy. *Science*, **249**: 1533-1544.

Morris, K. (2002). South Africa tests traditional medicines. *The Lancet Infectious Diseases*, **2**: 319.

Murakami, A., Kondo, A., Nakamura, Y., Ohigashi, H. and Koshimizu, K. (1993). Possible anti-tumor promoting properties of edible plants from Thailand and identification of an active constituent, Cardamonin, of *Boesenbergia pandurata*. *Bioscience Biotechnology Biochemistry*, **57**: 1971-1973.

Pathong, A., Tassaneeyakul, W., Kanjanapothi, D., Tuntiwachwuttikul, P. and Reutrakul, V. (1989). Anti-inflammatory activity of 5,7-dimethoxyflavone. *Planta Medica*, **55**: 133-136.

Premanathan, M., Rajendran, S., Ramanathan, T., Kathiresan, K., Nakashima, H. and Yamamoto, N. (2000). A survey of some Indian medicinal plants for anti-human immunodeficiency virus (HIV) activity. *Indian Journal of Medical Research*, **112**: 73-77.

Tantillo, C., Ding, J., Jacobo-Molina, A., Nanni, R.G., Boyer, P.L., Hughes, S.H., Pauwels, R., Andries, K., Janssen, P.A.J. and Arnold, E. (1994). Locations of anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure of HIV-1 reverse transcriptase. Implications for mechanisms of drug inhibition and resistance. *Journal of Molecular Biology*, **243**: 369-387.

Tewtrakul, S., Itharat, A. and Rattanasuwan, P. (2006). Anti-HIV-1 protease and HIV-1 integrase activities of Thai medicinal plants known as Hua-Khao-Yen. *Journal of Ethnopharmacology*, **105**: 312-315.

UNAIDS and World Health Organization, (2006). AIDS epidemic update.

Ungsurungsie, M., Sutheinkul, D. and Paovalo, C. (1982). *Food Cosmetic Toxicology*, **120**: 527.

Zhang, G.H., Wang, Q., Chen, J.J., Zhang, X.M., Tam, S.C. and Zheng, Y.T. (2005). The anti-HIV-1 effect of scutellarin. *Biochemical and Biophysical Research Communications*, **334**: 812-816.

"This page is Intentionally Left Blank"

15

New Fungal Metabolites, as Antifungal, Herbicides and Insecticides for Biocontrol of Agrarian Plants Pests*

A. EVIDENTE[1*]

ABSTRACT

Toxins produced by phytopathogenic fungi have assumed great importance because of their involvement in several plant diseases. These pathogeneses have seriously damaged plants of agrarian interest. Frequently, the fungal metabolites, as mycotoxins, represent a serious harmful for the animal and human health. Considering their social and economical impact, many efforts have been made to avoid losses in the agrarian production and contamination of foods. Plants infesting economical important crops are another important problem causing marked losses in the agrarian production. Weed pests have always being recognised as one of the most serious agricultural and environmental problems. Insect, and in particular aphids are a major cause of loss of agricultural produce and reduction of its quality. In fact, aphids have both a direct noxious effect on crop health, caused by subtraction of sap, and an indirect effect related to the spread of insect-transmitted virus diseases. In agriculture, the control of agrarian plant disease, of insects, and of weed diffusion is usually achieved by using agrochemicals belonging to different classes of organic compounds, often in large amounts. These cause serious problems to human and animal health and produce heavy environmental pollution. On the contrary, biological agents offer the advantage of being fully compatible with the environment, often with high specificity, and represent a long term solution also to control

1. Dipartimento di Scienze del Suolo, della Pianta, dell'Ambiente e delle Produzioni Animali, Università di Napoli Federico II, Via Università 110, 80055 Portici, Italy.

* Dedicated to my friend Frà Luigi Campoli, Monastero di Santa Chiara, Napoli, Italy.

* *Corresponding author* : E-mail : evidente@unina.it

pests particularly resistant to chemical herbicides. Therefore, many efforts have been made to develope new strategies using natural antagonists, mainly fungal bioactive metabolites. The isolation, chemical and biological charaterisation of bioactive fungal metabolites with potential antifungal, insecticide and herbicide activities against pathogenic fungi of important agrarian crops, including the mycotoxins producers, insects and weeds infesting in particular cereals, are reported. The results of studies on the structure activity relationships and mode of action of some bioactive metabolites, using natural analogues and chemical derivatives, are also discussed.

Key words : Fungal metabolites, antifungals, herbicides, insecticides, biological pest plant control

INTRODUCTION

Phytopathogenic fungi have assumed great importance because of their involvement in several plant diseases. These pathogeneses have seriously damaged plants of agrarian, forestall and environmental interest. Considering the economical importance of some agrarian crop, several studies have tried to understand the role of bioactive microbial metabolites in the pathogenic process. The chemical nature of these toxins ranging from low molecular weight compounds, including all classes of natural products as terpenes, chromanones, butenolides, pyrones, macrolides, aromatic derivatives, aminoacids etc., to high molecular compounds as proteins, glycoproteins and polysaccharides. As a result, many new phytotoxins, have been reported (Strobel, 1982; Graniti *et al.*, 1989; Ballio & Graniti, 1991; Evidente & Motta, 2001).

Frequently, the same microorganisms also produce mycotoxins, metabolites with acutely toxicity against humans and animals such as aflatoxns, ochratoxins and trichotechenes produced by *Aspergillus*, *Baccharis*, *Calonectria*, *Cephalosporium*, *Cylindrocarpon*, *Fusarium*, *Myrothecium*, *Penicillium*, *Stachybotrytis*, *Trichoderma*, *Verticimonosporium* spp. by infestation essentially in post-harvested diseased crops (Cole & Cox, 1981; Maxwell *et al.*, 2006; Leung *et al.*, 2006; Frisvad *et al.*, 2007; Magan & Aldred, 2007; Morgavi & Riley, 2007).

Plants infesting economical important crops are another important problem causing marked losses in the agrarian production. Weed pests have always being recognised as one of the most serious agricultural and environmental problems due to competition with the growth of agrarian crops by subtraction of water, nutrients, light and by the serious obstacles they represents for the agronomic activities.

Insects that parasiticzed important cultivations are another heavy problem for agriculture activity. Their diffusions represents a serious damages for the quantity and quality of the agrarian products which in

some cases determine the whole loss of the production. The pest status of an insect population depens on the abundance of individuals as the type of nuisance or injury that the insect inflict. Injury is the usually deleterious effect of insect activities (mostly feeding) on host physiology, whereas damage is the measurable loss of host usefulness, such as yield quality or quantity or aesthetic (Gullan & Crastan, 2005; De Lucia *et al.*, 2008; Inbar & Gerling, 2008).

In agriculture, the control of pathogenic and toxigenic fungi, weeds and insects diffusion is usually achieved by using agrochemicals belonging to different classes of organic compounds, often in large amounts. This causes serious problems to human and animal health and produces heavy environmental pollution. Frequently, the treatments have been repeated and the development of resistance was observed against several synthesized pesticides. On the contrary, biological agents offer the advantage of being fully compatible with the environment, often with high specificity, and represent a long term solution also to control pests particularly resistant to chemical herbicides. Therefore, many efforts have been made for pests control using their natural antagonists, mainly microscopic fungi and/or secondary bioactive metabolites they produce. Microbial metabolites are expected to overcome the increase of the level of resistance to synthetic pesticides that has been found in populations of some major plant pathogens and pests, and are generally regarded as more environment-friendly than their synthetic counterparts (Strobel *et al.*, 1987; Graniti *et al.*, 1989; Delfosse, 1990; Koltin *et al.*, 1993; Ghisalberti, 2000; Evidente & Motta, 2001; Duke *et al.*, 2003; Opender, 2005; Santino *et al.*, 2005; Evidente, 2006; Gullan & Cranstaton, 2005; Evidente & Abouzeid, 2006).

The isolation, chemical and biological charaterisation of several fungal metabolites with fungicide, herbicide, and insecticide activities against pests for plants with agrarian interest, were described. Studies on the structure activity relationships of some fungicides and phytotoxins were also investigated by using natural analogues and preparing key derivatives by their chemical derivatization.

FUNGICIDES

Fungi are a well-known source of bioactive compounds, and the research for isolation of novel fungal antibiotics and agrochemicals that blossomed more than forty years ago is still very active today. In addition to their potential use as agrochemicals or chemotherapeutics, bioactive compounds are also useful as lead molecules, by serving as structural models for the design of new synthetic compounds with higher activity or stability. In

particular, these compounds may disclose novel mechanisms of action capable of overcoming the acquired resistance of plant pathogens to known antibiotics. Moreover, the combined use of bioactive natural compounds with biocontrol agents, *e.g.,* antagonistic yeasts, has been proposed as a profitable approach to achieve additive and/or synergistic effects and provide greater consistency and efficacy of disease control (El-Ghaouth *et al.*, 2000).

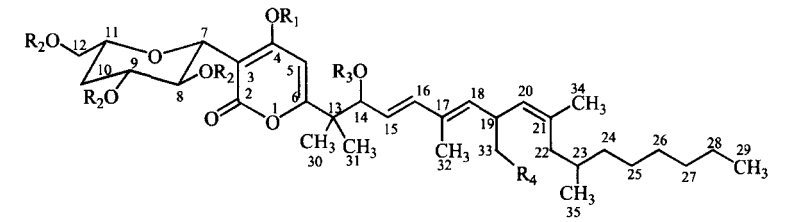
Members of the genus *Fusarium* are widespread throughout the world as soil inhabitants, plant pathogens, and food and feed contaminants. (Boott, 1971; Marasas *et al.*, 1983). Many *Fusarium* species have been investigated for their capability to produce bioactive secondary metabolites, and a number of molecules exhibiting a variety of structures as well as chemical and biological properties have been described so far (Vesonder & Golinski, 1989; Gelderblom *et al.*, 1992). Because of the obvious health and economic implications, *Fusarium* metabolites toxic to animals (mycotoxins) or plants (phytotoxins) have been the focus of most of our research. However, a number of bioactive compounds whose ecological significance and natural occurrence are not yet completely understood have also been isolated and structurally characterized.

α-Pyrones from *Fusarium semitectum*

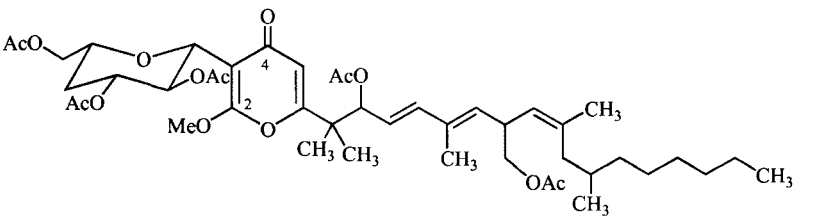
Isolation of fusapyrone and deoxyfusapyrone

During an extensive screening program for the isolation of toxigenic *Fusarium* strains and identification of toxins from cereals collected all over the Mediterranean area, a strain (ITEM-393) of *F. semitectum* Berk. & Rav., whose culture extract exerted a strong antifungal activity towards *Geothricum candidum* Link *ex* Pers. was isolated. A strain of this fungus, isolated from maize stalk rot in southern Italy produced bioactive metabolites when cultured on autroclaved rice kernels. The organic extracs of fungal cultures showed a strong antibiotic activity towards *G. candidum* in disk diffusion assay.

Two new α-pyrones, named Fusapyrone (FP) {3-(4-deoxy-β-*xylo*-hexopyranosyl)-4-hydroxy-6-[2-hydroxy-7-hydroxymethyl-1,1,5,9,11-pentamethyl-3,5,3,5,8-heptadecatrienys heptadecatrienyl]-2H-pyran-2-one} and deoxyfusapyrone (DFP), its 6-[2-hydroxy-1,1,5,7,9,11-hexamethyl] (**1** and **2**, Fig 1) were isolated from this organic extract by chromatographic methods and using the growth inhibition of *G. candidum* as a bioassay to guide the isolation process. The structure of the two α-pyrones was determined by spectroscopic metods (essentially 1D and 2D NMR). In particular, the attachment points of both the alkyl side chain and the 4-deoxy-β-xylo-hexopyranosyl on the 2-pyrone ring were deduced from the clear correlations observed in the 2D $^{13}C,^{1}H$ long range spectra (Evidente *et al.*, 1994).



1 R_1=R_2=R_3=H, R_4=OH
2 R_1=R_2=R_3=R_4=H
3 R_1=R_2=R_3=Ac, R_4=OAc
4 R_1=R_2=R_3=Ac, R_4=H
5 R_1=Me, R_2=R_3=Ac, R_4=OAc



6

Fig 1. The structures of fusapyrone and deoxyfusapyrone (**1** and **2**), their penta- and tetra-acetyl derivatives (**3** and **4**), the 4-*O*-methylpentacetyl derivative of fusapyrones and its 2-*O*-methyl isomers (**5** and **6**)

The structures of the two new α-pyrones (**1** and **2**) were supported by extensive MS investigation carried out using FAB in positive and negative modality and EI ionisation techniques integrated by mass analysed ion kinetic energy (MIKE) experiments with and without collision activation (CAD) (Evidente *et al.*, 1994). The structures of **1** and **2** was confirmed by preparing some key derivatives as the corresponding penta and the teracetyl derivatives (**3** and **4**, Fig 1), the 4-O-methylpentacetylfusapyrone (**5**, Fig 1) and its isomer 2-O-methyl-4-pyrone (**6**, Fig 1), formed during the esterification of **3** with diazomethane (Evidente *et al.*, 1994).

When assayed on *G. candidum* the minimum inhibition concentration (MIC) measured was 1.2 and 2.4 μg/disk for fusapyrone and deoxyfusapyrone, respectively (Evidente *et al.*, 1994).

Other secondary metabolites containing the pyran-2-one moiety (α-pyrones) have been reported to be produced by fungi belonging to several genera, including *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, and *Trichoderma*, and exhibit a wide range of biological activities, such as antibiotic, antifungal, cytotoxic, neurotoxic, and phytotoxic activity (Dickinson, 1993). Members of this class of compounds have also been investigated for their potent antitumor (Suzuki *et al.*, 1997; Kondoh *et al.*, 1998) and HIV protease-inhibiting (Thaisrivongs *et al.*, 1996; Poppe *et al.*, 1997; Turner *et al.*, 1998) properties, and for their plant growth-regulating activity (Nakajima *et al.*, 1987; Kobayashi *et al.*, 1994; Tsuchiya *et al.*, 1997). The chemistry, biochemistry, and toxicology of microbial α-pyrones

have been reviewed by Dickinson (1993). Besides FP and DFP, other α-pyrones isolated and characterized from *Fusarium* cultures include fusalanipyrone (Abraham & Arfmann, 1988), acuminatopyrone (Visconti *et al.*, 1994), the mycotoxin chlamidosporol (Abbas *et al.*, 1992) and its analogues (Solfrizzo *et al.*, 1994), and the phytotoxins poaefusarin and sporofusarin (Dickinson, 1993) and from other fungal species as the 6-substituted 5,6-dihydropyra-2-one from *piper reticulatum* (Maxwell *et al.*, 1998), as well as, natural bioactive 2-pyrones and mimetics (Fairlamb *et al.*, 2004; Mcglacken & Fairlamb, 2005).

HPLC Method for the Analysis of Fusapyrones and Deoxyfusapyrones

Prelimary biological characterization showed that fusapyrone and deoxy fusapyrone exhibited a notherworthy toxic acitivity towards a broad spectrum of filamentous fungi, including some important plant pathogenic and mycotoxigenic fungi. *e.g., Alternaria alternate*, *Aspergillus flavus*, *Penicillium* spp., *Phoma tracheiphila*, *Ascochyta rabiei*, *Cladosporium* spp. and *Botrytis cinerea* (Perrone *et al.*, 1995). They also showed a considerable inhibitory activity on several strains of human pathogenic fungi, including *Candida* spp. and *Aspergillus fumigates*. The activity of fusapyrones against *B. cinerea* is particularly intresting. It is evaluated by experiments on grapes artificially infected with this phytopathogenic fungus. Inoculated grapes, previously treated with a solution (100 mg/l) of **1**, showed significantly reduction of grey mould development on injured grapes and of conidia production invaded tissue (Altomare *et al.*, 1998; Ibidem, 1998b). As **1** and **2** appear also scarsely active towards yeasts and exhibited very low phytotoxicity in leaf-puncture assays, they appear to be promising candidates for practical usage as pesticides and good model for studies on antifungal acitivity mechanisms and structure-activity relationships.

These reasons, along with the potential perspective of commercial use of **1** and **2**, have prompted the development of a rapid, sensitive and quantitative method for their detection in crude sample.

A simple, very sensitive and rapid HPLC method was developed for the simultaneous quantitative analysis of fusapyrone and deoxyfusapyrone in crude extract. Such method was optimised on C-18 reverse phase column, using the isolated metabolites as standards, with a sequence of linear elution steps with MeOH-H_2O mixture and using an ultraviolet detector fixed at 285 nm, where both α-pyrones showed a characteristics absorption maximum. This method was used to quantify the bioactive metabolites in crude organic extracts from two *F. semitectum* strains. The chromatogram (Fig 2) of the crude extractds showed peaks on head and on queue, but those of **1** and **2**

remained well resolved. The recovery values ranged from 84% to 99% for **1** and from 99% to 101% for **2**, indicanting that the method was close to quantitative recovery (Evidente *et al.*, 1999).

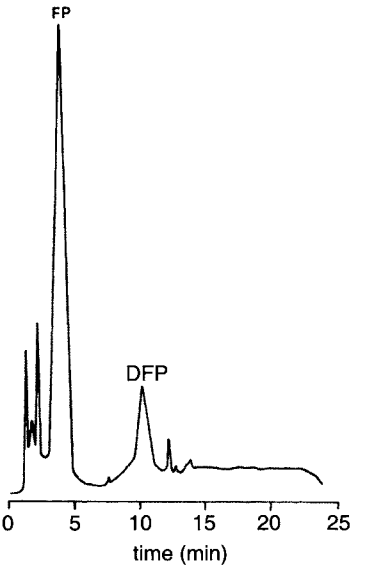


Fig 2. Chromatographic profile of the organic crude extract of a wheat culture of *F. semitectum* ITEM 1623

The developed HPLC analysis method proved to be efficient and sensitive for the identification and quantitative estimation of the two α-pyrones in crude samples. As far as potential practical applications is concerned. These procedures could be of interest for large scale production of the two antifungals, since their synthesis might be very complex, and for evaluation of their impact, such as persistence in agricultural products and soil.

An alternative production procedure on wheat kernels and a new purification procedure of the two α-pyrone were developed. The last was based on the use of an efficient medium pressure chromatography and TLC combined method (Evidente *et al.*, 1999).

Biological characterization of fusapyrones and deoxyfusapyrones

The zootoxicities of **1** and **2** were evaluated using the brine shrimp (*Artemia salina* L.) larvae mortality bioassay. This assay has long been utilized as a simple, rapid, and reliable method to detect antitumor or cytotoxic activity. Compounds **1** and **2** were tested in concentrations ranging from 11.7 to 500 μM. Compound **1** was not toxic to *A. salina* larvae at the highest concentration tested. The LC_{50} value of deoxyfusapyrone (**2**) was 37.1 μM (21.8 μg/ml) (Fig 3) (Altomare *et al.*, 2000).

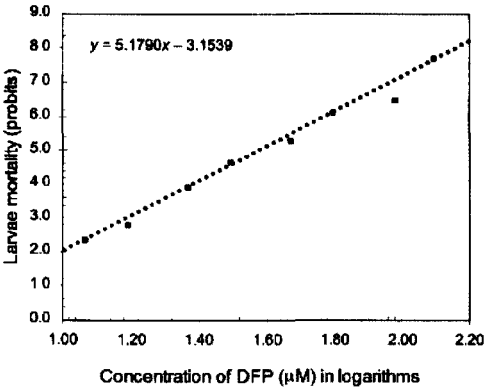


Fig 3. Regression line of the transformed dosage-mortality curve of *A. salina* larvae exposed to deoxyfusapyrone (**2**). Mortality frequencies in terms of probits are plotted vs. logarithms of **2** concentrations. No significant differences in mortality of larvae exposed to **2** for 24 or 36 h were observed (data not shown), hence only the 24 h data sets were used for calculation of the dosage-mortality curve. Data shown are the means of six independent experiments. LC_{50} 37.1 μM (21.8 μg/ml)

The antifungal activities of **1** and **2** were tested on 18 species (24 strains) of plant pathogenic and/or mycotoxigenic filamentous fungi, 11 strains of yeasts isolated from plants, and 10 agents of human mycoses. Both **1** and **2** showed considerable antifungal activity against several filamentous fungi but were inactive against yeasts at the assay dose of 15 μg/disk (Table 1). A wide variability of susceptibilities to the two pyrones was observed, hence their activities seemed to be species- and strain-specific. *Fusarium* species, with the exception of *F. graminearum*, were the least sensitive, while *Alternaria alternata*, *Ascochyta rabiei*, *Aspergillus flavus*, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Phoma tracheiphila*, *Penicillium verrucosum*, and *Penicillium brevicompactum* were the most sensitive species. Compound **1** was consistently more active than **2**. In several cases, **1** was as active or more active than nystatin against *P. tracheiphila* and *P. brevicompactum*. Compound **1** was mostly fungistatic at 5 μg/disk and fungicidal at 15 μg/disk (Altomare *et al.*, 2000).

Table 1. Antifungal activity of fusapyrone (**1**) and deoxyfusapyrone (**2**) in comparison with nystatin toward some filamentous fungi and yeasts (paper disk assay [15 μg/disk])

Species and strains[b]	**Diameter of the inhibition zone (mm)**[a]		
	1	**2**	**Nystatin**
Filamentous fungi			
Alternaria alternata ITEM 468	18.8 ++	10.8 ++	30.2 +++
A. alternata ITEM 511	18.0 +++	11.0 +	32.2 +++

Table 1. *Contd.*

Species and strains[b]	Diameter of the inhibition zone (mm)[a]		
	1	2	Nystatin
A. alternata ITEM 526	20.3 +++	11.7 +	28.7 +++
A. alternata ITEM 750	18.7 ++	10.5 +	29.2 +++
A. citri ITEM 466	18.0 ++	9.2 +	30.0 +++
Ascochyta rabiei ITEM 1067	35.8 +++	12.0 +++	38.5 +++
Aspergillus flavus ITEM 9	27.3 ++	12.4 ++	29.0 +++
A. parasiticus ITEM 11	27.5 ++	12.7 ++	28.7 +++
Botrytis cinerea ITEM 966	29.3 +++	13.5 +++	31.2 +++
B. cinerea	30.0 +++	17.0 +	31.0 +++
Cladosporium cladosporioides ITEM 2079	28.7 ++	16.2 +	37.3 +++
C. cucumerinum ITEM 2095	33.3 +++	13.5 ++	32.7 +++
Colletotrichum gloeosporioides ITEM 1729	31.8 +++	13.8 ++	38.2 +++
Fusarium acuminatum ITEM 795	17.8 +	0	20.8 +++
F. graminearum ITEM 2	26.5 +++	10.8 ++	25.9 +++
F. moniliforme ITEM 1497	20.1 +	0	18.7 +++
F. oxysporum ITEM 149	27.7 +	4.0 +	19.5 +++
F. oxysporum ITEM 1462	28.3 +	0	20.0 +++
F. oxysporum f. sp. lycopersici ITEM 1586	30.0 +	4.0 +	21.7 +++
F. subglutinans	6.0 +	0	24.0 +++
F. semitectum ITEM 393	28.7 ++	8.0 +	27.0 +++
Phoma tracheiphila ITEM 1605	40.0 +++	16.3 +++	31.8 +++
Penicillium verrucosum ITEM 439	29.7 +++	11.0 ++	28.2 +++
P. brevi-compactum ITEM 449	30.0 +++	10.5 ++	18.3 +++
Yeasts			
Candida guilliermondii ITEM 1638	0	0	20.0 +++
C. maltosa ITEM 1639	0	0	20.0 +++
Kluyveromyces fragilis ITEM 1657	0	0	19.5 +++
K. lactis ITEM 165	0	0	18.0 +++
Pichia anomala ITEM 1625	0	0	18.8 +++
P. anomala ITEM 1661	0	0	15.0 +++
P. dryadoides ITEM 1663	0	0	19.0 +++
P. guilliermondii ITEM 1644	0	0	18.5 +++
P. kluyveri ITEM 1649	0	0	16.5 +++
Saccharomyces cerevisiae ITEM 1633	0	0	21.0 +++
Rhodotorula pilimanae	0	0	28.5 +++

[a]Activity is classified as: 0) no effect; +) weakly fungistatic (reduced density of fungal growth); ++) fungistatic (no growth); +++) fungicidal. [b] ITEM codes refer to the Istituto Tossine e Micotossine da Parassiti Vegetali Culture Collection.

Among the agents of human mycoses that were examined, *Aspergilli* were the most sensitive to **1** and **2**, while some species-specific variability was found among the yeasts (Table 2). In particular, *Cryptococcus*

neoformans was inhibited by both **1** and **2**, *Candida kefyr* only by **1**, and *Candida albicans* and *Candida glabrata* were inhibited by neither compound at doses as high as 50 μg/ml (Altomare *et al.*, 2000).

The phytotoxicity of **1** and **2** to plant cells and organs was also determined and compared to fusaric acid (FA), a well-known *Fusarium* phytotoxin. The results of the cytotoxicity bioassay performed on chickpea cells are shown in Fig 4. Compounds **1** and **2** were much less phytotoxic than FA, their LC_{50} values being about one tenth that of FA (Fig 4). Neither α-pyrone was toxic at a concentration of 10^{-5} M.

Table 2. Antifungal activity of fusapyrone (**1**) and deoxyfusapyrone (**2**) toward yeasts and filamentous fungi on human mycoses

Species and strain		Minimum inhibitory concentration (μg/ml)		
		24 h	48h	72 h
Candida kefyr Y0601	1	0.78	1.56	3.12
	2	>50.00		
C. albicans Y01009	1	>50.00		
	2	>50.00		
*C. albicans*1	1	>50.00		
	2	>50.00		
*C. albicans*2	1	>50.00		
	2	>50.00		
C. glabrata 12	1	50.00	>50.00	
	2	>50.00		
Cryptococcus neoformans 13	1	6.25	6.25	6.25
	2	6.25	6.25	6.25
Cryptococcus neoformans 14	1	3.12	3.12	3.12
	2	3.12	3.12	3.12
Aspergillus fumigatus 1	1	1.56	1.56	1.56
	2	3.12	3.12	3.12
A. niger 2	1	1.56	3.12	3.12
	2	1.56	1.56	1.56
A. flavus 3	1	1.56	1.56	1.56
	2	1.56	1.56	1.56

In the tomato-leaf puncture assay, only **1** was active, causing necrotic spots at concentrations of 10^{-2} and 10^{-3} M (2-mm and 1-mm diameter, respectively). When the leaf puncture assay was performed on chickpea leaves, both toxins caused chlorosis and necrosis at concentrations $\geq 10^{-3}$ M. By comparison, FA caused severe symptoms on both tomato and chickpea leaves at 5×10^{-4} and 10^{-4} M, respectively. No symptoms were observed on tomato cuttings treated with 10^{-3} M of either **1** or **2**, while the first symptom of phytotoxicity (chlorosis of leaf veins) to cuttings treated with FA was detected at the concentration of 10^{-5} M, and complete wilting (leaves and

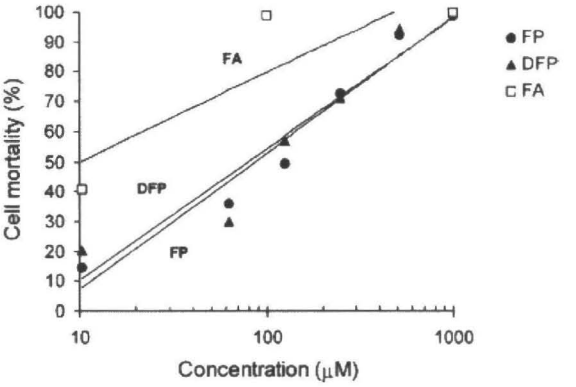


Fig 4. Toxicity of fusapyrone (**1**), deoxyfusapyrone (**2**), and fusaric acid to chickpea cells. Data shown are the means from three independent experiments

stems) was observed at 10^{-4} M. Neither **1** nor **2** showed phytotoxic activity in the tomato seedling germination assay. On the contrary, at the doses of 10^{-4} and 10^{-5} M, **2** stimulated the elongation of rootlets, the values being more than 120% of the control (Fig 5). Shoot length was unaffected by treatment with either **1** or **2** (Altomare *et al.*, 2000).

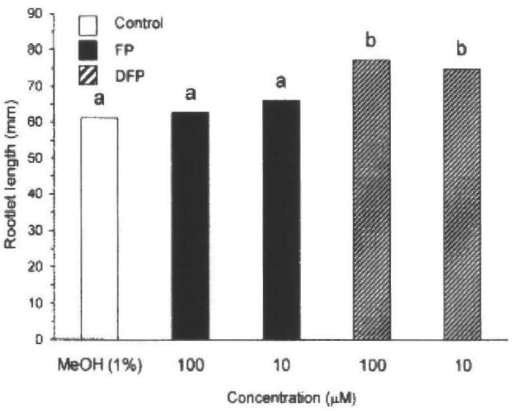


Fig 5. Tomato seedling growth assay. Bars with the same letter ere not different according to Duncan's multiple range test ($p<0.05$)

While **1** was not toxic to *A. salina*, the toxicity of **2** was similar to fusaproliferin (LC_{50}) (23.7 μg/ml), but 10- to >100-fold lower than the beauvericin and the trichothecenes (Smith, 1989). To evaluate the actual mycotoxicological significance of **2**, it would be useful to investigate the production of this toxin by other *Fusarium* species and its occurrence in naturally infested agricultural commodities. According to McLaughlin (1991) compounds with LC_{50} < 1000 ppm in the brine-shrimp lethality

assay are considered active and potentially cytotoxic against tumor cell lines. DFP (**2**) showed a noteworthy toxicity in this assay, indicating that it may be an interesting compound to be tested in more specific antitumor systems.

Compounds **1** and **2** showed antifungal activity against filamentous fungi, while no activity was observed against yeasts isolated from plants. In addition, in an agar diffusion assay, both **1** and **2** were inactive toward the Gram-positive bacterium *Bacillus megaterium* at the dose of 30 μg/disk (data not shown). Compounds **1** and **2** also showed a differential antifungal activity toward difficult-to-treat human pathogenic fungi such as *Aspergillus* spp. Interestingly, *C. kefyr*, an emergent opportunistic pathogen, showed a remarkable sensitivity only to **1**.

Neither **1** nor **2** were phytotoxic in a panel of assays that evaluated wilt-, chlorosis-, and necrosis-inducing activity. Furthermore, DFP (**2**) showed plant growth-regulating activity, as it stimulated the root elongation of tomato seedlings. Plant growth regulating activity has also been reported for other molecules belonging to the chemical family of α-pyrones, although with different findings. While neovasinone, a metabolite from *Neocomospora vasinfecta*, was reported to promote the root growth of lettuce seedlings (Nakajima *et al.*, 1987), 6-pentyl-α-pyrone from *Trichoderma harzianum* inhibited coleoptile elongation of etiolated wheat germlings (Cutler *et al.*, 1986). Interestingly, the phytotoxic activity of α-pyrones is greatly affected by different moieties bound to the common active core, the α-pyrone ring (Altomare *et al.*, 2000).

In the past decades, bioactive metabolites of microbial origin have been the subject of scientific research in several fields, including pharmacology, food science, mycotoxicology, and plant pathology. A relatively novel and promising field of study is the application of these compounds in agriculture, as pesticides, herbicides, or plant-growth regulators (Tanaka *et al.*, 1993; Evidente *et al.*, 2003b). In fact, microbial metabolites are expected to overcome the resistance and pollution that have accompanied the use of synthetic pesticides and can inspire the synthesis of new environmental friendly molecules. The considerable antifungal activity of FP (**1**) is of some interest for its possible use in agriculture, especially in consideration of its low phytotoxicity and mycotoxicity evidenced.

Structure-activity relatonships of fusapyrone derivatives

In spite of the close structural similarity, **1** and **2** exhibit distinct biological activities. In particular, **1** has been shown to be a potent antifungal compound with low zootoxicity in *Artemia salina* L. larvae mortality bioassays, whereas

2 had higher zootoxicity (LC_{50} to *A. salina* = 37.1 μm) (Altomare *et al.*, 2000). Therefore, in addition to the well-known bioactive properties of the α-pyrone ring, the functionalities of the aliphatic chain also seems to have a role in affecting, both quantitatively and qualitatively, the biological activity of these natural compounds.

A structure-activity relationships study, with respect to both antifungal and zootoxic activity, of these compounds and seven different chemical derivatives of **1** and one derivative of **2** was carried out. The aims of this study were: 1) verify if new molecules with increased antifungal activity could be generated; 2) evaluate the toxicity of the derivatives in the perspective of the potential use of these molecules as agrochemicals or chemotherapeutics.

Chemical transformation were conducted in order to modify the three chemical moieties present in fusapyrone (**1**) (the glycosyl residue, the 2-pyrone ring, the aliphatic chain or a combination thereof) and to prepare several key derivatives to investigate structure-activity relationship in comparison to **1** and **2**. By reaction with diazomethane, **1** was converted into the 4-*O*-methyl derivative (**7**, Fig 6) which showed a modification of the pyrone moiety. The oxidation with sodium periodate of the sugar residue at C-3 followed by the reduction of the oxidized intermediate gave the 3-(2-hydroxyethyl)-derivative of fusapyrone (**8**, Fig 6) showing the degradation of the glycosyl moiety. Finally, the catalytic hydrogenation of **1** allowed the preparation of two derivatives modified on the alkyl side chain attached at C-6. Derivatives **9** and **10** (Fig 6) were obtained from this reaction and showed the partial and the total saturation of the three olefinic bonds of polysubstituted heptadecatrienyl residue at C-6, respectively (Altomare *et al.*, 2004). The latter was also modified in deoxyfusapyrone (**2**), which presents a methyl group at C-19 in respect to the hydroxymethyl group of **1**. The three structural components present in fusapyrone (**1**) were all modified, although in a reversible form, by acetylation of all the hydroxy groups carried out under standard conditions, thereby converting **1** into the pentacetyl derivative (**3**, Fig 1). The same chemical transformation was performed on deoxyfusapyrone (**2**) to obtain the corresponding tetracetyl derivative (**4**, Fig 1), which is also modified on the same three moieties. Finally, the pentacetyl derivative of **1** (**3**) by reaction with diazomethane was converted in part into the corresponding 4-*O*-methyl derivative (**5**, Fig 1) and in part in the 2-*O*-methyl derivative of the isomeric γ-pyrone (**6**, Fig 1), which allowed us to test also a derivative of a 2-hydroxy-4-pyrone (Altomare *et al.*, 2002).

In order to compare biological activity of **1**, **2** and their derivatives, we used 50% lethal concentration (LC_{50}) to *A. salina* larvae, and minimum inhibitory

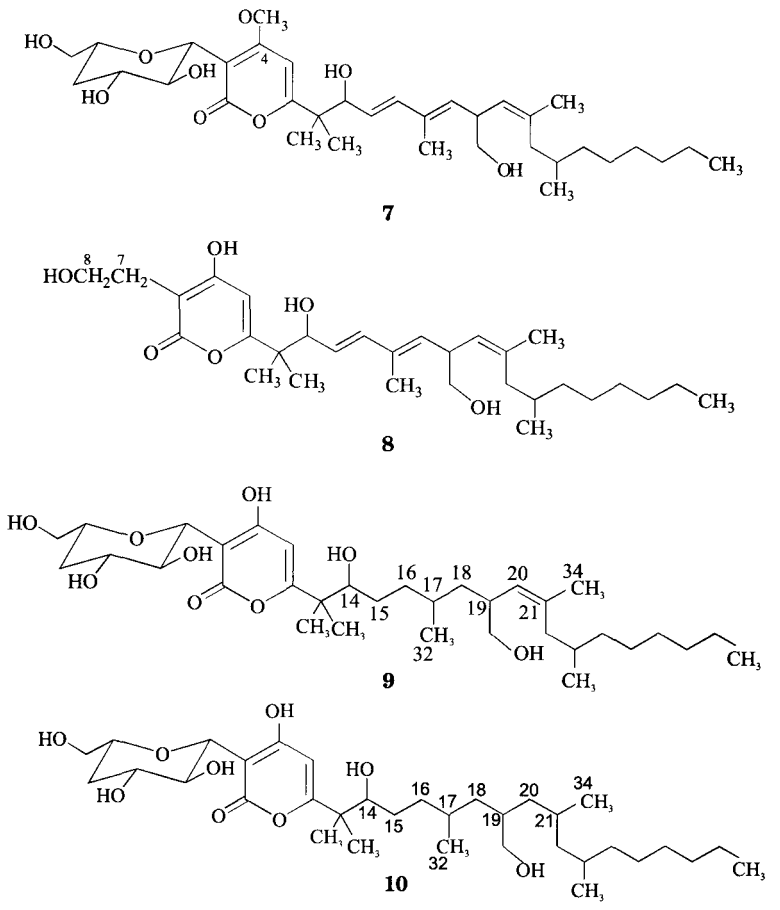


Fig 6. The structures of some derivatives prepared by chemical medication of the three moieties (α-pyrone, sugar and alkyl side chain) of fusapyrones (**7-10**)

concentration (MIC) to several filamentous fungi and yeasts. Toxicity data of compounds **3-6**, compared to **1** and **2** are summarized in Table 3. Among the fusapyrone (**1**) derivatives, compounds **7-10** were scarcely toxic to *A. salina*, similarly to **1** (LC_{50} > 200 μM). Acetylation of **1** into **3**, **5** and **6** resulted in an increase of toxicity, to a higher extent in the two latter derivatives containing also a methylation, respectively in α- and γ-position. Compound **4**, the tetracetyl derivative of deoxyfusapyrone (**2**), did not show a significative difference in toxicity from the lead molecule (Altomare *et al.*, 2004).

The only structural changes of **1** that resulted in an increase of biological activity in *A. salina* bioassay were those that affected mainly the water solubility of the molecule. In fact, the conversion of the three hydroxy groups of the glycosyl residue, as well as those of the side chain, resulting in the corresponding pentacetyl derivative **3**, lowered the polarity of the molecule. Moreover, the derivatization of the hydroxy pyrone group into

the corresponding methyl ether resulting in derivatives **5** and **6** further lowered the polarity and, consequently, the water solubility of these compounds. Hence, it appears that toxicity is correlated to hydrophobicity (Altomare *et al.*, 2004).

Table 3. Toxicity of fusapyrone (**1**), deoxyfusapyrone (**2**) and their derivatives (**3-6**) to *Artemia salina* larvae after 24 h exposure

Compound[a]	MW	LC_{50} (μm)	95% Confidence Limits (μm)	Slope	LC_{50} (μg/mL)
1	606	>200.0	-	-	-
2	590	37.1[b]	-	-	21.9
3	816	85.6	71.7 - 105.9	1.721	69.8
4	758	39.7	31.4 - 49.9	1.791	30.1
5	830	46.1	35.1 - 65.4	1.167	38.3
6	830	51.9	40.6 - 70.5	1.459	43.1

[a] Compounds not listed were not toxic at the highest concentration tested (200 μm).
[b] Altomare *et al.*, 2000.

The brine shrimp (*A. salina*) lethality assay has been reported to be useful as a preliminary test for identification of cytotoxic substances (McLauglin, 1991; Logrieco *et al.*, 1996). As above cited, according to McLaughlin (1991), compounds with LC_{50} < 1000 ppm are considered potentially toxic against tumor cell lines and worthy of being evaluated in more specific antitumor systems. Based on the results of the present study, compounds **2-6** could be evaluated for this use (Altomare *et al.*, 2004).

The antifungal bioassays showed the same results in the three independent experiments performed. The antifungal activity tests showed that all of the modifications made to **1** and **2** resulted in a loss of toxicity towards filamentous fungi. Among derivatives **3-10**, only compounds **7**, **9** and **10** retained some activity, limited to *B. cinerea* and only at high concentration (Table 4), while all the others were inactive. Antifungal activity of **1** and **2** showed to be considerably higher than either benomyl and dicloran on *A. parasiticus* and *P. brevi-compactum*, where only benomyl was able to reduce the germ tube elongation but not to inhibit the germination of conidia. Compounds **1** and **2** were also more active than dicloran, but not than benomyl, toward *B. cinerea*.

None of the compounds **1-10** inhibited the growth of yeasts at the highest concentration tested (50 μg/ml) (Altomare *et al.*, 2004).

1 and **2** structurally consist of a hydrophilic sugar residue opposed to a long hydrophobic chain, and therefore possess an amphiphilic nature that in other cases has been linked to antifungal and antibiotic properties (Stanghellini *et al.*, 1997; Vollenbroich *et al.*, 1997). These molecules, known as biosurfactants, interact with plasma membranes and cause loss of membrane integrity and cell lysis. A similar mechanism may be involved in

Table 4. Antifungal activity of fusapyrone (**1**), deoxyfusapyrone (**2**) and some derivatives, in comparison with chemical fungicides benomyl and dicloran, towards filamentous fungi

Species and strain	Compound[b]	MIC[a] (μg/mL)		
		24 h	48 h	72 h
Aspergillus parasiticus ITEM-11	**1**	6.25	6.25	12.50
	2	0.78	0.78	0.78
	Benomyl	>25.00	>25.00	>25.00
	Dicloran	>25.00	>25.00	>25.00
Botrytis cinerea ITEM-966	**1**	1.56	3.12	6.25
	2	0.78	1.56	1.56
	7	25.00	50.00	50.00
	9	50.00	50.00	50.00
	10	50.00	50.00	50.00
	Benomyl	0.19	0.19	0.19
	Dicloran	3.12	6.25	6.25
Penicillium brevi-compactum ITEM-449	**1**	0.78	3.12	6.25
	2	0.78	1.56	1.56
	Benomyl	>25.00	>25.00	>25.00
	Dicloran	12.50	25.00	>25.00

[a] Minimum inhibitory concentration

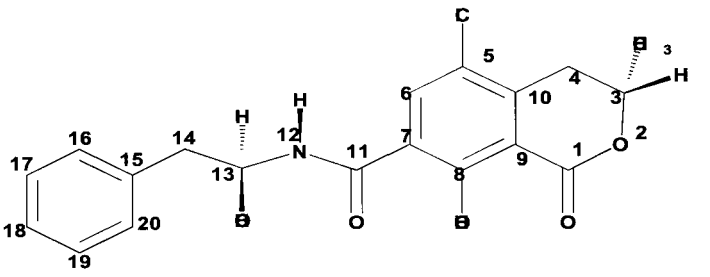
[b] Compounds not listed did not inhibit fungal growth at the highest concentration tested (50 μg/ml). The highest concentration of chemical fungicides tested was 25 μg/ml.

antifungal activity of **1**, **2** and, in a lesser extent, of **7**, **9** and **10**. The different sensitivity exhibited by filamentous fungi and yeasts is likely due to diversity in cell wall structure and permeability to these molecules, or to a variable fatty acid composition of plasma membranes (Gruiz, 1996). Should be this the mechanism of action of α-pyrones, the probability of developing insensitive strains would be low, since development of resistance would require a major structural change in the chemical makeup of the plasma membrane. This is particularly important for those phytopathogenic fungi, such as *B. cinerea*, that easily develop resistance to chemical pesticides. Fusapyrone (**1**), applied as a 100 μg/ml solution, was found to be very active in inhibiting the development of grey mold (*B. cinerea*) on wounded grapes *in vitro* (Altomare *et al.*, 1998).

The two yeasts (*P. guilliermondii*, and *R. glutinis*) that were utilised in our bioassays are effective biological control agents that have been successfully used for the control of post-harvest diseases of citrus fruits (Arras *et al.*, 2002). These particular strains were chosen as test organisms in order to assess their tolerance to the α-pyrones for a possible use of α-pyrones in combination with biocontrol agents for plant disease control. Therefore the combined use of **1** and **2** with antagonistic yeasts appears to be a feasible approach for reduction of synthetic pesticides. **1** appears to be particularly interesting because of its lower zootoxicity (Altomare *et al.*, 2004).

Inhibition of fungi species ochratoxin a producers

Ochratoxin A (OTA, **11**, Fig 7) is a mycotoxin, with nephrotoxic, teratogenic, immunosuppressive, and carcinogenic properties (Walker & Larsen, 2005), that has been classified by the International Agency for Research on Cancer (1993) as a possible human carcinogen (group 2B). OTA was first isolated from moldy corn meal in South Africa (Van der Merve *et al.*, 1965). Subsequently, OTA has been found in a number of agricultural commodities and foodstuffs, including cereal, coffee beans, and beer, as a by-product of contamination with fungi of the yeast genera *Aspergillus* and *Penicillium*, mainly *Aspergillus ochraceus* (also known as *Aspergillus alutaceus*) and *Penicillium verrucosum*. In recent years, there has been a growing interest in the occurrence of OTA in grapes and grape derivatives. In particular, concern has been raised for OTA contamination of wine grapes, due to the large and increasing consumption of wine and the economical relevance of wine industry (Zimmerli & Dick, 1996; Burdaspal & Legarda, 1999; Battilani & Pietri, 2002; Tjamos *et al.*, 2004). Several reports have indicated that members of the *Aspergillus* section *Nigri*, the so-called black aspergilli, are the dominant ochratoxigenic species on wine grapes worldwide (Battilani *et al.*, 2006; Bau *et al.*, 2006; Chulze *et al.*, 2006; LeOng *et al.*, 2006). Among these species, *Aspergillus carbonarius* seems to be the most important source of OTA because of the high proportion of producing strains and high amounts produced (Battilani *et al.*, 2003; Perrone *et al.*, 2006). There are evidences that grapes are already contaminated with OTA before harvest (Battilani *et al.*, 2003; Magnoli *et al.*, 2003; Bau *et al.*, 2005; Serra *et al.*, 2006), although its concentration may increase substantially in the time between harvest and alcoholic fermentation (Zimmerli & Dick, 1996; Grazioli *et al.*, 2006). Therefore, management of the sanitary state of grapes is a critical point in a strategy aimed at the prevention of OTA occurrence in wine. Unfortunately, very few chemical pesticides seem to be effective (Tjamos *et al.*, 2004; Leong *et al.*, 2006). In addition, the intensive use of these compounds may cause different important drawbacks, such as a loss of natural competitors, arising from resistant pathogen populations, and the presence of residues in the products and in the environment. In this context, the availability of alternative methods to control of black aspergilli would be highly desirable. A relatively novel and promising field of study is the application of antimicrobial compounds of microbial origin as an alternative to synthetic pesticides (Tanaka & Omura, 1993). Fusapyrone exhibited considerable antifungal activity against several plant pathogenic, mycotoxigenic, and human pathogenic filamentous fungi, including *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus niger*, and *Aspergillus fumigatus* (Altomare *et al.*, 2000). In consideration of these features, we examined the ability of **1** to inhibit the growth of the main ochratoxigenic species of *Aspergillus* section *Nigri*, *viz.*, *A. carbonarius*, *A. niger*, and *Aspergillus tubingensis*, and to prevent fungal colonization and OTA production in grapes.



11

Fig 7. Structure of ochratoxin A(**11**)

Inhibitory activity of fusapyrone to ochratoxigenic *Aspergillus* spp.

The antifungal activity of FP was tested against 16 *Aspergillus* isolates belonging to three different ochratoxigenic species, *viz.*, *A.carbonarius*, *A. niger*, and *A. tubingensis*. The *in vitro* inhibitory activity of FP to OTA-producing aspergilli is summarized in Table 5. The MIC method is subject to inherent variability, and therefore, the procedure is generally considered accurate within ±1 twofold dilution (Wexler *et al.*, 1990). The data are presented in Table 5 in the form of a MIC range for each isolate tested. In addition, average MICs of different species were calculated to allow for comparisons of susceptibility. Different susceptibilities to fusapyrone of the species tested were found. *A. carbonarius* was the most sensitive, whereas *A. niger* and *A. tubingensis* were less susceptible. Inhibitory effects (reduction of percentage of germinated conidia and germ tube length) were also found at concentrations lower than MICs. Strong inhibition of growth and morphological changes were still observed at half the MIC (sub-MIC) after 7 days (Fig 8) (Favilla *et al.*, 2008).

Under these conditions, germ tubes of *A. niger* and *A. tubingensis* exhibited severe thickening and irregular growth. Based on the amphiphilic nature of fusapyrone, it was hypothesized that **1** has an effect on plasma membrane function and integrity, similar to that of antifungal biosurfactants (Altomare *et al.*, 2004). Variation in sensitivity exhibited by different species in the *Aspergillus* section *Nigri* is likely due to diversity in cell wall structure, composition, and permeability to **1** as well as to a variable fatty acid composition of plasma membranes (Gruiz, 1996). Differences in the cell wall composition of species in the *Aspergillus* section *Nigri*, besides being a useful character for taxonomy, systematics, and phylogeny studies (Bartnicki-Gracía, 1987; Ahrazem *et al.*, 2001) of this group, might also have practical implications. Some fungicides (*e.g.*, organophosphorus and carboxylic acid amides) act as inhibitors of phospholipids and cell wall component biosynthesis and deposition. Therefore, it is conceivable that variations in plasma membrane and cell wall composition may result in

different efficacies of these fungicides in controlling of different ochratoxigenic species.

Table 5. MIC of fusapyrone (**1**) to ochratoxigenic strains belonging to *Aspergillus* section *Nigri*

Species and isolate[a]	Origin	MIC range (μg/mL) at[b]:			
		24 h	48 h	72 h	7 days
A. carbonarious					
ITEM 4167	Italy	3.1–6.2	6.2–12.5	6.2–12.5	6.2–25
ITEM 4550 (IMI 387223)	Portugal	3.1–6.2	6.2–12.5	6.2–12.5	6.2–12.5
ITEM 4838 (IMI 388654)	Italy	3.1–12.5	6.2–12.5	6.2–12.5	6.2–25
ITEM 4854	Italy	3.1–12.5	3.1–12.5	3.1–25	3.1–25
ITEM 4968 (IMI 387411)	Israel	6.2	12.5	12.5	12.5
ITEM 4929 (IMI 388716)	Spain	3.1–12.5	3.1–12.5	6.2–25	6.2–25
ITEM 4930 (IMI 388717)	Spain	3.1–12.5	6.2–12.5	6.2–12.5	6.2–25
ITEM 5000	Italy	3.1–12.5	6.2–12.5	6.2–25	6.2–25
ITEM 5007	Italy	3.1–6.2	6.2–12.5	6.2–12.5	6.2–25
ITEM 5012 5	Italy	3.1–12.5	6.2–12.	6.2–12.5	6.2–25
Geomtric mean ± SE		6.0 ± 0.7[a]	9.5 ± 0.8[a]	10.4 ± 0.9[a]	12.5 ± 0.8[a]
A. niger					
ITEM 7096	Italy	12.5–25	12.5–50	25–50	50
ITEM 7097	Italy	6.2–12.5	12.5–25	12.5–25	25–50
ITEM 7098	Italy	6.2–12.5	6.2–12.5	12.5–25	12.5–50
Geometric mean ± SE		11.6 ± 1.9[b]	15.7 ± 4.4[b]	25 ± 6.6[b]	36.7 ± 6.2[b]
A. tubingensis					
ITEM 4496	Italy	6.2–12.5	6.2–12.5	12.5–25	12.5–50
ITEM 4709	Italy	6.2–12.5	12.5–25	12.5–25	12.5–50
ITEM 4861	Italy	6.2–12.5	12.5–25	12.5–25	12.5–50
Geometric mean ± SE		9.9 ± 0[b]	13.5 ± 1.9[ab]	19.8 ± 0[b]	25 ± 0[c]

[a]ITEM, culture collections from the Institute of Sciences of Food Production, Bari, Italy; IMI, culture collections CABI Bioscience Genetic Resource Collection, Egham, United Kingdom. *Aspergillus* section *Nigri* species were isolated from grapes.

[b]Range of MIC found in three independent experiments. Geometric means were adopted since in twofold dilutions the arithmetic mean is not an accurate measure of central tendency (40). MICs were transformed into base 2 logs to approximate a normal distribution and subject to one-way analysis of variance. Means within columns followed by different letters are significantly different ($p<0.05$) by Tukey's multiple comparison test.

Inhibition of *A. carbonarius* by fusapyrone on artificially inoculated grape berries and effect on OTA content

Bunches of red wine grape variety Negroamaro were collected from vineyards located in the Salento area (southern Apulia, Italy) 7 to 10 days before the regular harvest date. Berries were wounded with a sterile needle

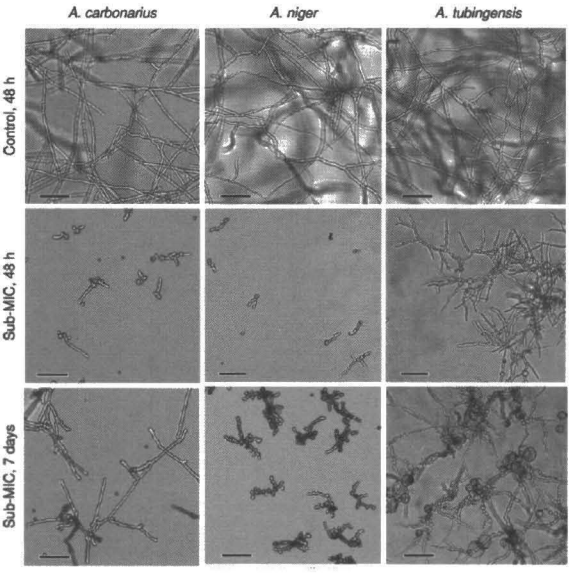


Fig 8. Effects of sublethal concentrations (sub-MIC) of fusapyrone on the growth of germinated conidia of *A. carbonarius*, *A. niger*, and *A. tubingensis*. Germinated conidia of *A. niger* and *A. tubingensis* showed severe alterations of morphology that consisted of thickening and irregular growth of hyphae that formed pronounced bulges. Abnormal swelling of cells was observed in the *A. tubingensis* isolates. (Top) Controls at 48 h. (Middle) Half MIC, 48 h of exposure. (Bottom) half MIC, 7 days of exposure. Bar =100 μm

in two symmetrical abaxial points and sprayed with sterile distilled water (negative control) or the conidial suspension of *A. carbonarius* ITEM 4167. Growth of the OTA-producing strain *A. carbonarius* ITEM 4167 in artificially inoculated grapes was evaluated by determination of CFU.

Three independent trials were carried out in which FP was applied at both 100-μg/mL and 50-μg/mL rates. Interestingly, in these experiments, *A. carbonarius* was isolated from the surface sterilized berries of negative controls. This result suggests endophytic behavior of the pathogen, which may be of importance for optimizing an effective field control strategy. The variability among different experiments in the development of *A. carbonarius* infections and in the level of natural contamination of the negative control prompted us to evaluate the results of these experiments separately (Table 6). In spite of the above variability, a consistent trend was found among different experiments. FP applied at either a 100- or a 50-μg/ml rate resulted in a significant ($p<0.05$) reduction of *A. carbonarius* infections. The treatment of berries with a solution of fusapyrone at 100 μg/ml resulted in a reduction of *A. carbonarius* biomass from 2 to 6 orders of magnitude. In two out of three experiments, the reduction of *A. carbonarius* growth achieved with fusapyrone at 50 μg/ml was smaller

Table 6. Effect of FP on *A. carbonarius* growth and OTA production in artificially inoculated grapes[a]

Treatment	Expt 1		Expt 2		Expt 3	
	***A. carbonarius* growth (CFU/g)**	**OTA production (ng/g ± SD)**	***A. carbonarius* growth (CFU/g)**	**OTA production (ng/g ± SD)**	***A. carbonarius* growth (CFU/g)**	**OTA production (ng/g ± SD)**
Water (negative control)	0^{a}	0.2 ± 0.2^{a}	1.5 ± 10^{5a}	2.8 ± 4.3^{a}	0^{a}	0.03 ± 0.01^{a}
A. carbonarius	1.7 ± 10^{8c}	54.7 ± 3.3^{c}	2.5 ± 10^{8b}	166.3 ± 59.1^{b}	3.3 ± 10^{8d}	116.6 ± 47.8^{b}
A. carbonarius ± FP 100 μg/ml	8.0 ± 10^{5b}	0.5 ± 0.2^{ab}	1.5 ± 10^{2a}	0.02 ± 0.05^{a}	1.0 ± 10^{2b}	1.3 ± 0.7^{a}
A. carbonarius ± FP 50 μg/ml	1.0 ± 10^{6b}	11.0 ± 8.5^{b}	6.8 ± 10^{2a}	0.5 ± 0.5^{a}	6.3 ± 10^{5c}	3.8 ± 3.5^{a}

[a]Values are the means of at least three replicates. Data of CFU and OTA content were subjected to one-way analysis of variance. Mean values within columns followed by different letters are significantly different ($P<0.05$) by Tukey's multiple comparison test.

but not statistically different ($p<0.05$) from that achieved with FP at 100 μg/ml (Favilla *et al.*, 2008).

The data of OTA content in artificially inoculated grapes treated with fusapyrone are shown in Table 6. In all three experiments, berries treated with either 100 or 50 μg/ml of FP showed asignificant ($p<0.05$) reduction of OTA content compared to the level for the positive control. The treatment with 100 μg/ml of fusapyrone prevented the accumulation of OTA in the berries to level comparable to the negative control (0.01 to 1% of the positive control) (Favilla *et al.*, 2008).

Fusapyrone proved to possess a strong inhibitory activity toward three ochratoxigenic *Aspergillus* species belonging to the section *Niger* that are the major source of OTA in grapes and grape-derived foods and beverages.

Dramatic reductions of the OTA content, compared to the level for the positive control (112.5 ng/g of grape), were obtained with application of either 100 or 50 μg/ml of fusapyrone.

In conclusion, our results show that fusapyrone is highly effective ininhibiting the growth of black aspergilli, particularly *A. carbonarius*, and prevent OTA occurrence in infected grape berries. These findings warrant further studies to assess whether the use of fusapyrone is a feasible strategy for the prevention of OTA occurrence in grapes and grape-derived products under field conditions (Favilla *et al.*, 2008).

Antigungal Metabolites from *Trichoderma viride*

α-pyrones from *Trichoderma viride*

Seeking for fungi suitable for the biological control of soil-borne plant pathogens, we found a strain of *Trichoderma viride* showing antagonistic activity, *in vitro* and *in vivo*, towards *Sclerotium rolfsii*, the causal agent of crown and stem rot of artichoke (Marras *et al.*, 1994; Spiga *et al.*, 1998). The antagonistic activity exhibited by *Trichoderma* spp. strains may in part be explained by the production of different classes of bioactive metabolites, including antibiotics, which are inhibitors of fungal growth and enzymes (Papavizas, 1985; Ghisalberti & Sivasithamparam, 1991; Sivasithamparam & Ghisalberti, 1998).

Previously isoharziandione, a new tetracyclic diterpene, was isolated and characterized from the culture filtrates of strain IPVS 1817 of *T. viride* able to inhibit fungal growth of *S. rolfsii* (Mannina *et al.*, 1997), together with 6-*n*-pentyl-2H-pyran-2-one (**12**, Fig 9) (Cooney *et al.*, 1997).

A new antifungal 6-substituted 2H-pyran-2-one, named viridepyronone (**13**, Fig 9), has been isolated from a cultural filtrate of a strain of *Trichoderma viride* showing antagonistic activity *in vitro* towards *Sclerotium*

rolfsii, which is the causal agent of crown and stem rot of artichoke. Viridepyronone was characterized as 6-(4-oxopentyl)-2H-2-pyranone (**13**) using spectroscopic methods.

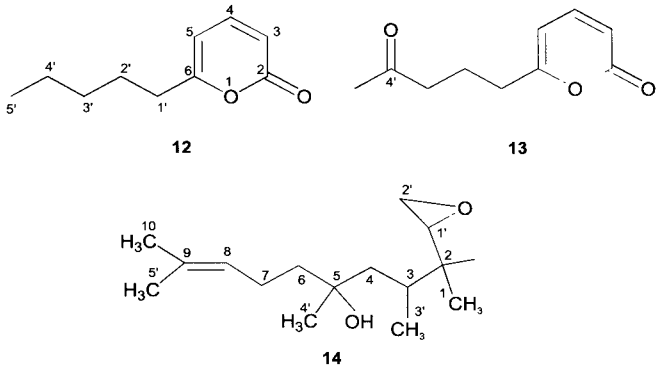


Fig 9. Structure of 6-*n*-pentyl-2H-pyran-2-one, viridepyronone and viridenepoxydiol (**12, 13** and **14**) produced by *Trichoderma viride*

The structure of **13** was supported by the $^{1}H,^{13}C$ long range correlations observed in the HMBC spectrum, and by MS spectra (Evidente *et al.*, 2003b).

Pyran-2-ones (α-pyrones) are a group of naturally occurring compounds which are broadly distributed in nature as plant, animal, marine organism and microbial metabolites, often with interesting biological activity (Dean, 1963; Thomson, 1985; Moreno-Manas & Pleixats, 1992) and the total synthesis of some of them has been achieved (Moreno-Manas & Pleixats, 1992). Other secondary metabolites containing the pyran-2-one moiety are produced by fungi belonging to several genera including *Alternaria*, *Aspergillus*, *Fusarium* and *Trichoderma*, and exhibit antibiotic, antifungal, cytotoxic, neurotoxic, and phytotoxic activities (Dickinson, 1993). α-Pyrones structurally related to **13** have been reported as products obtained by microbial transformation of **1** by *Botrytis cinerea* (Cooney *et al.*, 1997), *Sclerotinia sclerotiorum*, *Fusarium crookwellens* and a number of *Penicillium* isolates (Cooney & Lauren, 1999) and above cited as the bioactive 6-substituted-5,6-dihydropyran-2-ones, which were isolated from aerial parts of *Piper reticulatum* (Maxwell *et al.*, 1998).

The paper disk assay showed that viridepyronone was effective in inhibiting the growth of *S. rolfsii* by 48%. The culture filtrate of strain IPVS-1817 of *T. viride* and its crude organic extract showed an inhibition of 19 and 28%, respectively (Evidente *et al.* 2003b).

The results of this study provide new information on the production *in vitro* of antifungal metabolites by *Trichoderma viride* commonly employed as a biological control agent of plant pathogens.

Oxiranyldecene from *Trichoderma viride*

Successively, a new pentasubstituted oxiranyldecene, named viridenepoxydiol, has been isolated (0.9 mg/L) from the culture filtrate of the same strain of *T. viride*. Viridenepoxydiol was characterized as 3,5,9-trimethyl-2-oxiranyl-dec-8-ene-2,5-diol (**14**, Fig 9) using spectroscopic methods.

The structure of **14** was supported by the $^{1}H,^{13}C$ long range correlations observed in the HMBC spectrum, and by MS spectra (Evidente *et al.*, 2006c).

Knowing the absolute configuration of natural products has become crucial because it provides essential information for both total synthesis and molecular mode action of bioactive metabolites. The absolute configurations of natural compounds have, in some cases, been determined by X-ray analysis or synthetic work. Unfortunately, viridenepoxydiol is an oil resistant to crystallization and is laborious to synthesize because, of its numerous chiral centers. NOE-based methods in combination with molecular mechanics calculation have been proposed for configuration assignment of flexible molecules, particularly for macrocyclic compounds, as macrolides and other compounds. However, even with new NOE-based techniques it is still very difficult to assign the stereochemical configuration of highly flexible carbon chains because the presence of multiple conformers, in which minor populations often make disproportionately large contributions to NOE intensity, occasionally leads to contradictory distance constraints. This is the case of viridenepoxydiol for which the NOESY effects recorded between the protons of different moieties can only strongly support the structure assigned to **14**. Furthermore, the two new methods developed for the stereochemical determination of acyclic and small organic compounds, respectively based on carbon-proton spin-coupling constants (Matsumori *et al.*, 1999) and residual dipolar couplings (Mangoni *et al.*, 2003) were inapplicable for **3**. In fact, the first method requires the presence of stereogenic methine carbons substituted with a methyl or a hydroxy (alkoxy) group in the analyzed compound, while the latter one is still restricted only to water soluble compounds. Unfortunately, viridenepoxydiol contains two stereogenic suitable functionalized methines (C-3 and C-1'), two chiral quaternary carbons, and is not soluble in water.

Antifungal bioassay indicated that viridenepoxydiol was effective in inhibiting the growth of *S. rolfsii* by 100% at the concentration tested (5 μg/mL). The results of MIC test showed that the concentration of viridenepoxydiol was correlated ($R^2 = 0.98$) with the inhibition percentage of mycelial growth of *S. rolfsii* in PDA plates (Evidente *et al.*, 2006c).

Some decene derivatives are reported as component of spice flavours (Kawasaki, 2005), as metabolites of insects (Blum *et al.*, 2000) and fungi (Cambie *et al.,* 1963), while the oxiran group is present as structural feature important for the activity in several classes of bioactive fungal metabolites such as sesquiterpene eremophilanes (Capasso *et al.*, 1984), trichothecenes, verrucarins and cytochalasins (Cole & Cox, 1981; Vurro *et al.*, 1997; Andolfi *et al.*, 2005) and sphaeropsidones (Evidente *et al.*, 1998). The oxiranyldecenes have only been reported as synthetic compounds (Kenji *et al.*, 1992). Therefore, viridenepoxydiol is the first example of naturally occurring oxiranyldecene. To our knowledge, cyclonerodiol, a sesquiterpenoid produced by different fungi, including *Giberella*, *Fusarium*, *Trichoderma* and *Trichothecium* species but lacking antifungal activity (Li *et al.*, 2004), is the microbial metabolite closest to viridenepoxydiol.

The isolation of viridenepoxydiol provides new information on the production *in vitro* of antibiotic metabolites by *Trichoderma viride* commonly employed for developing suitable strategies of biological control against several plant pathogens such as *Colletotrichum capsici*, *Sclerotinia sclerotiorum*, *Pythium aphanidermatum*, *Fusarium oxysporum* f. sp. *lycopersici*, *Alternaria brassicola*, *A. alternata*, *Phomopsis vexans*, *Macrophomina phaseolina* and *Rhizoctonia solani* isolated from chili (*Capsicum* spp.), cauliflower (*Brassica oleracea*), tomato (*Solanum lycopersicum*) and eggplant (*Solanum melongena*) plants (Sharma & Dreja, 2004).

T. viride synthesizes bioactive metabolites belonging to different classes of natural compounds such as the 2*H*-pyran-2-ones, tetracyclic diterpenes and the oxiranyldecenes. The biological activities of secondary metabolites containing the 2*H*-pyran-2-one moiety is well documented, and they have been reported to be produced by fungi belonging to several genera (Dickinson, 1993; Evidente *et al.*, 2003c; Altomare *et al.*, 2004; Fairlamb *et al.*, 2004; McGlaken & Fairlamb, 2005). The oxiran group also plays an important role in the biological activity of different natural compounds (Cole & Cox, 1981; Capasso *et al.*, 1984; Vurro *et al.*, 1997; Evidente *et al.*, 1998; Andolfi *et al.*, 2005), so its presence could be essential to the antifungal activity of viridenepoxydiol.

Future investigations should be addressed to clarify the role of bioactive metabolites produced by this strain of *T. viride* in the biocontrol process, and the nature of interactions between them or with enzymes.

HERBICIDES

The weed pest is one of the most serious problems for agriculture and environment. Infesting plants generate a great obstacle to the normal flow

of superficial waters, destroy the natural habitat, seriously damage the archaeological and monumental area, and cause heavy losses to crop production and to pasture industry. Many plants of agrarian interest may dieback when the weed grows in the same field absorbing water, food substances, and sun light. Furthermore, they represent a serious impediment to the normal agrarian activity. The diffusion of weed reduces the pasture areas with consequent deterioration of animal food.

The control of weed diffusion has been achieved with agrochemicals belonging to different class of organic compounds. They are usually used in very large amounts in agriculture, thus causing serious problems to human and animal health and producing heavy environmental pollution. In fact, these substances have frequently low specificity and are weakly or not biodegradable, accumulate in food plants and in layer, and drinkable water. Furthermore, the chemical controls have short-life and must usually be repeated on an annual or semi-annual basis.

The biological agents offer the advantage of being compatible with the environment, often with high specificity and represent a long term solution also in the control of weed particularly resistant to chemical herbicides. Therefore, many efforts have been made to biologically control the weed using their natural antagonists as insect and/or microorganisms. Among the microorganisms, fungi are the most common pathogens of plants and therefore for weeds also. Some insects and fungi, which satisfy the criteria of efficacy, specificity and long-time persistence, have been already commercialised. Researches have performed to isolate several phytotoxins produced by some fungi pathogenic for weed and use them as natural herbicides. The goal of such a project is to use natural substances, their derivatives or synthetic analogues with increased efficacy and specificity to avoid the release of microorganisms, and the possibility that they became host of other organisms. Since many phytotoxins isolated from fungi pathogenic for agrarian plants are not specific, they may be considered as potential natural herbicides in native forms or as derivatives and analogues (Graniti *et al.*, 1989; Delfosse, 1990; Strobel, 1991; Strobel *et al.*, 1991).

The first approach is the isolation of microorganisms from tissues of infected infesting plants, followed by selection of the strain with higher specificity and virulence. The second step is to find appropriate conditions for the *in vitro* growth of the fungus to obtain culture filtrates with high phytoxicity against the host plant. Next, the phytotoxins are isolated, characterised and in some cases derivatized before to be tested as potential herbicide. Finally, the knowledge of the chemical structure of these substances may allow the partial or total synthesis of the most appropriate natural herbicide, therefore avoiding the collection, preservation and growth of pathogen agents. Furthermore the toxins could be used in indirect mode as biomarkers, to select the best fungal strain (if they are a virulent factor)

or to optimise for their large scale production (Evidente, 2006; Evidente & Abouzeid, 2006).

Fungal Phytotoxins

Studies on involvement of toxins in plant disease caused by pathogenic microorganisms date from the second half of the 19th century. Phytotoxins are defined as microbial metabolites that are harmful to plants at very low concentrations. Most of the plant pathogenic fungi produce toxins in culture and in their hosts. Frequently, these compounds play a role in the pathogenesis and reproduce some or even all of the symptoms of the disease. In many cases the toxins are low molecular weight compounds belonging to a variety of class of natural products. They are able to diffuse from the site of the infection to sorrounding tissues or are translocable within the plant. The virulence of the plant pathogen may depend on its capability to synthesize one or more toxins. Only few phytotoxins are kown as host-specific toxins, more frequently they are phytotoxic for a broad range of plant species. In some cases studies on their mode of action and their role as "vivo-toxin" have also been carried out (Strobel, 1982; Graniti *et al.*, 1989; Ballio & Graniti, 1991; Evidente, 1997; Upadhyay & Mukerji, 1997; Evidente & Motta, 2001).

Biological control of grass weeds

In many countries, annual and perennial grasses are among the most problematic weeds for various crops (Holm *et al.*, 1977). Of all the possible causes of loss in cereal yields, weeds, such as annual grasses are one of the most important; this is due to their similarity in morphology, physiology and ecology to the crop species.

Such weeds are difficult to control because of their prodigious seed production, which is responsible for their reproduction and diffusion, their tolerance to the chemical herbicides available, and their growth habits that can enable them to escape from chemical and mechanical control practices. Tactics that reduce the input of seed can improve long-term control of infesting grasses.

Considering the increasing number of weed species that are tolerant or resistant to the use of herbicides (Naylor, 2002), and the difficulties in finding new chemical active compounds, biocontrol microorganisms and new herbicides from natural sources are receiving a renewed interest.

One such strategy could be the massive application of seed-borne pathogens as bioherbicides. Pathogens damaging the seed in the inflorescence or preventing flowering have also potential for biological control.

Some promising fungal pathogens have been identified, and their use as inundative agents has been proposed (Zhan & Watson, 1997;

Chandramohan & Charudattan, 2001); furthermore, some fungal phytotoxins have been identified and considered as possible natural herbicides (Hallock *et al.*, 1988; Kastanias & Tokousbalides, 2000). Pathogenic fungi isolated from grass weeds were found in several fungal collections and many strains were collected (Fracchiolla, 2003). Such investigation was aimed at finding producers of toxic metabolites with herbicidal activities against grass weeds.

Biological control of *Bromus* spp.

Pathogens damaging the seed in the inflorescence or preventing flowering have also potential for biological control. Agents that attack the reproductive output of weeds are frequently used in biological control programmes against weeds in pastures, rangeland and natural habitats. *Pyrenophora semeniperda* (Brittlebank & Adam) Shoemaker, a seed-borne pathogen that causes several symptoms in infected plants, has been proposed as a bioherbicide (Campbell *et al.*, 1996). *P. semeniperda* was first described in Europe in 1841, and later in Australia, New Zealand, North America and South Africa. The fungus infects seeds and leaves of over 35 genera of grasses including all the winter cereals and six dicotyledonous genera (Medd, 1992). In brome grass (*Bromus* spp.) and wheat (*Triticum aestivum* L.) it has been reported to cause death of seed primordia and subsequent abortion of seed (Neergard, 1979). The most striking symptom is the production of vegetative fungal stromata on infected seeds, which can lead to a reduction in the germination capacity or a decrease in seedling vigour. The ability of *P. semeniperda* to infect seeds when applied as conidial suspension to the inflorescence of several grassy weed-species has also been demonstrated. Since some annual grasses may occur in pastures or crops used as forage, any potential bioherbicidal agent should be devoid of toxic effects on livestock. Equally, there should be no risk of introducing toxins to grains that are harvested for human consumption.

It is well known that other species of *Pyrenophora* produce toxins, some of which are potentially dangerous (Bach *et al.*, 1979; Friis *et al.*, 1991). Considering the interest for bioactive metabolites produced by weed pathogens to employed either in addition or as an alternative to the use of the pathogen, it seemed interesting to investigate the production of toxins by this species of *Pyrenophora*. Preliminary *in vitro* experiments showed that the fungus in liquid culture produces low-molecular lipophilic phytototoxins. When grown on wheat kernels it does not produce phytotoxins but cytochalasins, a large group of fungal metabolites having different biological activities (Cole & Cox, 1981; Natori & Yahara, 1991; Abate *et al.*, 1997; Vurro *et al.*, 1997; Evidente & Motta, 2001).

Three new cytochalasans, named cytochalasins Z1, Z2 and Z3 (**15**, **16** and **17**, Fig 10) were isolated from the wheat culture of *P. semeniperda*, a fungus proposed to biologically control grass weeds.

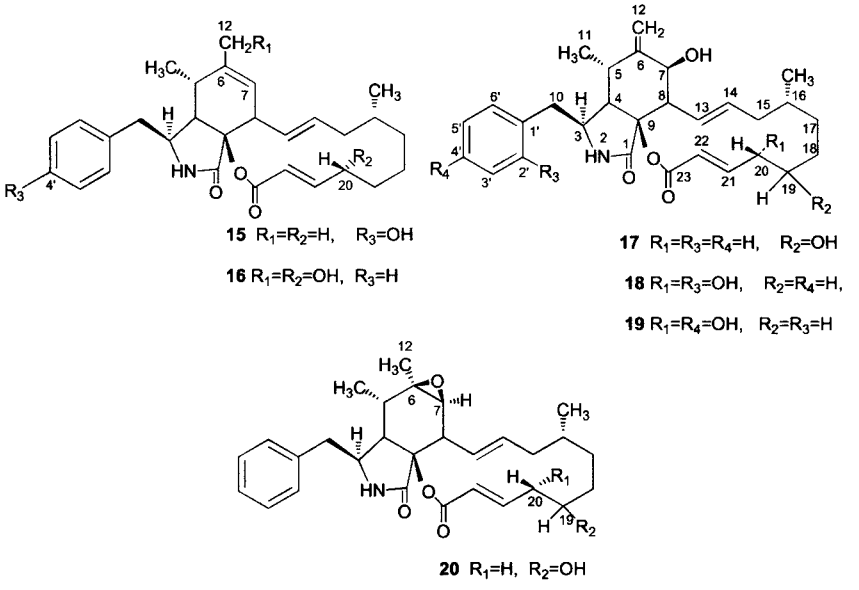


Fig 10. New cytochalasins Z1-Z6 (**15-20**) produced by both *Pyrenophora semeniperda* and *Phoma exigua* var. *heteromorpha*

The structure determination of the three new cytochalsins Z1, Z2 and Z3, which was all characterised as 24-oxa[14]cytochalasans, was performed out by spesctroscopic analysis carried out also in comparison with the spectral data of the several cytochalasins already known (Cole & Cox, 1981; Vurro *el al.*, 1997; Evidente & Motta, 2001). Other cytochalasins isolated from the same organic extract were identified, using the same spectroscopic techniques, as the already known cytochalasins F, T, deoxaphomin and cytochalasins B (**24**, **25**, **26** and **22**, Fig 11), the latter being produced in very large amounts. Cytochalasins Z1 and Z2 proved to be structurally related to cytochalasin T, whereas cytochalasin Z3 was related to cytochalasin (Evidente *et al.*, 2002).

In seedling assays (Fig 12) on wheat and on tomato, the most active compounds were cytochalasin B (CB), its 21, 22-dihydroderivative (diHCB, **27**, Fig 11), prepared by $NaBH_4$ reduction of **22** (Bottalico *et al.*, 1990), cytochalasins F and Z3 (CF and CZ3), and deoxaphomin (DEOXA). They were all able to reduce the root length by about 50%. In the puncture assay, only deoxaphomin, at the used concentration, showed the ability to produce

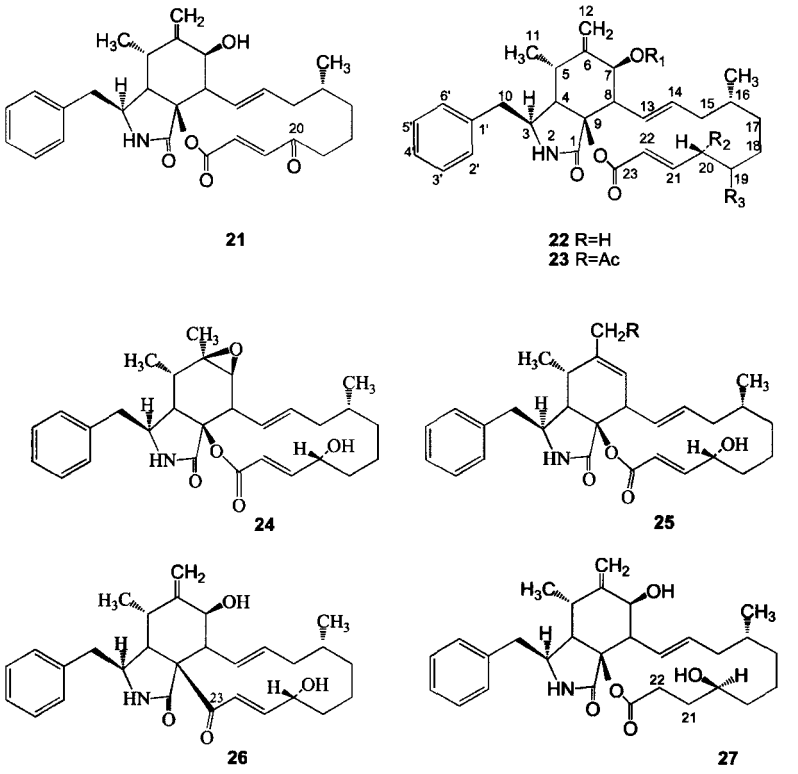


Fig 11. Structure of known cytochalasins A, B, 7-O-acetyl B, F, T and deoxaphomin (**21**-**26**) isolated from both *Pyrenophora semeniperda* and *Phoma exigua* var. *heteromorpha*, and the 21, 22 dihydroderivatives of cytochalasins B (**27**)

small necrotic lesions, whereas no effects were produced in the immersion assay by any of the tested cytochalasins. This, together with the observed phytotoxicity of liquid culture filtrates, could mean that other metabolites are responsible for phytotoxic effects caused by the pathogen. The existing structural correlation of cytochalasins Z1 and Z2 with cytochalasin T, and of cytochalasin Z3 with CB was also observed biologically. The first two were inactive, whereas the other two proved to be active in the root elongation assay (Evidente *et al.*, 2002).

Considering the potential applications and the availability of large amount of solid cultures of *Phoma exigua* var. *heteromopha* (Schulzer *et* Sacc.) Noordeloos *et* Boerema, wich is a good producer of cytochalasins is solid and liquid culture (Vurro *et al.*, 1997), an investigation was carried out to look for new cytochalasins yielded by this fungus. *Phoma exigua* var. *heteromorpha*, was the causal agent of a severe disease of Oleander (*Nerium oleander* L.) observed in 1985 in a nursery near Bari Italy (Vurro *et al.*, 1997).

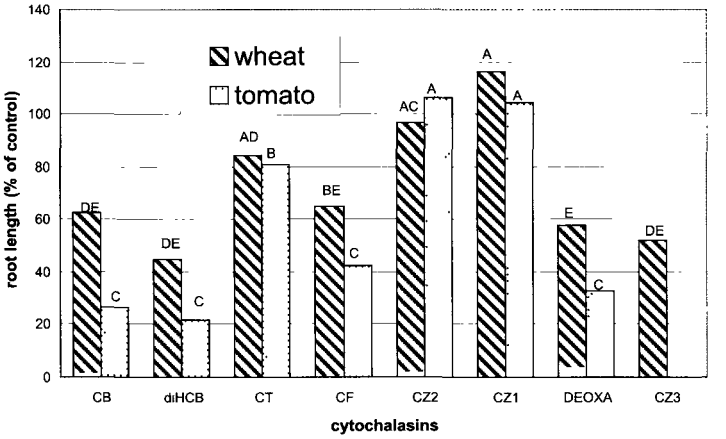


Fig 12. Effect of cytochalasins on root elongation of tomato and wheat seedlings. Root length was measured 3 days after treatment, and expressed as percentage with respect to the control. Means of root length was 20.5 (± 10.6) and 52.1 (± 10.9) mm for wheat and tomato, respectively. Values denoted by different letters are significantly different at $P = 0.05$. Abbreviations: CB, CF, CT, CZ1, CZ2 and CZ3 = cytochalasins B, F, T, Z1, Z2 and Z3, respectively; diHCB= 21,22-dihydrocytochalasin B; DEOXA= deoxyphomin

Three new cytochalasans, named cytochalasins Z4, Z5, and Z6 (**18-20**, Fig 10) were isolated from the wheat culture of *P. exigua* var. *heteromorpha* together with the known cytochalasin A, B, F, T, Z2, Z3, 7-*O*-acetylcytochalasin B, and deoxaphomin (**21-25**, Fig 11, **16** and **17**, Fig 10, and **23** and **26**, Fig 11). All three new cytochalasins were characterised as 24-oxa[14]cytochalasans by extensive use of NMR and MS techniques. Cytochalasins Z4 and Z5 proved to be structurally related to cytochalasin B, whereas Z6 was related to cytochalasin F (Evidente *et al.*, 2003a).

Cytochalasins Z1 and Z5 represents the first two examples of a 24-oxa[14]cytochalasan bearing a *p*-hydroxybenzyl residue at C-3 of the perhydroisoindolyl-1-one moiety, and therefore, differed from the other [14] cytochalasans showing a phenyl, isopropyl or an indol-3-yl residue at C-10 and having a different functionalised macrocyclic ring (Cole & Cox, 1981; Natori & Yahara, 1991; Vurro *et al.*, 1997). The most closely related cytochalasins are phenolchalasins A and B, two 21,23-dioxa[13]cytochalasans with a lactonic macrocyclic ring, produced by *Phomopsis* sp. (Tomoda *et al.*, 1999), pyrichalasin H, a phytotoxic [11]cytochalasan with a carbocyclic macrocyclic ring produced from *Pyricularia grisea* (Nukina, 1987) and phomopsischalasin, an antimicrobial [13]cytochalasan in which the macrocyclic ring arranges into a tricyclic carbocyclic system fused to the perhydroisoindolone unit, produced by an endophytic *Phomopsis* sp. (Horn *et al.*, 1995). The phenolchalasin A and phomopsischalasin, together with cytochalasins Z1 and Z5, have a *p*-hydroxybenzyl at C-3, which should be biosynthesized from tyrosine,

whereas phenolchalasin B and pyrichalasin H have a *p*-methoxybenzyl, which should derive from tyrosine methyl ether. With regard to the *o*-hydroxybenzyl residue attached to the C-3 of Z4 (**18**), a different and new biosynthetic origin should be hypothesized.

Cytochalasins Z1 and Z3, structurally related to cytochalasins T and B, respectively, are the first two 24-oxa[14]cytochalasans with a lactonic macrocyclic ring deoxygenated at C-20 (Cole & Cox, 1981; Natori & Yahara, 1991; Vurro *et al.*, 1997). Cytochalasin Z2, closely related to the well known cytochalasin T, is a new 24-oxa-[14]cytochalasan showing for the first time, among all the [11], [13] and [14]cytochalasans, a hydroxymethyl group on the C-6, (Cole & Cox, 1981; Natori & Yahara, 1991; Vurro *et al.*, 1997).

Furthermore, Z6 (**20**) is the first 24-oxa[14]cytochalasan showing the epoxy group located between C-6 and C-7 of the perhydroisondolyl-1-one residue, the deoxygenation of C-20, and the hydroxylation of C-19, as already observed for Z3 (Cole & Cox, 1981; Natori & Yahara, 1991; Vurro *et al.*, 1997).

In tomato seedling assay, at 10^{-4} M, only Z6 proved to be slightly active causing 30% inhibition of root elongation, whereas Z4 and Z5 were inactive. When assayed at the same concentration on brine shrimp, only Z5 caused a quite low mortality of larvae (21%), whereas Z4 and Z6 were both inactive (Evidente *et al.*, 2003a).

The results of structure-activity relationship studies (Bottalico *et al.*, 1990; Capasso *et al.*, 1991; Vurro *et al.*, 1997) and recent test results regarding the phytotoxicity of cytochalasin B, its 21,22-dihydro derivative, cytochalasins F, Z1, Z2, and Z3 and deoxaphomin (Evidente *et al.*, 2002) suggest the important role of the hydroxy group at C-7 in conferring the biological activity. This structural feature is present in Z4 and Z5 but not in Z6. Therefore, the lack of phytotoxicity in **18** and **19** is probably due to the *ortho*- or *para*-hydroxy substitution of the benzyl residue attached at C-3. In spite of the limited activity of Z6, the presence of an unsubstituted phenyl residue attached at C-10, the most common substituent in this group of natural products, is important for biological activity. This is in accordance with the activity shown by cytochalasin F in the same test (Evidente *et al.*, 2002) and with the results described previously (Bottalico *et al.*, 1990; Capasso *et al.*, 1991; Vurro *et al.*, 1997).

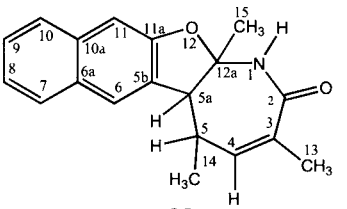
Cytochalasins have been considered as potential mycotoxins. If high level of toxins were really produced *in vivo*, this could, in practice, make it hazardous to use *P. semeniperda* as a biological control agent against grass weeds. Hence, studies need both to quantify the presence of such toxins in naturally infected seeds, as well as to estimate their stability and impact in the environment. In South Africa, *P. semeniperda* was one of several fungi isolated from leaf spots of grazing oats (*Avena sativa* L.) in association with field outbreaks of diarrhoea, photosensitivity and death in goats, diarrhoea and loss of milk production in dairy cattle. The fungus was highly toxic when fed freely as maize seed culture to ducklings, and as a pure meal drench to goats and sheep (Schneider *et al.*, 1985; Collett *et al.*, 1988), even

if there was no evidence that the fungus had mycotoxic properties other than at high dosage of almost pure cultures. Further observations are also need to identify the lipophilic phytotoxic compounds that the fungus is able to produce in liquid culture. If the found toxins were proved to be important in the disease caused by the fungus, an interesting alternative strategy could be the use of such metabolites as natural herbicide instead of the living agent.

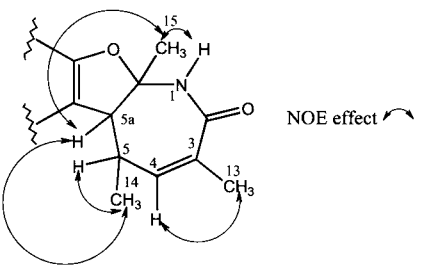
Biological control of *Lolium perenne*

Some of the selected fungal strains were able to produce highly phytotoxic culture filtrates, particularly one strain of *Drechslera siccans*, isolated from *Lolium perenne* L., another annual and perennial grass which are one of the most important causes of loss in cereal yields. *D. siccans* belong to a genus well known as phytotoxin producer. This strain is one of the best toxin producers among several grass pathogenic fungal strains collected and tested to find phytotoxins to be used as natural herbicides of monocot weeds.

In fact, when grown in a minimal defined medium it produced phytotoxic metabolites. From the culture filtrates a new phytotoxic trisubstituted naphthofuroazepinone was isolated and named drazepinone (**28a**, Fig 13), and characterised it as a 3,5,12a-trimethyl-2,5,5a,12a-



28a



28b

Fig 13. Structure and NOE correlations of drazapinone (**28**) produced by *Dechslera siccans*

tetrahydro-1*H*-naphtho[2',3':4,5]furo[2,3-*b*]azepin-2-one on the basis of its spectroscopic properties (Essentially NMR and MS). The structure assigned to drazapinone was supported by several ^{1}H,^{13}C long range correlations recorded for **1** in the HMBC spectrum, and by EI and ESI MS data (Evidente *et al.*, 2005).

The relative stereochemistry of drazepinone was based on NOESY correlations. Among them (**28b**, Fig 13), the following correlations determine the spatial arrangement of the azepinone ring. A strong NOE effect was observed between the H-5a and the Me-15, both linked to the bridgehead carbons between the dihydrofuran and the azepinone rings, which consequently should be a *cis*-jointed. The strong interaction between H-5a and the adjacent methyl group Me-14 confirms the *cis*-configuration. Furthermore, the strong effect between H-5 and H-5a justifies the small value observed for their coupling in the ^{1}H-NMR spectrum. Finally, significant effects were observed between NH-1 and naphthalene H-6, and the H-4 and Me-15.

All of the observed NOEs were used for molecular mechanics and dynamics calculations, based on a distance calibration (r^{-6}, two-spin approximation) to convert the volumes of the most intense and significant NOESY cross-peaks of the azepinone ring into internuclear distances. All the calculated structures show that the azepinone ring takes up a boat-like conformation with the amidic proton pointing toward the naphthalene ring, therefore justifying the observed NOE effects between NH-1 and H-6, and H-4 and Me-15. The pattern of the found NOEs ruled out the other possible boat-like conformation, with the amidic proton pointing away of the naphthalene ring (Evidente *et al.*, 2005).

Drazepinone is structurally related in part to a group of naturally occurring compounds that are broadly distributed in nature as plant and marine organism metabolites. Most of them show interesting biological activity (Mattia *et al.*, 1982; Sekine *et al.*, 1989; Xu *et al.*, 1997; Baudion *et al.*, 2002) and the total synthesis of some of them has been achieved (Xu *et al.*, 1997; Baudoin *et al.*, 2002). Natural compounds containing the naphthoazepin skeleton have not been reported yet, and those having furoazepine are described only as synthetic derivatives with important pharmacological activity (Cortes *et al.*, 1994; Cho *et al.*, 2004). Therefore, drazepinone is the first natural compound presenting all three moieties jointed in a new and interesting bioactive fungal metabolite.

The fungal culture filtrate proved to be particularly effective, when applied by infiltration both to the host and non host plant tested, causing quick chlorosis in the injected leaf tissues, followed by wide necrosis along the leaves. The culture filtrate produced by *D. siccans*, assayed with this screening procedure, proved to be one of the most toxic filtrates among those produced by tens of grass pathogens, collected and tested with the aim to find producers of toxic metabolites having herbicidal activity (Fracchiolla, 2003).

Applied to wounded leaves, the toxin caused necrosis on almost all the species tested (Table 7). Necrosis severity ranged from very wide, as in the case of *Urtica dioica*, to small ones as those observable applying the

toxin to *Setaria viridis* and *L. perenne* leaves. The necrosis on *Euphorbia helioscopia* and *Mercurialis annua* leaves, both Euphorbiaceae, and *Chenopodium album* were also interesting. On the opposite, *Amaranthus retroflexus* and *Bromus* sp. were completely unaffected by the toxin. The symptoms caused by the pure drazepinone and by the culture filtrate appear to be almost the same, both in term of speed of appearance and size of necrosis, although the concentration of drazepinone in the culture filtrate is much lower with respect to the pure solution. This could mean that, besides drazepinone, the main toxin in the culture extracts, the fungus could produce other bioactive compounds. Their possible presence and role are under ascertainment (Evidente *et al.*, 2005).

Table 7. Toxicity of drazepinone (**28**) in the leaf-puncture assay

Common name	Scientific name	Family	Toxicity[a]
Alligator weed	*Alternanthera philoxeroides* (Mart.) Griseb.	Amaranthaceae	+
Annual mercury	*Mercurialis annua* L.	Euphorbiaceae	+++
Brome	*Bromus sp.*	Poaceae	-
Common Chickweed	*Stellaria media* L.	Caryophyllaceae	++
Common mallow	*Malva silvestris* L.	Malvaceae	+
Durum Wheat	*Triticum durum* Desf.	Poaceae	++
Fat hen	*Chenopodium album L.*	Chenopodiaceae	+++
Green bristlegrass	*Setaria viridis* L. Beauv.	Poaceae	+
Madwoman's milk	*Euphorbia helioscopia L.*	Euphorbiaceae	+++
Perennial ryegrass	*Lolium perenne* L.	Poaceae	+
Pigweed	*Amaranthus retroflexus* L.	Amaranthaceae	-
Sowthistle	*Sonchus arvensis* L.	Asteraceae	++
Stinging nettle	*Urtica dioica* L.	Urticaceae	++++

[a]Toxicity scale: - no symptoms; + necrosis with diameter 1-2 mm; ++ necrosis 2-3 mm; +++ necrosis 3-5 mm; ++++ wider necrosis

Assayed up to 50 μg/disk on *Geotrichum candidum*, drazepinone showed a weak fungistatic activity, causing only a slight reduction of the fungal growth. The toxin proved to be completely inactive when tested up to 50 μg/disk on *Pseudomonas syringae* and *Lactobacillus plantarum* (a Gram- and a Gram+ bacterium, respectively). Assayed for zootoxic activity at 10^{-3} M, the metabolite caused the total mortality of shrimp larvae, which decreased to 81% and 12% when assayed at 10^{-4} and 10^{-5} M, respectively (Evidente *et al.*, 2005).

As above cited, *Drechslera* is a well-known genus producing phytotoxic metabolites. Most of those pathogens and their toxins have been deeply studied being agents of very severe diseases of cropped cereals (Tatum, 1971; Padmanabhan, 1973; Strobel *et al.*, 1988). Some species were also isolated from grass weeds (Chandramohan & Charudattan, 2001), and their toxins proposed as potential natural herbicides (Kastanias & Tokousbalides,

2000; Kenfield *et al.*, 1989a, 1989b). Toxins with structure completely different from drazepinone were previously isolated from other strains of the same fungus, such as de-*O*-methyldiaporthin (Hallock *et al.*, 1998) and siccanol (Lim *et al.*, 1996), an isocoumarin and a bicyclic sesterterpene, respectively. Siccanol completely inhibited the root of Italian ryegrass (*L. multiflorum* Lam.) seedlings at a level of 100 ppm (Lim *et al.*, 1996). De-*O*-methyldiaporthin was almost inactive when assayed on host plants (*L. perenne* L. and *A. sativa* L.), whereas it was toxic when assayed on corn, crabgrass, and soybean (at 4 mmol), and on Barnyard grass and spiny amaranth (8 and 21 nmol, respectively) (Hallock *et al.*, 1998), with a toxicity resembling that caused by drazepinone.

The original chemical structure of drazepinone, the interesting phytotoxic activity, the low activity against fungi and bacteria, and the relatively low zootoxicity, suggest further studies for its use as an environmentally friendly and safe herbicide (Evidente & Motta, 2001).

Phytotoxins produced by *Drechslera gigantea*, a potential mycoherbicide for grass weeds

Drechslera gigantea Heald & Wolf is a cosmopolitan fungal pathogen found throughout North and South America, Japan, and other regions (Sivanesan, 1992). The extensive studies carried out over the past seven years have shown that this fungus is effective for grass management under field conditions, alone and in combination with two other grass pathogens, *Exserohilum longirostratum* and *E. rostratum* (Chandramohan & Charudattan, 2001; Chandramohan *et al.*, 2002). Typically, symptoms of *D. gigantea* leaf blight appear in about one week after the fungus is sprayed on the grass foliage and the disease progresses steadily over the following two to three weeks. The treated foliage is killed and the control lasts for 10 weeks or more. Rhizomes are not killed and the grasses will re-grow after a period of mycoherbicide-caused suppression. Evaluations over the past seven years have confirmed that these fungi are effective for grass management under field conditions (Chandramohan & Charudattan, 2001; Chandramohan *et al.*, 2002).

As above anticipated for *D. siccans,* many of these species have also been studied for the production of phytotoxic secondary metabolites, which are often involved in the infection processes, and some phytotoxins also have been isolated from pathogens of grass weeds and proposed as potential natural herbicides (Kastanias & Chrysayi-Tokousbalides, 2000; Kenfield *et al.*, 1989a, 1989b; Evidente *et al.*, 2005).

A strain *D. gigantea,* isolated in Florida from diseased large crabgrass (*Digitaria sanguinalis*) produced in liquid culture four phytotoxic metabolites. The main metabolite was identified by spectroscopic methods and optical properties as ophiobolin A (**29**, Fig 14) (Evidente *et al.*, 2006a), a

well known phytotoxic sesterterpene produced by several phytopathogenic fungi of important crops, already extensively studied for its interesting biological activities (Au *et al.*, 2000). The physical and the spectroscopic data were similar to those previously reported in literature (Nozoe, *et al.*, 1965; Li *et al.*, 1995; Canales & Gray, 1988). This result was also confirmed by a direct X-ray analysis carried out on the natural metabolite and below reported.

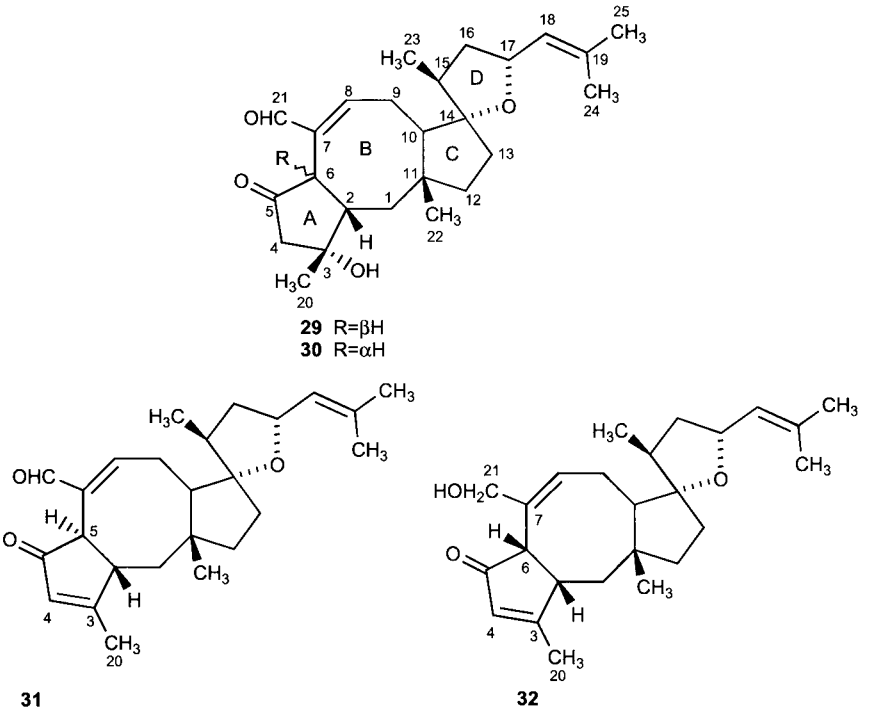


Fig 14. Structures of ophiobolin A, 6-*epi*-ohpiobolin A, 3-anhydro-6-*epi*-ophiobolin A and ophiobolin I (**29**-**32**) produced by *Drechslera gigantea*

The other three compounds were isolated as amorphous solids but in lower amounts (1.5, 1.1 and 0.3 mg/l for **30**, **31** and **32**, respectively) compared to **1** and appeared to be closely related to ophiobolin A, by preliminary spectroscopic investigation. They were identified by comparison of their spectral data, essentially ^{1}H and ^{13}C NMR and MS data, as 6-*epi*-ophiobolin A, 3-anhydro-6-*epi*-ophiobolin A, and ophiobolin I (**30**, **31** and **32**, Fig 14) (Evidente *et al.*, 2006a). Their physical and the spectroscopic data are similar to those reported in literature (Kim *et al.*, 1984; Sugawar *et al.*, 1987; Li *et al.*, 1995; Canales *et al.*, 1988; Sugawara *et al.*, 1988).

Ophiobolin A (**29**) proved to be highly toxic to almost all the plant species tested (Table 8), already at the lowest concentration used (1.25 10^{-4}

M; 3.2 μg/roplet). Among dicotyledons, annual sowthistle appeared to be particularly sensitive, whereas canarygrass was the most sensitive among monocotyledons. On the opposite, even at the highest concentration used, the toxin was almost inactive to bermudagrass. Compared to ophiobolin A, 6-*epi*-ophiobolin A (**30**) proved to have almost the same spectrum of plant sensitivity, but at a lower intensity. With regard to 3-anhydro-6-*epi*-ophiobolin A (**31**), it was almost inactive to most of the plant tested, with the exception of green foxtail and rocket. Ophiobolin I (**32**) proved to be inactive, even at the highest concentration, to all the plants tested (Evidente *et al.*, 2006a).

It is interesting to note a certain level of selectivity of the toxins. In fact, on average ophiobolins proved to be more active to grass weeds respect to dicotyledonous species. The ophiobolins are a group of polycyclic sesterterpenoid with a common basic structure. They are secondary phytotoxic metabolites produced by pathogenic fungi attacking several crops, such as rice, maize and sorghum. Ophiobolin A was the first member of the group to be isolated and characterized independently by Canonica (1966a) and Nozoe (1966). In addition to ophiobolin A, several analogs were isolated in the late sixties and their structures determined. These include ophiobolin B from *B. oryzae* (Canonica *et al.*, 1966b), ophiobolin C from *B. zizanie* (Nozoe *et al.*, 1966), ophiobolin D from *Cephalosporium caerulens* (Itai *et al.*, 1967; Nozoe *et al.*, 1967), and ophiobolin F from *B. maydis* (Nozoe *et al.*, 1968). A wealth of information has been accumulated regarding the biological activities of ophiobolins as well as on their biosynthesis, even if neither the enzymes nor the genes responsible have been identified (Au *et al.*, 2000). They share the same carbotryciclic diterpenoid ring with fucicoccins and cotylenins, other two groups of microbial metabolites produced by *Fusicoccum amygdali*, the causal agent of almond and peach diseases (Ballio & Graniti, 1991), and by *Cladiosporum* sp. 501-7W. (Sassa, 1971; Sassa *et al.*, 1972). Although ophiobolins were quite deeply studied for their interesting effects on plant physiology and for their biological activities, only limited information are reported on their potential herbicidal activity. Furthermore, there are only few studies on structure-activity relationships. One of them was carried out on four ophiobolins, namely **29**, **30**, **31** and 3-anhydroophiobolin A, isolated from the culture filtrates of the *B. sorghicola,* a fungal pathogen of Johnsongrass (*Sorghum halepense* L.), using a leaf-spot assay on several plants. The results showed that ophiobolin A and its 6-epimir were more phytotoxic than their anhydro derivatives against sorghum, sicklepod, and maize. 6-*Epi*-ophiobolin A at a high concentration produced the largest necrosis on leaves of all plants tested except morning-glory. The anhydrous derivatives were generally less phytotoxic and no toxic at all to morning glory leaves, even at concentrations of 2 mg/ml (Pena-Rodriguez & Chilton, 1989). The results are in agreement with our findings, because also in our assays ophiobolin A proved to be more active to almost all the plant species tested, in comparison with 6-*epi*-ophiobolin

Table 8. Effect of ophiobolins **29-31** in the leaf puncture assay on different weed species[a]

			Compound											
			(29)			(30)			(31)			(32)		
			Concentration (μg/ml)											
Species	**Common name**	**Family**	**250**	**100**	**50**	**250**	**100**	**50**	**250**	**100**	**50**	**250**	**100**	**50**
Monocotyledons														
Avena ludoviciana Dur.	Sterile oat	Gramineae	3	3	3	2	2	2	0	0	0	0	0	0
Bromus sterilis L.	Poverty brome	Gramineae	3	2	1	2	1	1	1	1	1	0	0	0
Cynodon dactylon (L.) Pers.	Bermuda grass	Gramineae	1	0	0	2	1	0	0	0	0	0	0	0
Digitaria sanguinalis (L.) Scop.	Large crab grass	Gramineae	3	3	3	3	2	2	1	1	0	0	0	0
Echinochloa crus-galli (L.) Beauv.	Barnyard grass	Gramineae	3	3	3	2	2	1	1	1	1	0	0	0
Oryzopsis miliacea (L.) Aschers	Smilo grass	Gramineae	3	2	1	2	1	1	1	1	1	0	0	0
Phalaris canariensis L.	Canary grass	Gramineae	3	3	3	3	2	1	1	1	1	0	0	0
Setaria viridis (L.) Beauv.	Green foxtail	Gramineae	3	3	3	3	3	3	2	2	2	0	0	0
Dicotyledons														
Amaranthus retroflexus L.	Redroot pigweed	Amaranthaceae	3	2	2	1	1	1	0	0	0	0	0	0
Chenopodium album L.	Common lambsquarter	Chenopodiaceae	3	2	2	3	2	2	1	1	0	0	0	0
Convolvulus arvensis L.	Field bindweed	Convolvulaceae	2	2	2	2	2	2	0	0	0	0	0	0
Diplotaxis erucoides (L.) DC.	Rocket	Cruciferae	2	2	2	2	2	2	3	3	2	0	0	0
Sonchus oleraceus L.	Annual sowthistle	Asteraceae	3	3	3	3	3	3	1	1	0	0	0	0

[a]Diameter of necrosis on leaves: 3 = necrosis > 3 mm; 2 = necrosis between 2 and 3 mm; 1 = necrosis between 1 and 2 mm; 0 = no necrosis

A, whereas the 3-anhydro compound was much less toxic, being almost inactive to many of the plant tested, even at the highest concentration used. Furthermore, the ophiobolin I proved to be inactive to all the species tested. On the basis of these results structural features important for the phytotoxicity appear to be the hydroxy group at C-3, the stereochemistry at C-6 and the aldehyde group at C-7 (Evidente *et al.*, 2006a).

The isolation of ophiobolins from this strain of *D. gigantea* isolated by *Digitaria sanguinalis* is enough surprising considering Kenfield *et al.* (1989a) had previously studied the metabolites produced by another strain of *D. gigantea* and reported only the isolation of gigantenone as the main toxin. Although being both terpenoids, gigantenone belongs to the chemical subgroup of sesquiterpenes, whereas ophiobolins belong to that of sesterterpenoids. Several biological investigations have also described gigantenone as a promising compound in different areas of research such as pathological physiology, photosynthetic efficiency, senescence, vegetation propagation, and development of selective herbicides (Kenfield *et al.*, 1989b). Many biological properties were reported for ophiobolins, too. For example, they can reduce root and coleoptile growth of wheat seedlings, inhibit seed germination, change cell membrane permeability, stimulate leakage of electrolytes and glucose, or cause respiratory changes (Au *et al.*, 2000). In our assays, the necrotic spot lesions on leaves induced by the application of drops of toxins resemble those caused by the pathogen, even if those symptoms are not as specific as the pathogen. For this reason, further studies are in progress to evaluate the possibility of enhancing the efficacy of the promising mycoherbicide *D. gigantea* with the joint application of sublethal doses of the toxins.

Ophiobolins are also toxic to animals. For example, the LD_{50} doses of ophiobolin A for mice are 238 mg/kg when administered subcutaneously, or 73 mg/kg, orally (Nakamura & Ishibashi, 1958). Even if they are much less toxic (as acute toxicity) compared to other powerful mycotoxins [*e.g.*, oral LD_{50} for T-2 toxin and aflatoxin B_1 is ranging between 0.6 and 6.1 mg/kg, and between 0.4 and 18 mg/kg, respectively, depending on the animal species (Bottalico, 2004)], their real impact in the environment should be evaluated, as well as their effect to non-target organisms, and their fate after the introduction in the environment, if considered as possible natural herbicides. Furthermore, new ophiobolins could be isolated by other fungal strains, or new derivatives could be obtained starting from the natural compounds, having a lower undesired toxicity or stronger phytotoxic properties.

The determination of the structure of ophiobolin A by X-ray analysis was carried out to contribute to a better understanding of the discriminant

role of the stereochemistry assumed by the carbotricyclic ring in the affinity with the receptors and so its changes consequently to the modification of some functional groups. Intensity diffraction data were collected at low temperature allowing us to better determine geometry and conformation of the molecule in the solid state and establish the geometry of the double bond of the terminal moiety (Fig 15) (Andolfi *et al.*, 2006).

The molecular structure of ophiobolin A is essentially trycyclic and consist of five-, eight-, five-membered rings (rings A, B, C in Fig 14) forming a unique C25 terpenoid skeleton type of ring system found only in this class of naturally occurring compounds. A side chain 2-isopropylidene-4-methyltetrahydrofuran is attached to the ring C in a spiro form at the position 5 of the tetrahydrofuran ring (ring D in the Fig 14). The molecule is also characterized by the presence of hydroxyl, α,β-unsaturated carbonyl and olefinic groups and a ketone function in a five member A ring.

The molecule presents 8 chiral centres, and absolute configuration was assigned in accordance with literature data for ophiobolin methoxybromide (Morisaki *et al.*, 1968) as *R*, *S*, *S*, *R*, *R*, *S*, *R* and *S* and at the C17, C15, C14, C11, C10, C6, C3 and C2 atoms, respectively.

The overall conformation of the molecule is similar to that observed for the ophiobolin methoxybromide derivative (Morisaki *et al.*, 1968), revealing that the juncture between the cyclopentanone ring, namely A ring, and the eight membered ring, namely B ring, is in the *cis* form, while that between the B and C rings is in the *trans* form.

The five membered rings A and C adopt a distorted envelope conformation, with a more pronounced deformation in A, probably because of the presence of an intramolecular hydrogen bond between the O1-H and O3 atoms with a distance and bond angle of 1,77Å and 151°, respectively. The eight membered B ring adopts a distorted chair conformation, with the distortion present at the junction between the A and D rings, while the D ring presents an envelope type conformation, with the mean plane of its atoms being quite orthogonal to that formed by the C ring (Fig 15). The two double bonds C7-C8 and C21-O3 are both in a *trans* position and the plane of the double bond at C18=C19 is perpendicular to the mean plane of the fing D (Fig 15) (Andolfi *et al.*, 2006).

Considering the interesting results obtained, the same organic extract was reinvestigated in order to identify further minor metabolites. Furthermore, the fungus was also grown on a solid medium, and the extract obtained by organic solvent extraction was analysed with the aim of finding new phytotoxic metabolites. In fact, changes in culturing conditions can

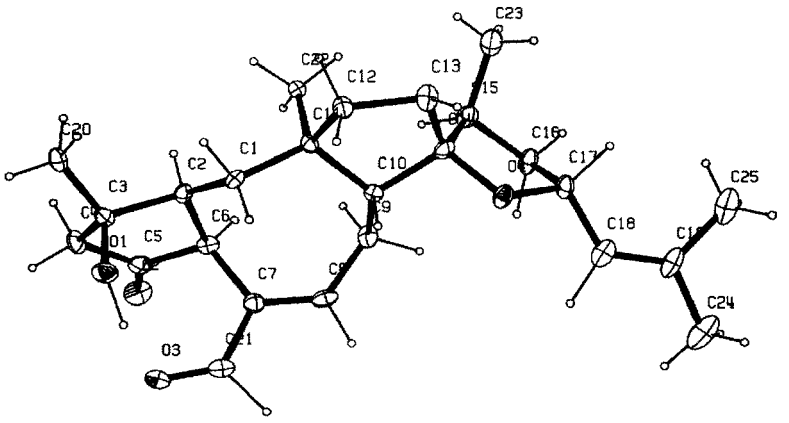


Fig 15. A view of the molecular structure of ophiobolin A. Displacement ellipsoids are drawn at the 50% probability level

strongly influence the biosynthetic production of ophiobolins. For example, *B. maydis* was able to produce ophiobolin A, 3-anhydroophiobolin A, ophiobolin B and ophiobolin L when grown in liquid conditions (Li *et al.*, 1995), whereas it produced ophiobolin M, 6-*epi*-ophiobolin M, ophiobolin C, 6-*epi*-ophiobolin C, ophiobolin K and 6-*epi*-ophiobolin K when grown on solid media (Perna-Rodriguez & Chilton, 1989)

Four further ophiobolins were isolated and two of them being new natural compounds were named E and 8-*epi*-ophiobolin J (**34** and **36**, Fig 16), respectively. Their structure was essentially determined by extensive use of spectroscopic methods (essentially NMR and MS techniques), as well as those of the two well-known ophiobolins B and J (**33** and **35**, Fig 16) isolated together with **36** from the fungal solid culture (Evidente *et al.*, 2006b). The physical and spectroscopic data of these latter were very similar to those reported in the literature (Li *et al.*, 1995; Sugawara *et al.*, 1988).

The structure of ophiobolin E and 8-*epi*-ophiobolin J were supported by several $^{1}H,^{13}C$ long-range correlations and the effects recorded for **6** in the HMBC and NOESY spectra (Evidente *et al.*, 2006b).

Tested on four weedy plants using the leaf-puncture assay, at 0.5 mg/ml-droplets 15 ml, only ophiobolins B and J proved to be toxic (Table 9), whereas the two new ophiobolins, ophiobolin E and 8-*epi*-ophiobolin J, appeared to be inactive on all the tested plant species. In particular, ophiobolin B was highly toxic to *Bromus* sp. and *Hordeum marimum* leaves, but less toxic to the other two weed species. The same range of toxicity, but at a lower level, was observed for ophiobolin J (Evidente *et al.*, 2006b).

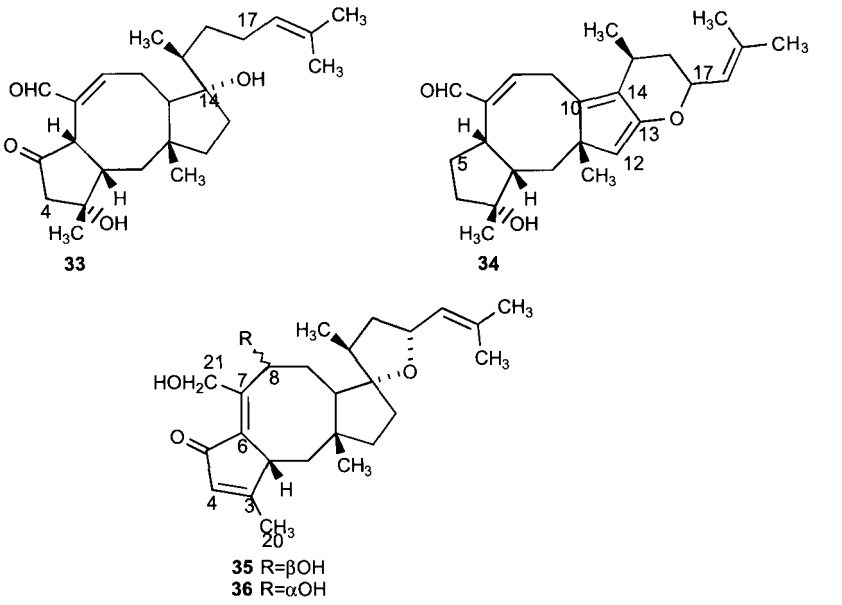


Fig 16. Structures of ophiobolins B, E, J, and 8-*epi*-ohpiobolin J (**33-36**) produced by *Drechslera gigantea*

Table 9. Effect of ophiobolins B and J, (**33**, and **35**, respectively) on various weed species tested by leaf puncture assay. Observations were made 2 days after droplet application

Species	Compound[a]	
	Ophiobolin B (33)	**Ophiobolin J (35)**
Avena sterilis	++[b]	+
Bromus sp.	++++	++
Hordeum murinum	++++	++
Oryzopsis miliacea	+	-

[a] 0.5 mg mL^{-1} - droplets 15 µl

[b] Diameter of necrosis on leaves: ++++ = necrosis diameter > 6 mm; ++ = necrosis between 4 and 2 mm; + = necrosis between 2 and 1 mm; - = no necrosis

The modulated activity of ophiobolin B (**33**) on the different tested plants appears to be similar to that previously reported for ophiobolin A (**29**). This result was predictable because the two ophiobolins are structurally closely related. Moreover ophiobolin J (**35**), having reduced or no activity, is related to ophiobolin I (**32**), which had proved to be inactive (Evidente *et al.*, 2006a). This activity is in agreement with the phytotoxicity previously observed for the same toxin (Sugawara *et al.*, 1988). The different phytotoxicity shown by the two ophiobolins J and I could be attributed to the different conformation that the octacyclic B ring can assume, as a consequence of the different position of the double bond, which is located

between C-7 and C-8 in **32**, and between C-6 and C-7 in **35**. Probably, when present, the epimerization of the hydroxy group of C-8, observed for the first time in **36**, imparts the total loss of the activity. The noteworthy structural differences present in the ophiobolin E could justify the observed inactivity on the tested plants. In fact, this latter ophiobolin showed the conversion of the cyclopentane C ring, present in all the other ophiobolins, into a 1,3-cyclopentadiene joined with the D ring, which in turn is present for the first time as a tetrasubstituted dihydropyran ring. Consequently, the configuration of the octacyclic B ring as well as that of the 2,2-dimethylvinylidene residue at C-17 should be substantially changed. Moreover, as a further difference in respect to the other ophiobolins, **34** showed the lack of the ketone group at C-5, which determines a different A ring conformation (Evidente *et al.*, 2006b).

In conclusion, when grown in solid culture, *D. gigantea* produces ophiobolin A, but at a very low level compared to the amount recovered from the liquid culture, and some different related metabolites, which show novel chemical structures and different biological activities. The availability of different compounds structurally related could contribute to clarify the structure-activity relationships within the ophiobolin family, which includes sesterterpenoids with a common basic structure produced by different phytopathogenic fungi widely studied for their interesting biological activity (Au *et al.*, 2000). This knowledge can be of great importance in understanding the site of action of the toxins, in preparing new derivatives with enhanced or modified biological activities, and for the synthesis of bioactive compounds, in view of a potential practical application of those compounds as natural and selective herbicides.

INSECTICIDES

It is well known that some plant secondary metabolites act as defense against phytophagous insects. Defense chemical such as insect antifeedant, insecticidal compounds, repellents have various mode of action (Norris *et al.*, 1981). Natural products have been used as pest control agents by ancient civilization, these days, these biologically active natural products are used as lead compounds in the development of agrochemicals (Hedin, 1997). Physostgimine, which is an alkaloid found in the seed of *Physostigma venerosum* (Leguminosae), was used as lead compound for the carbamate insecticides (Kuhr & Dorough, 1976). Natural pyrethroids are insecticidal terpenoids produced by the pyrethrum flower, *Chrysanthemum cinerariaefolium* (Asteraceae) (Katsuda, 1999). Nicotine is a major alkaloid produced by tobacco, *Nicotiana rustica* (Solanaceae) and is non-specific compounds against both mammals and insects (Shiokawa *et al.*, 1992).

Some natural products have insecticidal and commercial use in pest management. Spinosad is an insect neuro-toxin produced by soil

actinomycete *Saccharopolyspora spinosa* (Thompson *et al.*, 2000). Azaridachtin is a famous phytophagous insect antifeedant produced by the neen tree *Azadirachta indica* (Meliaceae) (Mordue, 1993). The terpenoids has various biological activities, and has been incorporated in commercial products sold as an emulsifiable concentrate and in form of solid extract. Hexane, ether and methanol extracts of 108 species of Japanes wild plant were tested for their antifeedant activity against *Spodotera litura*. Some hexane extract of *Cyperus* spp. and *Gnapahlium affine* revealed insect antifeedant activity in the screening test (Morimoto & Komai, 2006).

The search for microbial bioactive molecules is aimed both to the direct use of these compounds as agrochemicals and to the discovery of new structural models and modes of action that can lead the synthesis of molecules with improved efficacy, stability or biodegradability.

Fungi are an endless source of secondary metabolites with many different biological activities. In particular, it is known that some fungi and fungal metabolites can affect several traits of insect biology, such as survival, development, fecundity, and feeding activity. In the case of crop pests, this latter, the so-called antifeedant activity, may result in reduction of both the direct feeding damage and the indirect damage due to infection of crop plants with insect-transmitted viruses (Vey *et al.*, 2001).

However, data on the antifeedant effect of fungal metabolites towards insect pests are still sparse (Wright *et al.*, 1982; Mulè *et al.*, 1992; Amiri *et al.*, 1999; Ekesi *et al.*, 2001; Quesada-Moraga *et al.*, 2006).

Metabolites produced by *Trichoderma citroviride* with potential antifeedant activity

Aphids are a major cause of loss of agricultural production and reduction of its quality. They induce a direct noxious effect on crop health, caused by subtraction of sap, and an indirect effect related to the spread of insect-transmitted virus diseases. Aphid management relies mainly on the use of synthetic insecticides, being almost all biological control options available are only intended for greenhouse aphid pests. However, the extensive use of synthetic pesticides has caused major drawbacks, such as environmental pollution, development of resistant populations of pests, killing of beneficial insects and contamination of food. Therefore, there is a demand for alternative means of aphid control, with low environmental impact.

Ganassi *et al.* (2007) showed that fungal isolates of genus *Trichoderma* can influence the feeding preference of *Schizaphis graminum,* one of the most important pests of cereal crops, and a potential antifeedant activity of these fungi towards aphids was conceived.

Trichoderma citronoviride strain ITEM 4484 has molecular characterized by sequence analysis of the internal transcribed spacer

regions ITS-1 and ITS-2 of the nuclear rDNA, and a fragment of the translation elongation factor gene TEF-1α, is also reported. The fungus grown on rice solid culture produces several metabolites that influenced the feeding preference of the alate morph of the aphid pest *Schizaphis graminum*, restraining individuals from feeding on treated leaves. Investigation of the fungal organic extract allowed isolation 5.8 and 8.9 mg/kg of two new bioactive metabolites, named citrantifidiene and citrantifidiol (**37** and **38**, Fig 17), whose structure was determined by using spectroscopic methods (essentially NMR and MS techniques) (Evidente *et al.*, 2008).

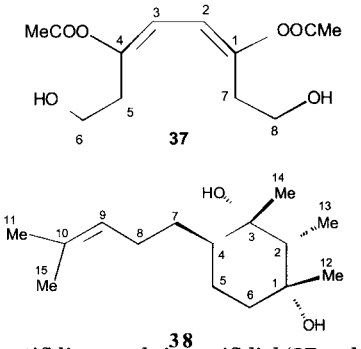


Fig 17. Structure of citrantifidiene and citrantifidiol (**37** and **38**) produced by *Trichoderma citronoviride*

Citrantifidiene is a symmetrical compound formulated as 4-acetoxy-6-hydroxy-1-(2-hydroxy-ethyl)-hexa-1,3-dienyl ester of acetic acid (**37**). The structure assigned to citrantifidiene was confirmed by couplings observed in the HMBC spectrum and supported by data from EI mass spectrum. Furthermore, the *E*-stereochemistry of the double bond could be assigned because of the clear effect observed in a NOESY spectrum (Beger & Braun, 2004) between the olefinic proton at δ 5.81 and the methyl of acetoxy group at δ 1.99. This was consistent with the typical allylic coupling (J=0.6 Hz) observed between the olefinic proton and the methylene group at δ 2.38 (Sternhell, 1969; Ptresch *et al.*, 2000; Evidente *et al.*, 2008).

Citrantifidiol was formulated as 1,2,3-trimethyl-4-(4-methyl-pent-3-enyl)-cyclohexane-1,3-diol (**38**). This structure was supported from the results of the HR ESI spectrum, and the couplings oserved in both the HMBC and NOESY spectra.

Furthermore, the NOESY data, supported by an inspection of a Dreiding model, suggested that the cyclohexane ring assumes a chair conformation and that of the methyl groups Me-12 Me-13 and Me-14, bonded at C-1, C-2 and C-3, are axial while the 4-methylpenten-3-enyl, located on C-4, is equatorial. This relative configuration is depicted in structure **38** (Evidente *et al.*, 2008).

The organic extract of *T. citrinoviride* and citrantifidiene and citrantifidiol exhibited a clear ability to influence the feeding preferences of the aphid *S. graminum* restraining them from visiting treated leaves. In feeding preference tests GLM (Generalised Linear Models) analysis revealed a treatment effect of both the organic extract (GLM organic extract: treatment $F_{1, 136} = 569.717$, $p<0.01$) and the pure metabolites: citrantifidiene (GLM: treatment $F_{1, 88} = 45.500$, $p<0.01$) and citrantifidiol (GLM: treatment $F_{1, 88} = 151.724$, $p<0.01$). GLM analysis did not revealed treatment*time interactions (GLM organic extract: treatment*time $F_{7, 136} = 0.416$ $p>0.05$), (GLM citrantifidiene: treatment*time $F_{7, 88} = 0.854$ $p>0.05$) and (GLM citrantifidiol: treatment*time $F_{7, 88} = 0.405$ $p>0.05$).

The Bonferroni adjustment for the number of comparisons revealed that the mean of the number of aphids counted on control leaves (M = 3.521, SE = 0.079) was significantly greater than that of the number of aphids on leaves dipped in organic extract (M = 0.674, SE = 0.056) over the time. Moreover, the same analysis revealed that the average number of aphids counted on leaves treated with either citrantifidiene (M = 1.458, SE = 0.114) or citrantifidiol (M = 0.958, SE = 0.76) was significantly smaller than the number of aphids counted on correspondent control leaves (M = 2.896, SE = 0.107 and M = 3.042, SE = 0.109, respectively) (Evidente *et al.*, 2008).

The organic extract of *T. citrovitide* solid culture showed to contain other antifeedant metabolites whose isolation and chemical characterization are in progress. By prelimimary spectroscopic investigation they showed to be different from **37** and **38** and appeared to belong to two different groups of natural compounds.

Citrantifidiene and citrantifidiol are two new fungal metabolites isolated from *T. citrinoviride* ITEM 4484 which shows an interesting antifeedant activity. They appear to have a very original carbon skeleton among the naturally occurring compounds. The compound most closely structurally related to citrantifidiol appear to be magydardienediol, a diterpene previously isolated from *Magydaris panacifolia* (Nagano *et al.*, 1984). Citrantifidiene, being a hexa-1,3-dienyl ester of acetic acid is quite different from citrantifidiol, which is a tetrasubstituted derivative of a cycloexane-1,3-diol. They also appeared very different from the other four metabolites produced by the same fungus which appeared close related to trichodimerol belonging to the complex structural group of bisorbicillinoids (Liu *et al.*, 2005). The antifeedant activity exhibited by citrantifidiene and citrantifidiol open an interesting perspective of practical use of these metabolites or the producing fungal strain in control of aphid pest *S. graminarum* and warrants further investingation on their mode of action, sistemicity, volatility, and performance under field conditions.

ACKNOWLEDGEMENTS

This work was carried out within the project "Enhancement and Exploitation of Soil Biocontrol Agents for Bio-Constraint Management in Crops" (contract n. FOOD-CT-2003-001687), which is financially supported by the European Commission within the 6th FP of RTD, Thematic Priority 5 Food Quality and Safety. The information/opinions provided in the paper do not necessarily represent the official position/opinion of the European Commission. The research was also supported by a grant from Regione Campania L.R. 5/02. Contribution DISSPAPA Book 4.

REFERENCES

Abate, D., Abraham, W.R. and Meyer, H. (1997). Cytochalasins and phytotoxins from the fungus *Xylaria obovata*. *Phytochemistry*, **44**: 1443-1448.

Abbas, H.K., Mirocha, C.J. and Shier, W.T. (1992). Isolation, identification and biological activity of chlamydosporol from *Fusarium culmorum* HM-8. *Mycopathologia*, **118**: 115-123.

Abraham, W.R. and Arfmann, H.A. (1988). Fusalanipyrone, a monoterpenoid from *Fusarium solani*. *Phytochemistry*, **27**: 3310-3311.

Ahrazem, O., Prieto, A., Gómez-Miranda, B., Bernabe´, M. and Leal, J.A. (2001). Comparison of cell-wall polysaccharides from *Nectria cinnabarina* with those from the group of *Nectria* with *Sesquicillium* anamorphs. *Microbiology*, **147**: 1839-1849.

Altomare, C., Pengue, R., Favilla, M., Evidente, A. and Visconti, A. (2004). Structure-activity relationships of derivatives of fusapyrone, an antifungal metabolite of *Fusarium semitectum*. *Journal of Agricultural and Food Chemistry*, **52**: 29973001.

Altomare, C., Perrone, G., Stornelli, C. and Bottalico, A. (1998a). Prove di lotta contro la muffa grigia dell'uva con fusapirone, un metabolita di *Fusarium*. *Quaderni della Scuola di specializzazione in Scienze viticole ed enologiche*, **22**: 59-66.

Altomare, C., Perrone, G., Zonno, M.C., Evidente, A. and Bottalico, A. (1998b). Fusapirone e deossifusapirone, due metaboliti di *Fusarium semitectum* di possibile impiego per la lotta contro funghi fitopatogeni e micotossigeni. *In*: Book of the Abstracts of the VI Meeting of the Italian Society of Plant Pathology (SIPaV), Campobasso, Italy, 17-18 September 1998, abs. 68.

Altomare, C., Perrone, G., Zonno, M.C., Evidente, A., Pengue, R., Fanti, F. and Polonelli, L. (2000). Biological characterization of fusapyrone and deoxyfusapyrone, two bioactive secondary metabolites of *Fusarium semitectum*. *Journal of Natural Products*, **63**: 1131-1135.

Amiri, B., Ibrahim, L. and Butt, T. (1999). Antifeedant properties of destruxins and their potential use with the entomogenous fungus *Metarhizium anisopliae* for improved control of crucifer pest. *Biocontrol Science and Technlogy*, **9**: 487-498.

Andolfi, A., Boari, A., Evidente, A. and Vurro, M. (2005). Metabolites inhibiting germination of *Orobanche ramosa* seeds produced by *Myrothecium verrucaria* and *Fusarium compactum*. *Journal of Agricultural and Food Chemistry*, **53**: 1598-1603.

Andolfi, A., Evidente, A., Santini, A. and Tuzi, A. (2006). Ophiobolin A. *Acta Crystallographic*, **62E**: 2915-2917.

Arras, G., Scherm, B. and Migheli, Q. (2002). Improving biocontrol activity of *Pichia guilliermondii* against post-harvest decay of oranges in commercial packing-houses by reduced concentrations of fungicide. *Biocontrol Science and Technology*, **12**: 547-553.

Au, T.K., Chick, W.S.H. and Leung, P.C. (2000). The biology of ophiobolins. *Life Science*, **67**: 733-742.

Bach, E., Christiansen, S., Dalgaard, L., Larse, P.O., Olsen, P.E. and Smedergard-Peterson, V. (1979). Structures, properties and relationship to the aspergillomarasmines of toxins produced by *Pyrenophora teres*. *Physiological Plant Pathology*, **14**: 41-46.

Ballio, A. and Graniti, A. (1991). Phytotoxins and their involvement in plant disease. *Experientia*, **47**: 751-826.

Bartnicki-García, S. (1987). The cell wall: a crucial structure in fungal evolution. *In*: Evolutionary Biology of the Fungi, *Eds.* Rayner, A.D.M., Brasier, C.M. and Moore D., Cambridge University Press, Cambridge, U.K. pp. 389-403.

Battilani, P., Giorni, P., Bertuzzi, T., Formenti, S. and Pietri. A. (2006). Black aspergilli and ochratoxin A in grapes in Italy, *International Journal of Food Microbiology*, **11**: S53-S60.

Battilani, P., Giorni, P. and A. Pietri. 2003. Epidemiology of toxin-producing fungi and ochratoxin A occurrence in grape. *European Journal of Plant Pathology*, **109**: 715-722.

Battilani, P. and Pietri. A. (2002). Ochratoxin A in grapes and wine. *European Journal of Plant Pathology*, **108**: 639-643.

Bau, M., Bragulat, M.R., Abarca, M.L., Minguez, S. and Cabanes, F.J. (2005). Ochratoxigenic species from Spanish wine grapes. *International Journal of Food Microbiology*, **98**: 125-130.

Bau, M., Castella, G., Bragulat, M.R. and Cabañes, J.F. (2006). RFLP characterization of *Aspergillus niger* aggregate species from grapes from Europe and Israel, *International Journal of Food Microbiology*, **111**: S18-S21.

Baudoin, O., Cesario, M., Guénard, D. and Guéritte, F. (2002). Application of the palladium-catalyzed borylation/suzuki coupling (BSC) reaction to the synthesis of biologically active biaryl lactams. *Journal of Organic Chemistry*, **67**: 1199-1207.

Berger, S. and Braun, S. ((2004). 200 and More Basic NMR experiments: A Practical Course. Wiley-VCH, Weinheim, Germany.

Booth, C. (1971). The Genus *Fusarium*. Commonwealth Institute, Kew, England.

Bottalico, A. (2004). Micotossine. *In*: Chimica degli Alimenti; Chapter 27, *Eds.* Cabras, P. and Martelli, A., Piccin, Padova, Italy.

Bottalico, A., Capasso, R., Evidente, A., Randazzo, G. and Vurro, M. (1990). Cytochalasins: structure-activity relationships. *Phytochemistry*, **29**: 93-96.

Blum, M.S., Fales, H.M. and Roger, A. (2000). Chemical characters of two related species of giant honeybeess (*Apis dorsata* and *A. laboriosa*): possible ecological significance. *Journal of Chemical Ecology*, **26**: 801-807.

Burdaspal, P. and. Legarda, T.M. (1999). Ochratoxin A from wines and grape products originating from Spain and other European countries. *Alimentaria*, **36**: 107-113.

Cambie, R.C., Hirschberg, A., Jones, E.R.H. and Lowe, G. (1963). Chemistry of the higher fungi. XVI. Polyacetylenic metabolites from *Aleurodiscus roseus*. *Journal of Chemical Society*, pp. 4120-4130.

Campbell, M.A., Medd, R.W. and Brown, J.F. (1996). Cultural and infection studies on *Pyrenophora semeniperda*, a possible bioherbicide for annual grass weeds. *In*: Proceedings of the 9th International Symposium on Biological Control of Weeds, Stellenbosch, South Africa, pp. 519-523.

Canales, M.W. and Gray, G.R. (1988). 6-epi-ophiobolin A and 3-anhydro-6-*epi*-ophiobolin A-host specific phytotoxins of *Drechslera maydis* (race T). *Phytochemistry*, **27**: 1653-1663.

Canonica, L., Fiecchi, A., Galli Kienle, M. and Scala, A. (1966a). The costitution of cochliobolin. *Tetrahedron Letters*, **11**: 1211-1218.

Canonica, L., Fiecchi, A., Galli Kienle, M. and Scala, A. (1966b). Isolation and constitution of cochliobolin. *Tetrahedron Letters*, **13**: 1329-1333.

Capasso, R., Evidente, A. and Vurro, M. (1991). Cytochalasins from *Phoma exigua* var. *heteromorpha*. *Phytochemistry*, **30**: 3945-3950.

Capasso, R., Iacobellis, N.S., Bottalico, A. and Randazzo, G. (1984). Structure-toxicity relationships of the eremophilane phomenone and PR-toxins. *Phytochemistry*, **23**: 2781-2784.

Chandramohan, S. and Charudattan, R. (2001). Control of seven grasses with a mixture of three fungal pathogen with restricted host range. *Biological Control*, **22**: 246-255.

Chandramohan, S., Charudattan, R., Sonoda, R. and Singh, M. (2002). Field evaluation of a fungal pathogen mixture for the control of seven weedy grasses. *Weed Science*, **50**: 204-213.

Cho, H., Murakami, K., Nakanishi, H., Fujisawa, A., Isoshima, H., Niwa, M., Hayakawa, K., Hase, Y., Uchida, I., Watanabe, H., Wakitani, K. and Aisaka, K. (2004). Synthesis and structure-activity relationships of 5,6,7,8-tetrahydro-4*H*-thieno[3,2-*b*]azepine derivatives: novel arginine vasopressin antagonists. *Journal of Medicinal Chemistry*, **47**: 101-109.

Chulze, S.N., Magnoli, C.E. and Dalcero, A.M. (2006). Occurrence of ochratoxin A in wine and ochratoxigenic mycoflora in grapes and dried vine fruits in South America. *International Journal of Food Microbiology*, **111**: S5-S9.

Cole, J.C. and Cox, R.H. (1881). Handbook of toxic fungal metabolites. Academic Press, New York.

Collett., M.G., Fincham, J.E., Tustin, R.C., Joubert, G. and Marasas, W.F.O. (1988). The pathology of chronic *Drechslera campanulata* toxicosis in inbred rats. *Onderstepoort Journal of Veterinary Research*, **55**: 51-57.

Cooney, J.M. and Lauren, D.R. (1999). Biotransformation of the *Trichoderma* metabolites 6-*n*-pentyl-2*H*-pyrone (6PAP) by selected fungal isolates. *Journal Natural of Products*, **62**: 681-683.

Cooney, J.M., Denis, R.L., Poole, P.R. and Whitaker, G. (1997). Microbial transformation of the *Trichoderma* metabolites 6-*n*-pentyl-2*H*-pyran-2-one. *Journal of Natural Products*, **60**: 1242-1244.

Cortes, C.E., Cortes, R.E. and Taylor, A.D. (1994). Synthesis and spectral properties of 4*H*-2,7,7-trimethyl-4-(phenylamino)-7,8-dihydrofuro[3,2-*c*]azepines. *Journal of Heterocyclic Chemistry*, **31**: 725-727.

Cutler, H.G., Cox, R.H., Crumley, F.G. and Cole, P.D. (1986). 6-pentyl-a-pyrone from *Trichoderma harzianum*: its plant growth inhibitory and antmicrobial properties. *Agricultural and Biological Chemistry*, **50**: 2943-2945.

Dean, F.M. (1983). Naturally Occurring Oxygen Ring Compounds. Butterworth: London, pp. 82-134.

Delfosse, E.S. (1990). Proceedings of the VII International Symposium on Biological Control of Weeds, Roma, Italy, March 6-11.

De Lucia, E.H., Casteel, C.L., Nabity, P.D. and O'Neill, B.F. (2008). Insects take a bigger bite out in a warmer, higher carbon dioxide world. *Proceedings of the National Academy of Sciences of the United States of America*, **105**: 1781-1782.

Dickinson, J.M. (1993). Microbial pyran-2-ones and dihydropyran-2-ones. *Natural Products Reports*, **10**: 71-98.

Duke, S.O., Baerson, S.R., Dayan, E., Rimando, A.M., Scheffler, B.E., Tellez, M.R., Wedge, D.E., Schrader, K.K., Akey, D.H., Arthur, F.H., De Lucca, A.J., Gibson, D.M., Harrison, H.F.Jr., Peterson, K., Gealy, D.R., Tworkoski, T., Wilson, C.L. and Morris, J.B. (2003). ARS research on natural products for pest management. *Pest Management Science*, **59**: 708-717.

El-Ghaouth, A., Smilanick, J.L., Brown, G.E., Ippolito, A., Wisniewski, M. and Wilson, C.L. (2000). Application of *Candida saitoana* and glycolchitosan for the control of postharvest diseases of apple and citrus fruit under semi-commercial conditions. *Plant Disease*, **84**: 243-248.

Ekesi, S., Egwurube, E.A., Akpa, A.D. and Onu, I. (2001). Laboratory evaluation of the entomopathogenic fungus, *Metarhizium anisopliae* for the control of the groundnut bruchid, *Caryedon serratus* on groundnut. *Journal of Stored Products Research*, **37**: 313-321.

Evidente, A. (1997). Bioactive metabolites from phytopathogenic fungi and bacteria. *In: Recent Research Developments in Phytochemistry*, *Ed.* Pandalai, S.G., Research Signpost, Trivandrum (India), pp. 255-292.

Evidente, A. (2006). Chemical and biological characterization of toxins produced by weed pathogenic fungi as potential natural herbicides. *In*: Natural Products for Pest Management, ACS Sympossium Series 927, *Eds.* Rimando, A.M. and Duke, S.O, ACS Division of Agricultural and Food Chemistry, Inc., Washington, USA, pp. 62-75.

Evidente, A. and Abouzeid, M.A. (2006). Characterization of phytotoxins from phytopathogenic fungi and their potential use as herbicides in integrated crop

management. *In*: Handbook of Sudtainable Weed Management, *Eds.* Singh, P.H., Batish, D.R. and Kohli, R.K., The Harworth Press Inc., New York, pp. 507-532.

Evidente, A., Amalfitano, C., Pengue, R. and Altomare, C. (1999). High performance liquid chromatography for the analysis of fusapyrone and deoxyfusapyrone, two antifungal α-pyrones from *Fusarium semitectum*. *Natural Toxins*, **7**: 133-137.

Evidente, A., Andolfi, A., Cimmino, A., Vurro, M., Fracchiolla, M. and Charudattan, R. (2006a) Herbicidal potential of ophiobolins produced by *Drechslera gigantea*. *Journal of Acgricultural and Foood Chemistry*, **53**: 1779-1783.

Evidente, A., Andolfi, A., Cimmino, A., Vurro, M., Fracchiolla, M., Charudattan, R. and Motta, A. (2006b). Ophiobolin E and *epi*-ophiobolin J produced by *Drechslera gigantean*, a potential mycoherbicide of weedy grasses. *Phytochemistry*, **67**: 2281-2287.

Evidente, A., Andolfi, A., Vurro, M., Fracchiolla, M., Zonno, M.C. and Motta, A. (2005). Drazepinone, a trisubstituted tetrahydronaphthofuroazepinone with herbicidal activity produced by *Drechslera siccans*. *Phytochemistry*, **66**: 715-721.

Evidente, A., Andolfi, A., Vurro, M., Zonno, M.C. and Motta, A. (2002). Cytochalasins Z1, Z2 and Z3, three 24-oxa[14]cytochalasans produced by *Pyrenophora semeniperda*. *Phytochemistry*, **60**: 45-53.

Evidente, A., Andolfi, A., Vurro, M., Zonno, M.C. and Motta, A. (2003a) Cytochalasins Z4, Z5 and Z6, three new 24-oxa[14]cytochalasans produced by *Phoma esigua* var. *heteromorpha*. *Journal of Natural Prducts*, **66**: 1540-1544.

Evidente, A., Cabras, A., Maddau, L., Marras, F., Andolfi, A., Melck, D. and Motta, A. (2006c). Viridenepoxydiol, a new penta substituted oxiranyldecene produced by *Trichoderma viride*. *Journal of Agricultural and Food Chemistry*, **54**: 6588-6592.

Evidente, A., Cabras, A., Maddau, L., Serra, S., Andolfi, A. and Motta, A. (2003b). Viridepyronone, a new antifungal 6-substituted 2*H*-pyran-2-one produced by *Trichoderma viride*. *Journal of Agricultural and Food Chemistry*, **51**: 6957-6960.

Evidente, A., Conti, L., Altomare, C., Bottalico, A., Sindona, G., Segre, A.L. and Logrieco, A. (1994). Fusapyrone and deoxyfusapyrone, two α-pyrones from *Fusarium semitectum*. *Natural Toxins*, **2**: 4-13.

Evidente, A., Maddau, L., Spanu, E., Franceschini, A., Lazzaroni, S. and Motta, A. (2003c). Diplopyrone, a new phytotoxic tetrahydropyrapyran-2-one produced by *Diplodia mutila*, fungus pathogen of cork oak. *Journal of Natural Products*, **66**: 3131-315.

Evidente, A. and Motta, A. (2001). Phytotoxins from fungi, pathogenic for agrarian, forestall and weedy plants. *In*: Biocative Compounds from Natural Products, *Ed.* Tringali, C. Taylor & Francis, London, pp. 473-526.

Evidente, A., Ricciardiello, G., Andolfi, A., Sabatini, M.A., Ganassi, S., Altomare, C., Favilla, M. and Melck, D. (2008). Citrantifiediene and citrantifidiol: bioactive metabolites produced by *Fusarium citrinoviride* with potential antifeedant activity toward aphids. *Journal of Agricultural and Food Chemistry*, **56**: 3569-3573.

Evidente, A., Sparapano, L., Fierro, O., Bruno, G., Giordano, F. and Motta, A. (1998). Sphaeropsidone and episphaeropsidone, phytotoxic dimedone methyl ethers produced by *Sphaeropsis sapinea* f. sp *cupressi* grown in liquid culture. *Phytochemistry*, **48**: 1139-1143.

Fairlamb, I.J.S., Marrison, L.R., Dickinson, J.M., Lu, F.J. and Schmidt, J.P. (2004). 2-Pyrones possessing antimicrobial and cytotoxic activities. *Biorganic Medicinal Chemistry*, **12**: 4285-4299.

Favilla, M., Pascale, M., Ricelli, A., Evidente, A., Amalfitano, C. and Altomare, C. (2008). Inhibition of species of the *Aspergillus* section *Nigri* and ochratoxin A production in grapes by fusapyrone. *Applied Environmental Mycrobiology*, **74**: 2248-2253.

Fracchiolla, M. (2003). Biological control of grass weeds by using bioherbicides. Ph.D. Thesis University of Bari, Italy (in Italian).

Friis, P., Olsen, C.E. and Moller, B.G. (1991). Toxin production in *Pyrenophora teres* the ascomycete causing the net-spot blotch disease of barley (*Hordeum vulgare* L.). *The Journal of Biological Chemistry*, **266**: 13329-13335.

Frisvad, J.C., Thrane, U. and Samson, R.A. (2007). Mycotoxin producers. *Mycology Series*, **25**: 135-159.

Ganassi, S., De Cristofaro, A., Grazioso, P., Altomare, C., Logrieco, A. and Sabatini, M.A. (2007). Detection of fungal metabolites of various *Trichoderma* species by the aphid *Schizaphis graminum*. *Entomology Experimental Applied*, **122**: 77–86.

Gedelrblom, W.C., Marasas, W.F., Vleggaar, R., Thiel, P.G. and Gawood, M.E. (1992). Fumonisins: isolation, chemical characterization and biological effects. *Mycopathologia*, **117**: 11-16.

Ghisalberti, E.L. (2000). Bioactive metabolites from soilborne fungi: natural fungicides and biological agents. *Studies in Natural Products Chemistry*, **21**: 181-250.

Ghisalberti, E.L. and Sivasithamparam, K. (1991). Antifungal antibiotics produced by *Trichoderma* spp. *Soil Biological and Biochem*istry, **23**: 1011-1020.

Graniti, A., Durbin, R.D. and Ballio, A. (1989). Phytotoxins and Plant Pathogenesis. Springer-Verlag, NATO ASI Series, Series H, Vol. 2, Berlin.

Grazioli, B., Fumi, M.D. and Silva, A. (2006). The role of processing on ochratoxin A content in Italian must and wine: a study on naturally contaminated grapes. *International Journal of Food Microbiology*, **111**: S93-S96.

Gruiz, K. (1996). Fungitoxic activity of saponins: practical use and fundamental principles. *Advanced in Experimental Medicinal Biology*, **404**: 527-534.

Gullan, P.G. and Cranston, P.S. (2005). The insects-An Outline of Entomology. Publishing Blackwell, Oxford.

Hallock, Y.F., Clardy, J., Kenfield, D.S. and Strobel, G. (1988). De-*O*-methyldiaporthine, a phytotoxin from *Dreschslera siccans*. *Phytochemistry*, **27**: 3123-3125.

Hedrin, P.A., Hollingworth, R.M., Masler, E.P., Miyamoto, J. and Thompson, D.G. (1997). Phytochemical for Pest Management, *Eds.*, *ACS* Symposium Series 658., American Chemical Society, Washington, DC, U.S.A.

Holm, L.G., Pluckett, D.L., Pancho, J.V. and Herberger, J.P. (1977). The World's Worst Weeds (Distribution and Biology). University Press of Hawaii, Honoloulou, pp. 84-91.

Horn, W.S., Simmonds, M.S.J., Schwartz, R.E. and Blaney, W.M. (1995). Phomopsichalasin, a novel antimicrobial agent from an endophytic *Phomopsis* sp. *Tetrahedron*, **51**: 3969-3978.

Inbar, M. and Gerling, D. (2008). Plant-mediated interactions between whiteflies, herbivores, and natural enemies. *Anuual Review of Entomology*, **53**: 431-448.

International Agency for Research on Cancer, (1993). Ochratoxin A. *In*: IARC monographs on the evaluation of carcinogenic risks to humans. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins, vol. 56. International Agency for Research on Cancer, Geneva, Switzerland, pp. 489-521.

Itai, A. Nozoe, S., Tsuda, K., Okuda, S., Iitaka, Y. and Makayama, Y. (1967). Structure of cephalonic acid, a pentaprenyl terpenoid. *Tetrahedron Letters*, **42**: 4111-4112.

Kastanias, M.A. and Tokousbalides, M.C. (2000). Herbicidal potential of pyrenophorol isolated from a *Drechslera avenae* pathotype. *Pest Management Science*, **56**: 227-232.

Katsuda, Y. (1999). Development of and future prospects for pyrethroid chemistry. *Pesticide Sciences*, **55**: 775-782.

Kawasaki, K. (2005). Spice flavor compositions, their manufacture by compounding synthetic flavor compounds with spice extracts, and food and containing them. *Japan. Kokai Tokio Koho*, 48 pp. *CODEN: JKXXAF JP 2005013138 A2 20050120.* Patent written in Japanese.

Kenfield, D., Bunkers, G., Wu, Y.H., Strobel, G., Sugawara, F., Hallock, Y. and Clardy, J. (1989a). Gigantenone, a novel sesquiterpene phytormone mimic. *Experientia*, **45**: 900-902.

Kenfield, D., Hallock, Y., Clardy, J. and Strobel, G. (1989b). Curvulin and *o*-methylcurvulinic acid: phytotoxic metabolites of *Drechslera indica* which cause necroses on purslane and spiny amaranth. *Plant Science*, **60**: 123-127.

Kenji, M., Mitsumutsu, F. and Toshihiro, H. (1992). Preparation of 2-alkylalkanediols. *Japan Kokai Tokio Kpho*, 5 pp. *CODEN: JKXXAF JP 04305545 A2 1992210228 Heisei.* Patent written in Japanese.

Kim, J.M., Hyeon, S.B., Isobai, A. and Suzuki, A. (1984). Isolation of ophiobolin A and its analogs to photosynthesis. *Agricultural Biological Chemistry*, **48**: 803-805.

Kondoh, M., Usui, T., Kobayashi, S., Tsuchiya, K., Nishikawa, K., Nishikiori, T., Mayumi, T. and Osada, H. (1998). Cell cycle and antitumor activity of pironetin and its derivatives. *Cancer Letters*, **126**: 29-32.

Kobayashi, S., Tsuchiya, K., Kurokawa, T., Nakagawa, T., Shimada, N. and Iitaka, Y. (1994). Pironetin, a novel plant-growth regulator produced by *Streptomyces* sp. *Journal of Antibiotics*, **47**: 703-707.

Koltin, Y., Ginzberg, I. and Finkler, A. (1993) Biotechnology Plant Disease. Wiley-Liss, New York: pp. 257-274.

Kuhr, R.J. and Dorough, H.W. (1976). Carbammate Insecticides: Chemistry Biochemistry and Toxicology. CRC Press, Cleveland, OH.

Leong, S.L., Hocking, A.D., Pitt, J.I., Kazi, B.A., Emmett, R.W. and Scott, E.S. (2006). Australian research on ochratoxigenic fungi and ochratoxin A. *International Journal of Food Microbiology*, **111**: S10-S17.

Leung, M.C.K., Díaz-Liano, G. and Smith, T.K. (2006). Mycotoxins in pet food: a review on worldwide prevalence and preventive strategies. *Journal of Agricultural and Food Chemistry*, **54**: 9623-9635.

Lim, C.H., Miyagawa, H., Ueno, T., Takenaka, H. and Sung, N.D. (1996). Siccanol: sesterterpene isolated from pathogenic fungus *Drechslera siccans*. *Han'guk Nonghwa Hakhoechi*, **39**: 241-244. From C.A.126: 131654 (1996).

Li, E., Clark, A.M., Rotella, D.P. and Hufford, C.D. (1995). Microbial metabolites of ophiobolin A and antimicrobial evaluation of ophiobolins. *Journal of Natural Products*, **58**: 74-81.

Li, X., Kim, S.K., Kang, J.S., Choi, H.D. and Son, B.W. (2004). Polyketide and sesquiterpenoid metabolites from a marine-derived fungus. *Bulletin of Korean Chemistry Society*, **25**: 607-608 (and references therein cited).

Liu, W., Gu, Q., Zhu, W., Cui, T. and Fan, G. (2005). Dihydrotrichodimerol and tetrahydrotrichodimerol, two new bisorbicillinoids, from a marine-derived *Penicillium terrestre*. *Journal of Antibiotic,* **58**: 621-624 (and literature therein cited).

Logrieco, A., Moretti, A., Fornelli, F., Fogliano, V., Ritieni, A., Caiaffa, M.F., Randazzo, G., Bottalico, A. and Macchia, L. (1996). Fusaproliferin production by *Fusarium subglutinans* and its toxicity to *Artemia salina*, SF-9 insect cells, and IARC/LCL 171 human B lymphocytes. *Applied Environmental Microbiology*, **62**: 3378-3384.

Magan, N. and Aldred, D. (2007). Why do fungi produce mycotoxins? *Mycology Series*, **25**: 121-133.

Magnoli, C., Violante, M., Combina, M., Palacio, G. and Dalcero, A. (2003). Mycoflora and ochratoxin-producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina. *Letter Applied Microbiology*, **37**: 179-184.

Mangoni, A., Esposito, V. and Randazzo, A. (2003). Configuration assignment in small organic molecules *via* residual dipolar couplings. *Chemical Communication*, 154-155.

Mannina, L., Segre, A.L., Ritieni, A., Fogliano, V., Vinale, F., Randazzo, G., Maddau, L. and Bottalico A. (1997). A new antifungal growth inhibitor from *Trichoderma viride*. *Tetrahedron*, **53**: 3135-3144.

Matsumori, N., Kaneno, D., Murata, M., Nakamura, H. and Tachibana, K. (1999). Stereochemical determination of acyclic structures based on carbon-proton spin-coupling constants. A method of configurational analysis for natural products. *Journal of Organic Chemistry*, **64**: 866-876.

Marasas, W.F.O., Nelson, P.E. and Tousson, T.A. (1983). Toxigenic *Fusarium* Species and Mycotoxicology. The Pennsylvania State University Press, University Park, USA.

Marras, F., Maddau, L., Franceschini, A., Corda, P. and Bottalico, A. (1994). Screening of antagonistic fungal strains for biological control of *Sclerotium* and *Sclerotinia* crown and stem rot of artichoke. *In*: Proceedings 9th Congress of the Mediterranean Phytopathological Union, September 18-24, 1994, Kusadasi-Aydin, Turkie, pp. 197-199.

Mattia, C.A., Mazzarella, L. and Puliti, L. (1982). 4-(2-Amino-4-oxo-2-imidazolin-5-ylidene)-2-bromo-4,5,6,7-tetrahydropyrrolo[2,3-*c*]-azepin-8-one methanol solvate: a new bromocompound from the sponge *Acanthella aurantiaca*. *Acta Crystallographic*, **38B**: 2513-2515.

Maxwell, A., Dabideen, D., Reynolds, W.F. and McLean, S. (1998). Two 6-substituted 5,6-dihydropyran-2-ones from *Piper reticulatum*. *Journal of Natural Products*, **61**: 815-816.

McGlacken, G.P. and Fairlamb, I.J.S. (2005). 2-Pyrone natural products and mimetics: isolation, characterization and biological activity. *Natural Products Reports*, **22**: 369-385.

McLaughlin, J.M. (1991). Crown gal tumours on potato discs and brine shrimp lethality: Two simple bioassays for higher plant screening and fractionation. *In*: Methods in Plant Biochemistry, Vol. 6 Chapeter, 1, *Ed*. Hostettmann, K., Academic Press, New York, USA, pp. 1-32.

Medd, R.H. (1992). A review of the world distribution and host range of *Pyrenophora semeniperda*. *Review of Plant Pathology*, **71**: 891-901.

Mordue, L.A.J. (1993). Azadirachtin: an update. *Journal of Insect Physiology*, **30**: 903-924.

Moreno-Manas, M. and Pleixats, R. (1992). Dehydroacetic acid, triacetic acid lactone and related pyrones. *In*: Advances in Heterocyclic Chemistry, Vol. 53, *Ed*. Katritzky, A.R., Academic Press, San Diego, CA, pp. 1-84.

Morgavi, D.P. and Riley, R.T. (2007). A historical overview of field disease outbreaks known or suspected to be caused by consumption of feeds contamined with *Fusarium toxins*. *Animal Feed Science and Technology*, **137**: 201-212.

Morimoto, M. and Komai, K. (2006). Insect antifeedant of natural products and the structure-activity relationships of their derivatives. *In*: Natural Products for Pest Management, ACS Symposium Series 927, *Eds*. Rimando, A.M. and Duke, S.O, American Chemical Society Washington, DC, pp. 182-193.

Morisaki , M., Nozoe, S. and Iitaka, Y. (1968). X-ray studies of C_{25} terpenoids. I. The crystal structure of ophiobolin methoxybromide. *Acta Crystallographica*, **B24**: 1293-1303.

Mulè, G., D'Ambrosio, A., Logrieco, A. and Bottalico, A. (1992). Toxicity of mycotoxins of *Fusarium sambucinum* for feeling in *Galleria melonella*. *Entomologia Experimentalis et Applicata*, **62**: 17-22.

Nagano, H., Tori, M., Shiota, M. and De Pascual, T.J. 400 MHz proton nuclear magnetic resonance study of magydardienediol, a diterpene from *Magydaris panacifolia*. *Bulletin of Chemistry Society Japan*, **57**: 2971-2975.

Nakajima, H., Nishimura, K., Hamasaki, T., Kimura, Y., Yokota, T. and Udagawa, S.-I. (1987). Structure of neovasinone, a new alpha-pyrone plant regulator produced by the fungus *Neocossmospora vasinfecta* E.F. Smith. *Agricultural Biological Chemistry*, **51**: 1221-1224.

Nakamura, M. and Ishibashi, K. (1958). New antibiotic ophiobolin, produced by *Ophiobolus miybeanus*. *Journal of Agricultural Chemistry Society Japan*, **32**: 739-744.

Natori, S. and Yahara, I. (1991). Cytochalasins. *In*: Mycotoxins and Phytoalexins, Chapter 12, *Eds*. Sharma, R.P. and Salunke, D.K., CRC Press, Boca Raton, pp. 291-336.

Naylor, R.E. (2002). Weed Management Handbook. Blackwell Publishing, Oxford.

Neergard, P. (1979). Seed Pathology, Vol. 1. The Macmillan Press, London.

Norris, D.M., Haug, G. and Hoffmann, H. (1981). Anti-feeding-Compounds. Springer-Verlag, Berlin, pp. 99-144.

Nozoe, S., Hirai, K. and Tsuda, K. (1966). The structure of zizanin-A and -B, C_{25}-terpenoids isolated from *Helminthosporium zizaniae*. *Tetrahedron Letters*, **20**: 2211-2216.

Nozoe, S., Itai, A., Tsuda, K. and Okuda, S. (1967). Chemical trasformation of cephalonic acid. *Tetrahedron Letters*, **42**: 4113-4117.

Nozoe, S., Morisaki, M., Tsude, K., Iitaka, Y., Tokahashi, N., Tamura, S., Ishibashi, K. and Shirasaka, M. (1965). Structure of ophiobolin, a C25 terpenoid having a novel skeleton. *Journal of American Chemical Society*, **87**: 4968-4970.

Nozoe, S., Morisaki, M., Fukushima, K. and Okuda, S. (1968). The isolation of an acyclic C25-isoprenoid alcohol, geranylnerolidol, and a new ophiobolin. *Tetrahedron Letters*, **42**: 4457-4458.

Nukina, M. (1987). Pyrichalasin H, a new phytotoxic metabolite belonging to cytochalasans from *Pycularia grisea* (Cooke) saccardo. *Agicultural and Biological Chemistry*, **51**: 2625-2628.

Opender, K. (1996). Natural product leads as anti-insect compound. Prospects for biotechnology. *Biotechnological Perspectives in Chemical Ecology of Insects*, 15-27.

Padmanabhan, S.Y. (1973). The great Bengal famine. *Annual Review of Phytopathology*, **11**: 11-26.

Papavizas, G.C. (1985). *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. *Annual Review of Phytopathology*, **23**: 23-54.

Pena-Rodriguez, L.M. and Chilton, W.S. 3-Anhydroophiobolin A and 3-anhydro-6-*epi*-ophiobolin A, phytotoxic metabolites of the johnson grass pathogen *Bipolaris sorghicola*. *Journal of Natural Products*, **52**: 1170-1172.

Perrone, G., Altomare, A., Bottalico, A. and Evidente, A. (1995). Biological characterization of fusapyrone and deoxyfusapyrone, two antifungal α-pyrones from *Fusarium semitectum*. *In*: Book of the Abstracts of the International Seminar on *Fusarium* Mycotoxins, Taxonomy, Pathogenicity, Martina Franca, Italy 9-13 May 1995, pp. 62.

Perrone, G., Mulè, G., Susca, A., Battilani, P., Pietri, A. and Logrieco, A. (2006). Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. *Applied Environmental Microbiology*, **72**: 680-685.

Poppe, S.M., Slade, D.E., Chong, K.T., Hinshaw, R.R., Pagano, J., Markowitz, M., Ho, D.D., Mo, H., Gorman, R.R. III, Dueweke, T.J., Thaisrivongs, S. and Tarpley, W.G. (1997). Antiviral activity of the dihydropyrone PNU-140690, a new non-peptidic human immunodeficiency virus protease inhibitor. *Antimicrobial Agents and Chemotherapy*, **41**: 1058-1063.

Pretsch, E., Bühlmann, P. and Affolter, C. (2000). Structure Determination of Organic Compounds -Tables of Spectral Data; Springer-Verlag, Berlin, pp. 161-244, 313-386.

Quesada-Moraga, E., Carrasco-Diaz, J.A. and Santiago-Alvarez, C. (2006). Insecticidal and antifeedant activities of protein secreted by entomopathogenic fungi against *Spodoptera littoralis* (Lep, Noctuidae). *Journal of Applied of Entomology*, **130**: 442-452.

Santino, A., Poltronieri, P. and Mita, G. (2005). Advances on plant products with potential to control toxigenic fungi: A review. *Food Additives & Contaminants*, **22**: 389-395.

Sassa, T. (1971). Cotylenines, leaf growth subatances produced by fungus. Part I. Isolation and characterization of cotylenins. *Agricultural Biololgical Chemistry*, **35**: 1415-1418.

Sassa, T., Neguro, T. and Ueki, H. (1972). Production and characterization of a new fungal metabolite, cotylenol. *Agricultural Biololgical Chemistry*, **36**: 2281-2285.

Schneider, D.J., Marasas, W.F.O., Collett, M.G. and van der Westhuizen, G.C.A. (1985). An experimental mycotoxicosis in sheep and goats caused by *Drechslera campanulata*, a fungal pathogen of green oats. *Onderstepoort Journal of Veterinary Research*, **52**: 93-100.

Sekine, T., Jiro, A., Kazuki, S., Fumio, I., Siripon, O. and Isamu, M. (1989). (+)-Acacialactam, a new seven-membered lactam from seeds of *Acacia concinna*. *Chemical Pharmaceutical Bulletin*, **37**: 3166-3165.

Serra, R., Mendonca, C. and Venancio, A. (2006). Ochratoxin A occurrence and formation in Portuguese wine grapes at various stages of maturation. *International Journal of Food Microbiology*, **111**: S35-S39.

Sharma, P. and Dreja, P. (2004). Evaluation of *Trichoderma harzianum* and *T. viride* isolates at BCA pathogen crop interface. *Journal of Mycology and Plant Pathology*, **34**: 47-55.

Shiokawa, K., Moriya, K., Shibuya, K., Hattori, Y., Tsuboi, S. and Kagabu, S. (1992). 3-(-6-Chloronicotinyl)-2-nitromethylene-thiazoline as a new class of insecticide acting against *Lepidoptera* species. *Bioscience Biotechnology and Biochemistry*, **52**: 1364-1365.

Sivanesan, A. (1992). IMI description of fungi and bacteria no. 1123. *Drechslera gigantea*. *Mycopathologia*, **119**: 40-50.

Sivasithamparam, K. and Ghisalberti, E.L. (1998). Secondary metabolism in *Trichoderma* and *Gliocladium*. *In*: Trichoderma & Gliocladium, *Eds*. Kubicek, C.P., Harman, G.E., Taylor & Francis: London, pp. 139-191.

Schmidt, R. (1989). The application of *Artemia salina* L. bioassay for screening of *Fusaria* toxins. *In*: Fusarium-Mycotoxins, Taxonomy and Pathogenicity, Chapter 6, *Ed.* Chelkowski, J., Elsevier, Amsterdam, The Netherlands, pp. 121-130.

Solfrizzo, M., Visconti, A., Savard, M.E., Blackwell, B.A. and Nelson, P.E. (1994). Isolation and characterization of new chlamydosporol related metabolites of *Fusarium chlamydosporum* and *Fusarium tricintum*. *Mycopathologia*, **127**: 95-101.

Spiga, D., Marras, F., Maddau, L., Franceschini, A. and Corda, P. (1998). Prove preliminari di lotta contro il "Marciume del colletto" del carciofo da *Sclerotium rolfsii* mediante *Trichoderma viride*. *Notiziario sulla protezione delle piante*, **8**: 173-179.

Stanghellini, M.E. and Miller, R.M. (1997). Biosurfactants: Their identity and potential efficacy in the biological control of zoosporic plant pathogens. *Plant Disease*, **81**: 4-12.

Sternhell, S. (1969). Correlation of interproton spin-spin coupling constants with structure. *Quarterly Review*, **23**: 237-269.

Strobel, G.A. (1982). Phytotoxins. *Annual Review of Biochemistry*, **51**: 309-333.

Strobel, G.A. (1991). 'Il controllo biologico delle erbe infestanti'. *Le Scienze*, **277**: 56-63, Italian edition of Scientific American, New York: Scientific American Inc.

Strobel, G.A., Kenfield, D., Bunkers, G., Sugawara, F. and Clardy, J. (1991). Phytotoxins as potential herbicides. *In*: Phytotoxins and their involvement in plant disease, *Eds.* Ballio, A. and Graniti, A. *Experientia*, **47**: 819-826.

Strobel, G., Kenfield, D. and Sugawara, F. (1988). The incredible fungal genus *Drechslera* and its phytotoxic ophiobolins. *Phytoparasitica*, **16**: 145-152.

Strobel, G.A., Sugawara, F. and Clardy, J. (1987). Phytotoxins from plant pathogens of weedy plants. *In*: Allelochemicals: Role in Agriculture and Forestry, ACS Symposium Series 330, *Ed.* Waller, G.R., American Chemical Society, Washington, USA, pp. 516-523.

Sugawara, F., Strobel, G., Strange, N.R., Siedow, J.N., Van Duyne, G.D. and Clardy, J. (1987). Phytotoxins from the pathogenic fungi *Drechslera maydis* and *Drechslera sorghicola*. *Proceedings of National Academy of Science U.S.A.*, **84**: 3081-3085.

Sugawara, F., Takahashi, N., Strobel, G., Yun, C.H., Gray, G., Fu, Y. and Clardy, J. (1988). Some new phytotoxic ophiobolins produced by *Drechslera oryzae*. *Journal of Organic Chemistry*, **53**: 2170-2172.

Suzuki, K., Kuwahara, A., Yoshida, H., Fujita, S., Nishikiori, T. and Nakagawa, T. (1997). NF0059A_1, A_2, A_3, B_1 and B_2, novel amtitumor antibiotics produced by *Aspergillus* sp. NF 00569. I. Taxonomy, fermentation, isolation and biological properties. *Journal of Antibiotics*, **50**: 314-317.

Tanaka, Y. and Omura, S. (1993). Agroactive compounds of microbial origin. *Annual Review of Microbiology*, **47**: 57-87.

Tatum, L.A. (1971). The Southern corn leaf blight. *Science*, **171**: 1113-1115.

Thaisrivongs, S., Romero, D.L., Tommasi, R.A., Janakiraman, N., Strohbach, J.W., Turner, S.R., Biles, C., Morge, R.R., Johnson, P.D., Aristoff, P.A., Tomich, P.K., Lynn, J.C., Horng, M.M., Chong, K.T., Hinshaw, R.R., Howe, W.J., Finzel, B.C. and Watenpaugh, K. (1996). Structure-based design of HIV protease inhibitors: 5,6-dihydro-4-hydroxy-2-pyrones as effective nonpeptidic inhibitors. *Journal of Medicinal Chemistry*, **39**: 4630-4642.

Tjamos, S.E., Antoniou, P.P., Kazantzidou, A., Antonopoulos, D.F., Papageorgiou, I. and Tjamos, E.C. (2004). *Aspergillus niger* and *Aspergillus carbonarius* in Corinth raisin and wine-producing vineyards in Greece: population composition, ochratoxin A production and chemical control. *J. Phytopathol.*, **152**: 250-255.

Thomson, R.H. (1985). The Chemistry of Natural Products. Blackie: Glasgow, pp. 107-153.

Thompson, G.D., Dutton, R. and Sparks, T.C. (2000). Spinosad - a case study: an example from a natural products discovery programme. *Pest Management Science*, **56**: 696-702.

Tomoda, H., Namatame, I., Tabata, N., Kawaguchi, K., Si, S. and Omura, S. (1999). Structure elucidation of fungal phenolchalasin, novel inhibitors of lipid droplet formation in mouse macrophages. *Journal of Antibiotics*, **52**: 857-861.

Tsuchiya, K., Kobayashi, S., Nishikiori, T., Nakagawa, T. and Tatsuta, K. (1997). NK10958P, a novel plant growth regulator produced by *Streptomyces* sp. *Journal of Antibiotics*, **50**: 259-260.

Turner, S.R., Strohbach, J.W., Tommasi, R.A., Aristoff, P., Johnson, P.D., Skulnick, H.I., Dolak, L.A., Seest, E.P., Tomich, K., Bohanon, M.J., Horng, M.M., Lynn, J.C., Chong, K.T., Hinshaw, R.R., Watenpaugh, K.D., Janakiraman, M.N. and Thaisrivongs, S. (1998). *Journal of Medicinal Chemistry*, **41**: 3467-3476.

Upadhyay, R.K. and Mukerji, K.G. (1997). Toxins in plant disease development and evolving Biotechnology, Oxford & IBH Publishing Co. Pvt. Ltd, New Delhi India.

van der Merwe, K.J., Steyn, P.S., Fourie, L., Scott, D.B. and Theron, J.J. (1965). Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus*. *Nature* **205**: 1112-1113.

Vey, A., Hoagland, R. and Butt, T.M. (2001). Toxic metabolites of fungal biocontrol agents. *In*: Fungi as Biocontrol Agents, *Eds*. Butt, T.M., Jackson, C. and Magan, N., CAB International, Wallingfors, U.K., pp. 311-340.

Vesonder, R.F. and Golinski, J. (1989). Titolo. *In*: Fusarium-Mycotoxins, Taxonomy and Pathogenicity, Chapter 1, *Ed*. Chelkowski, J., Elsevier, Amsterdam, The Netherlands, pp. 1-39.

Visconti, A., Solfrizzo, M., Fruchier, A. and ApSimon, J.W. (1994). Acuminatopyrone: revised structure and production by *Fusarium chlamydosporum* and *Fusarium tricinctum*. *Journal of Naural Products*, **57**: 695-699.

Vollenbroich, D., Pauli, G., Özel, M. and Vater, J. (1997). Antimycoplasma properties and application in cell culture of surfactin, a lipopeptide antibiotic from *Bacillus subtilis*. *Applied Environmental Microbiology*, **63**: 4-49.

Vurro, M., Bottalico, A., Capasso, R. and Evidente, A. (1997). *In*: Cytochalkasins from phytopathogenic *Ascochyta* and *Phoma* species. *In*: Toxins in Plant Disease Development and Evolving Biotechnology, *Eds*. Upahyay, R.K. and Mukerji, K.G., Oxford & IBH publishing Co. Pvt. Ltd., New Delhi, India, pp. 127-147.

Walker, R. and Larsen, J.C. (2005). Ochratoxin A: previous risk assessments and issues arising. *Food Additives and Contaminants*, **22**: 6-9.

Wexler, H.M., Lavin, P.T.E., Molitoris and Finegold, S.M. (1990). Statistical analysis of the effects of trial, reader, and replicates on MIC determination for cefoxitin. *Antimicrobial Agents Chemotherapy*, **34**: 2246-2249.

Wright, V.F., Vesonder, R.F. and Ciegler, A. (1982). Mycotoxins and other fungal metabolites as insecticides. *In*: Mycotoxins and Other Fungal Metabolites as Insecticides: Microbial and Viral Pesticides, *Ed*. Kurstak, E., Dekker: New York, Ny, USA & Basel, Switzerland, pp. 559-583.

Xu, Y., Yakushijin, K. and Horne, D.A. (1997). Synthesis of $C_{11}N_5$ marine sponge alkaloids: (±)-hymenin, stevensine, hymenialdisine, and debromohymenialdisine. *Journal of Organic Chemistry*, **62**: 457-464.

Zhan, W.M. and Watson, A.K. (1997). Host range of *Exserohilum monoceras*, a potential bioherbicide for the control of *Echinocloa* species. *Canadian Journal of Botany*, **75**: 685-692.

Zimmerli, B. and Dick, R. (1996). Ochratoxin A in table wine and grape-juice: Occurrence and risk assessment. *Food Additives and Contaminants*, **13**: 655-668.

"This page is Intentionally Left Blank"

16

On the Potential of Some Natural Colouring Agents: An Overview

ANNIE SHIRWAIKAR[1*], ARUN SHIRWAIKAR[2], RICHARD LOBO[1] AND KIRTI. S. PRABHU[1]

ABSTRACT

The natural colours, already used widely in the industry, will probably increase with the need to have colourants that produce the required colour but with the minimum risk to the consumer. Some synthetic food colourants have been withdrawn from the market because of their adverse effects on human health. There is a particular need for good colours that are stable at higher pH levels. The public perception of natural colours is that they are safer and better than man-made synthetic colours. The present article discusses the potential of natural colouring agents.

Key words : Colourants, carotenoids, betalain, anthocyanins, carminic acid, EU, USA

INTRODUCTION

Colour is an important part of life itself. Plants use it to lure insects to pollinate and to ensure the survival of species. Insects and other animals use it to attract members of the opposite sex for the same purpose. All colours except green help plants to utilize solar energy for life and growth. It is no wonder that for millennia, colour has played a dominant role in three areas important to man: food, physical appearance and art. Archeology and recent history are filled with accounts of the widespread use of colour in art and as an additive to cosmetic preparations. The use of colour in

1. Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal, India.
2. Dean, Gulf Medical College, Gulf Medical University, Ajman, U.A.E.
* *Corresponding author* : E-mail : annieshirwaikar@yahoo.com

cosmetics has been documented in the Egyptian pyramids and tombs dating back more than 5000 years. Egyptian woman used henna to dye their hair, and kohl (a poisonous antimony compound) to blacken their eyebrows, eyelids and lashes. The ancient Romans painted their faces with lead pigments and dyed their hair.

The natural coloring of foodstuffs is the healthiest way of colouring food although the cheapest way of colouring food is by the use of artificial colouring agents produced by synthesis. These artificial colouring agents are obtained from basic chemical materials and are above all considerably cheaper than natural colouring agents. Their number and the maximum quantities admissible as regulated by the law have dramatically dropped in recent years as a result of their toxic nature and because of their negative effects on health *viz.* cancer and other disorders. Natural colouring agents are extracted from intensely coloured natural raw materials *e.g.* curcuma root, paprika, stinging nettles, annatto seeds, marigolds, grape skins etc. These natural colouring agents provide very good stability with a wide range of colours and uses. However as these products are also treated as additives under the foodstuff law they must thus be included in the list of ingredients as colouring agents or with their E numbers. (Reference to the natural quality of the raw materials is not permitted). The Table 1. below illustrates the effects of different food colourants on health (GNT-Healthy colours for healthy life).

Table 1. Effects of different colourants on health

Food colour	Health effects
Red #3 (Erythrosine)	Thyroid tumors, chromosomal damage, hyperactivity.
Red #40 (Allura red AC)	Lymphatic tumors, hyperactivity.
Blue #1 (Brilliant blue FCF)	Chromosomal damage.
Blue #2 (Indigotine)	Brain tumor.
Green #3 (Fast green FCF)	Bladder tumors.
Yellow #5 (Tartrazine)	Thyroid and lymphatic tumors, allergy, hyperactivity.
Yellow #6 (Sunset yellow FCF)	Kidney tumors, chromosomal damage.

A pharmaceutical colouring agent may be defined as any material that is a dye, pigment or substance made by a process or synthesis, extracted, isolated from plant or otherwise derived from a suitable animal, mineral or other source that is employed in pharmaceutical product to impart a colour.

Colouring agents are used for the following reasons

They visually alter the appearance of a medicinal product by imparting a definite colour; They enhance easier identification and are hence beneficial to patients on multiple medication. The use of colours in medicinal products,

in conjunction with other factors, such as shape and packaging, additionally, serves to reinforce brand image and identity and colour helps to impart a uniformity of appearance to a product. Hence an ideal colouring agent should possess the following properties (Mortensen, 2006).

Ideal requirements for colouring agents

It should be harmless to health and should not have any physiological activity. It should be compatible with medicaments; should not be affected by oxidizing or reducing agents or by pH changes; should be non-toxic, non carcinogenic, stable over a wide range of temperature; should produce colour even in small quantities; should be soluble in water and oils in minimum quantities and should be economical.

As per the FDA colouring agents, are classified as

- F D and C colourants: - Colourants certifiable for use in colouring food, drugs and cosmetics.
- D and C colourants: - Dyes and pigments considered safe in drugs and cosmetics that can be ingested or used in direct contact with mucous membranes.

Generally colouring agents may be classified as

- Natural colouring agents: *Ex*: Curcumin, Orcein etc.
- Synthetic colouring agents: *Ex*: Sunset Yellow, Tartrazine.

Natural colouring agents

These are the chemical substances, which are obtained from natural sources like minerals, plants and animals and can be further classified.

1. **On the basis of their origin:**
   - Natural colours from plant sources: *Ex*: Curcumin, bixin, crocin.
   - Natural colours from animal sources: *Ex*: Cochineal, orcein.
   - Natural colours from mineral sources: *Ex*: Iron oxides and hydroxides, Titanium dioxide.
2. **On the basis of their chemical stucture:**
   - Isoprenoid derivatives: *Ex*: Carotenoides: α, β, γ- Carotenoids, Capsanthin, Capsorubin.
   - Tetrapyrrole derivatives: *Ex:* Chlorophyll.
   - Betalains: *Ex*: Betanin.
   - Anthocyanins: *Ex*: Cyanidin, Delphidin, Malvidin, Pelargonidin.
   - Quinones: Anthraquinones: *Ex*: Alizarin.

- Napthoquinones: *Ex*: Shikonin, Lawsone
- Others: *Ex*: Monascorubrin, Phycocyanobilin, Phycoerythrobilin, Vitisins, Calcium carbonate, Aluminium, Gold, Silver.

Carotenoids

The carotenoid group includes several hundred tetraterpenoid compounds consisting of a sequence of 8 isoprene units. Their characteristic chromophore of at least ten conjugated double bonds explain their yellow or orange colour and their tendency to get oxidized. Their colour originates due to the conjugation reactions *i.e.*, a reduction in conjugation results in yellow colour while an increase in conjugation results in a red colour.

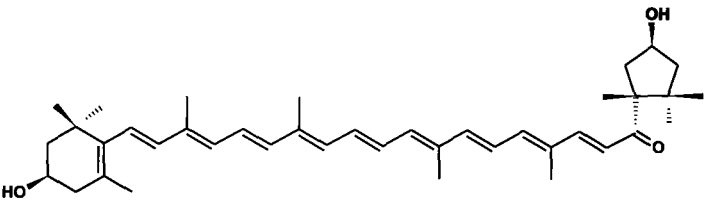
Table 1. Examples of oxygenated carotenoids

Carotenoids	Occurrence
Bixin	*Bixa orellana*
Capsanthin	*Capsicum* species
Capsorubin	*Capsicum* species
Crocin	Saffron
Crocetin	Saffron
Fucoxanthin	Brown Algae
Lutein	*Tagetes erecta*
Lycopene	*Lycopersicum esculentum*
Phycobiliproteins	Red algae

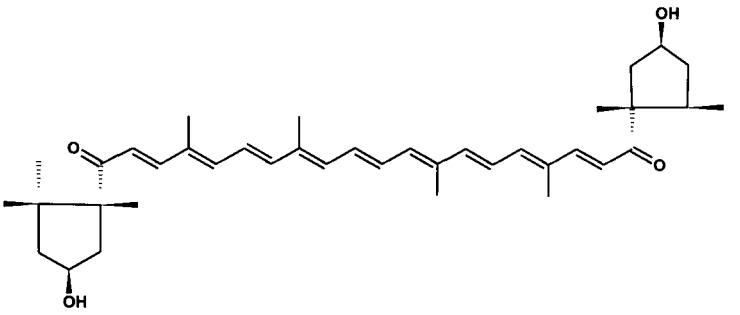
Carotenoids are found in flowers, petals, leaves and fruits. They are also found in yeast, fungi, moulds and bacteria. Pure carotenoids are available in 2 forms *i.e.,* as a micro-crystalline suspension in vegetable oil or as powders to be dispersed in water.

Capsanthin and Capsorubin

These principle colouring agents are obtained from the dried ripe fruits of *Capsicum annum* and *C. frutescens.* Family Solanaceae. Their colour is due to the presence of a terminal cyclopentane ring . Used as colours and flavors, they are oil soluble and impart an orange-red shade (Dweck, 2002; Bosland, 1996).



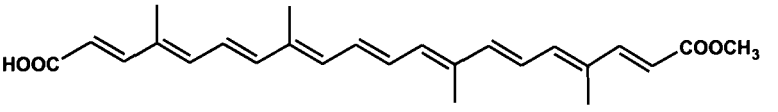
Capsanthin



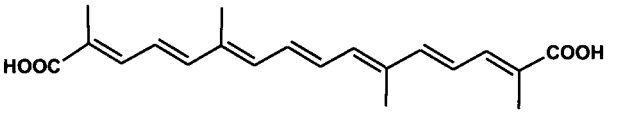
Capsorubin

Annatto

Annatto is a natural carotenoid pigment derived from the fruit of the tropical bush *Bixa orellane*, commonly known as the Lipstick tree. Family Bixaceae. The seed coat consists of mostly of carotenoids dyes (2%) and an essential oil. Bixin (10-30%) is the principal colouring matter. Bixin was isolated for the first time in 1875. Anatto pigment is incompatible with calcium salts, oxygen and carbon dioxide. They are alkali stable and precipitate in acid. They impart a yellow-orange shade. The acceptable daily intake is 2 mg/kg.



Bixin

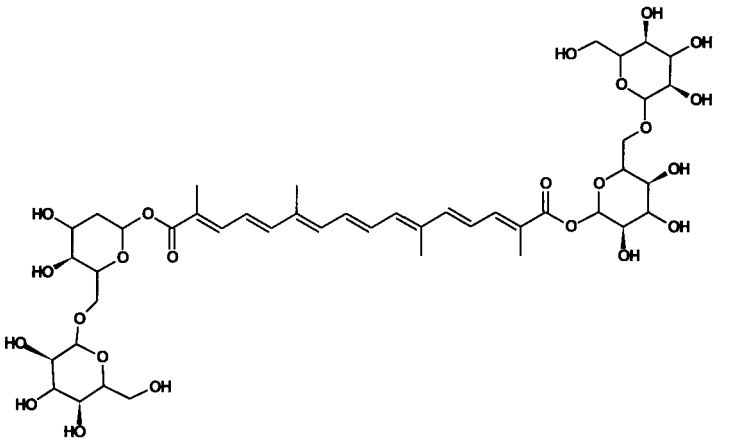


Norbixin

The annatto dye is non-toxic and is mainly used for colouring edible materials like butter, ghee, milk products, chocolates and cosmetics. It neither affects the colour nor the aroma of the concentrates and drinks. A yellow, transparent, water-soluble ink is prepared from the dye. The dye is used as an adsorption indicator in argentometric titrations. Coloured coating materials have been made using annatto and cellulose acetate phthalate and the same has been used for coating tablets, pills, granules and in herbal preparations. The addition of annatto seed-meal to poultry feed is reported to deepen the colour of the egg-yolk. Annatto dye is also used in floor polishes, shoe polishes and hair oils (*Off. J. Eur. Commun.* 1995, 1999, 2001; Code of Federal Regulations; Evans, 1996; Saraswathy, 2004).

Crocin

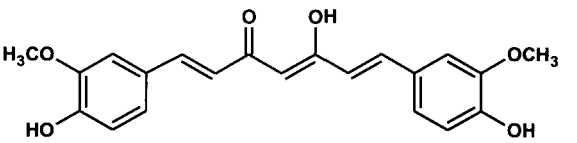
It is obtained from the dried stigmas and tops of the styles of *Crocus sativus* (saffron) Family Iridiaceae (Farrell, 1985). Colour of the drug is due to the carotenoids, chiefly represented by crocin, which is the diester of crocetin and gentiobiose. The colour is due to a pigment called crocin, so strong that 1 part crocin in a 100,000 parts of water imparts a deep golden colour. The flavour comes from a related compound called picrocrocin. Approved by the FDA in 1966 as a natural colourant, saffron is used as a cosmetic dye. Its tincture is used as a colouring agent at a level of 1 to 260 ppm in a wide range of culinary, bakery, alcoholic and non alcoholic beverages and confectionery. It is used to impart yellow colour to rice and other foods and also employed as a spice (Dweck, 2002).



Crocin

Curcumin

It is obtained by solvent extraction of the dried rhizomes of turmeric *Curcuma longa* (Zingiberaceae). This pigment of the spice turmeric gives a range of colours from yellow to deep orange colour. In USA, both turmeric oleoresin and curcumin are permitted colourants whereas only curcumin is recognized as a colourant in the EU. Curcumin is very prone to photobleaching. It is insoluble in water but is stable in acidic condition. It imparts an orange-yellow shade.



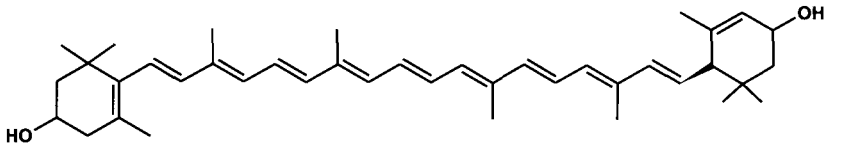
Curcumin

It is also used to compensate for fading of natural colouring in pre-packed food and serves as a colourant in foods, pharmaceuticals, cheese,

margarine, baked sweets and in fish fingers (Dweck, 2002; Mortensen, 2006).

Lutein

It is obtained from the florets of *Tagetes erecta* (Compositae). Lutein is a carotenoid whose name is derived from the Latin word for yellow. Commonly known as African marigold lutein is a dihydroxy derivative of α-carotene.



Lutein

Lutein is used in dietary supplements. Commercially it is used as an additive in chicken feed to increase the yellow colour of egg yolk (Mortensen, 2006).

Chlorophyll

Chlorophyll is the green pigment utilized by all higher plants. The name is derived from the Greek words for green and leaf. It is a green colouring matter of plants. It is very difficult to prepare chemically pure chlorophyll and the commercial product contains a mixture of the two chlorophylls a and b (in the ratio of 3:1) and several carotenoids.

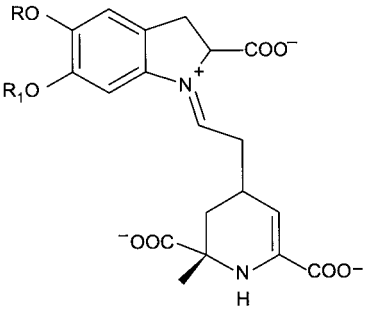
Chlorophyll 'A' is bluish-black in colour and chlorophyll 'B' is greenish-black in colour. The difference between the two chlorophylls is the methyl side chain in chlorophyll 'A' which is replaced by a formyl group in chlorophyll 'B'. Both of these are magnesium complexes with a porphyrine ring. Chlorophyll is not a permitted colourant in USA but is legal in the EU when extracted from edible plants.

Used as a colouring agent for fats and oils chlorophyll extract is consumed orally, for its antioxidant property. The dye is extracted from natural sources and is used extensively in colouring inks, resins, soaps and waxes, edible fats, cosmetics, liniments, lotions, perfumes, mouthwashes and leather. The dyestuff is known as CI Natural Green 3 and CI Number 75810 (Mortensen, 2006; Stevens & Verhe, 2004).

Betalains

These are obtained from the roots of *Beta vulgaris*. Family Chenopodiaceae. The purple root owes its colour to the presence of betalains. Beetroot is the only allowed source of betalain colourant in the EU and the USA. Betalains are actually comprised of two groups of pigments: the red–purple betacyanidins and the yellow betaxanthins, both of which are water-soluble.

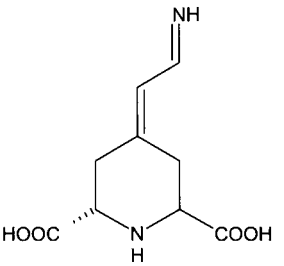
It is also obtained from the hairy root cultures of the plants, belonging to *Agrobacterium* or *Rhizobium* species, which release a red pigment to the culture medium. Betanin is a nitrogen-containing glycoside that on hydrolysis gives the aglycone betanidin and glucose. Betanin is the predominant colouring compound and represents 75-90% of total pigment.



Betacyanin

R=R_1=H- Betanidin

R= glucose, R_1=H- Betanin



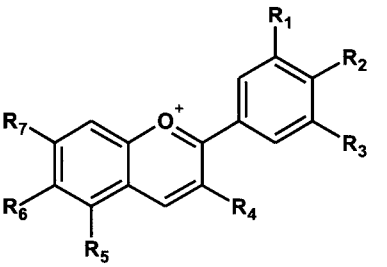
Betaxanthine

Betalains have potential biotechnological application in food industries because of its high water solubility. Betanin from red beet root has been used for a long time to colour yoghurt, ice-cream, sugar confectioneries, dry mixes and other products (*Off. J. Eur. Commun.*, 1995, 1999, 2001; Code of Federal Regulations; Stevens & Verhe, 2004; Mortensen, 2006).

Anthocyanins

Anthocyanins are red, blue or violet plant pigments present in the cell sap of many flowers, fruits and vegetables. Anthocyanins give rise to the blue–purple–red–orange colour of flowers and fruits, in particular, of many plants. The name comes from two Greek words meaning flower and dark blue. Anthocyanins are glycosides of anthocyanidins (also called aglycones). Anthocyanins are highly water soluble. Anthocyanin has a great impact on the colour. An increase in the number of hydroxyl groups yields a more

bluish colour, whereas methoxy groups give a more red colour. Thus, pelargonidin is orange, whereas delphinidin and malvidin are purple. At low pH (around 3), the anthocyanins are most strongly coloured, exhibiting their well-known purple–red colour. Around pH 5, anthocyanins turn almost colourless, and at neutral and alkaline pH the colour goes from blue to green. Acidity also affects the stability of anthocyanins, which are rather unstable at weakly acidic to alkaline pH.



Anthocyanidin

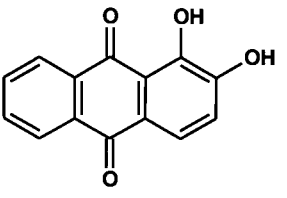
Anthocyanidins	R	R'	Occurrence of glycosides
Pelargonidin	H	H	Flowers of *Pelargonium* (Geraniaceae) and pomegranate
Cyanidin	OH	H	*Centurea anus* (Asteraceae), *Rosa* species and *Vitis vinifera* (Vitaceae)
Peonidin	OCH_3	H	*Vaccinium macrocarpon* (Ericaceae)
Delphinidin	OH	OH	*Ribes nigrum* (Grossulariaceae) and *Viola* species.
Petunidin	OH	OCH_3	*Petunia* species (Solanaceae)
Malvidin	OCH_3	OCH_3	*Malva* species, purple grades.

They are used to colour soft drinks, pickles, soup, dairy products, jelly and sweets (Mortensen, 2006).

Quinones: Anthraquinones

Alizarin

It is obtained from roots of *Rubia tinctorum* (Rubiaceae). Rubia means red and the plant has been used as the source of a permanent red dye. It contains several anthraquinone glycosides, the chief of which is ruberythric acid, which on hydrolysis yields alizarin and primeverose.

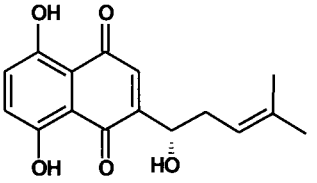


Alizarin

Napthaquinones

Alkannin

It is obtained from the dried root of *Alkanna tinctoria* (Boraginaceae). The chief colouring constituent is alkannin. It imparts a red shade.

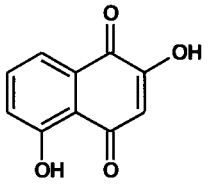


Alkannin

It is used for colouring oils and tars. In the form of tinctures, it is used for the microscopical detection of oils and fats. They are used in pharmaceutical preparations, as ingredients of cosmetic formulations or as food colourants and additives (Spyros *et al.*, 2005).

Lawsone

It is obtained from the fresh or dried leaves of the plant *Lawsonia inermis* (Lythraceae). Lawsone is the main colouring constituent, which is the degradation product of the primary glycosides hennosides A, B and C. Lawsone is responsible for imparting a red colour. Used as a hair dye either alone or in combination (Dweck, 2002).



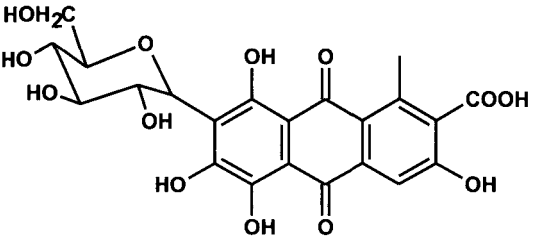
Lawsone

Colourants from Animal Source

Carminic acid

It is obtained from the dried female insect *Dactylopus coccus*, Order-Hemiptera, containing eggs and larvae. Cochineal contains about 10% of carminic acid, a brilliant purple, water-soluble colouring matter. It is a C-glycoside, an anthraquinone derivative. The dried pulverized bodies of these insects yield the red dyestuff cochineal. Carmine and Aluminium Lake is prepared by precipitation by adding aluminium and calcium ions to an extract of cochineal. Under acidic conditions, below pH 3, carmine becomes insoluble. This colour is banned in USA. It is soluble in water and precipitates below pH 3. The colour of carminic acid in solution changes

with pH, thus at low pH carminic acid is orange, changes to red at slightly acidic and neutral pH, and finally turns violet in alkaline solution.

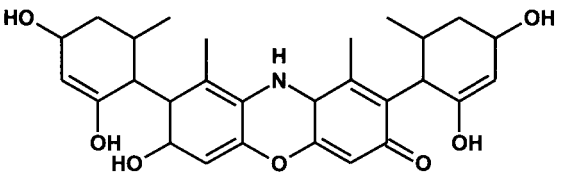


Carminic acid

Cochineal and carmine are used as colouring agents for both liquids and solids. They are also used as indicators. It is used to colour products like alcoholic beverages, dyed cheeses, icings, sweets etc. It has been used as a pigment and as a colouring agent in cosmetic, paints and in beverages. The homoeopathic tincture is prepared from the dried bodies of the female insect and is an important remedy for whooping cough (Dweck, 2002; Evans, 1996; Mortensen, 2006).

Orcein, Orchil

These colours are derived from archil, the lichen *Rocella tinctoria*, Family Rocellaceae. Orcein is a mixture of compounds with a phenoxazone structure, composed of hydroxyorceins, amino-orceins and amino-orcinimines. Orcein (a reddish-brown dye is obtained from orcinol by the action of aqueous ammonia and air. Orcein and Orchil impart a reddish – brown and purple-blue colour respectively (Food Additives Guide, 2008).



Orcein

Colourants from mineral sources

Minerals are frequently termed as pigments and are used to colour lotions and cosmetics.

Ferric oxide red

It contains not less than 90% of Fe_2O_3. It is prepared by heating native ferric oxide or hydroxide at a temperature, which yields a product of desired colour. The temperature, time of heating, and presence of other metals and particle size of the oxide govern the colour. It is used to impart colour to neocalamine and other cosmetics (Food Additives Guide, 2008).

Ferric oxide yellow

It contains not less than 97.5% of Fe_2O_3. It is prepared by heating ferrous hydroxide or ferrous carbonate in air at low temperatures. It is used to impart colour to neocalamine and other cosmetics (Food Additives Guide, 2008).

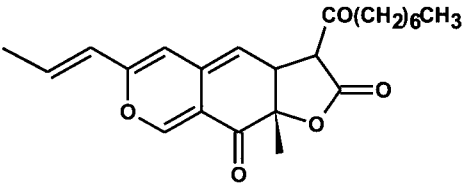
Titanium dioxide

It is a white, amorphous, odorless and tasteless pigment. It is prepared by adding ammonia or alkali carbonate to a solution of titanil sulfate; titanic acid is precipitated out and after filtration, washed, dried and ignited. It absorbs U.V rays and thus prevents sunburns, so it is used in cosmetics and face powders. It also improve the pearlness and elegance of product. It is used to impart white colour to film-coated and sugar-coated tablets and gelatin capsules. It is used in lakes as an opacifier,to extend the colour (Food Additives Guide, 2008).

Colourants from other sources

Monascorubrin

It is obtained from the mould, *Monascus purpureus*. *Monascus purpureus* is a red mould species which may be cultivated on a starch containing substrate. It is also known as Red yeast rice and Red mould in USA. Monascus is not used as a colourant in EU and USA. It gives a dark red shade.

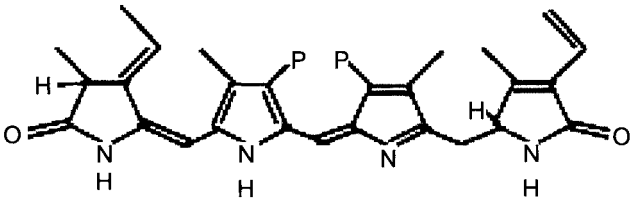


Monascorubin

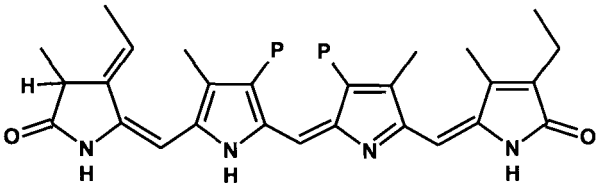
Monascorubin is a natural colour used for colouring and flavouring food stuff. It is used as a preservative for meat (Erdogrul, 2004; Mortensen, 2006).

Phycocyanobilin and Phycoerythrobilin:

These are characteristic of three types of algae; the Rhodophyta, the Cryptophyta and the Cyanophyta and are collectively known as phycobiliproteins. They are built up of bilins,which are open-chain tetrapyrroles, covalently linked via one or two thioether links to the cysteine residues in the apoproteins. These colours are stable at pH values in the range 5-9, but get precipitated at lower pH values. They are water soluble and impart a red or blue shade. Phycocyanins are photosynthetic pigment absorbing the red light, they are water soluble protein.



Phycoerythrobilin



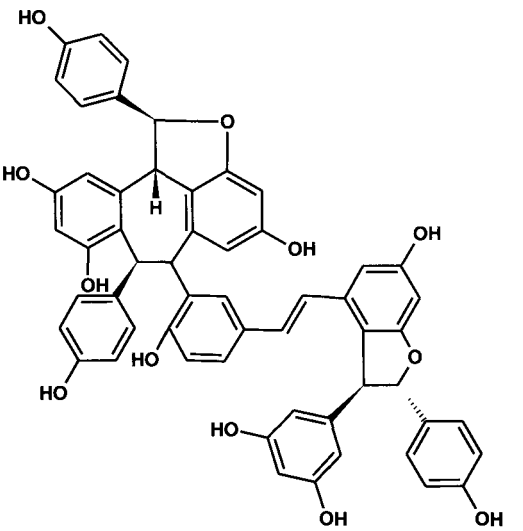
P = -CH_2CH_2COOH

Phycocyanobilin

They are used as colourants in the food and cosmetic industries with a concentration of the dye at 0.1% (Mortensen, 2006).

Vitisins

Vitisin A, Vitisin B, Vitisidin A and Vitisidin B are four new anthocyanins found in both red table wines and in fortified red wines in trace amounts. They are generated in wines during maturation. They are used as added colours in foods and drinks, but their use has been limited due to their sensitivity to bleaching by SO_2 and their limited capability at pH values above 3.5 (Stevens & Verhe, 2004).



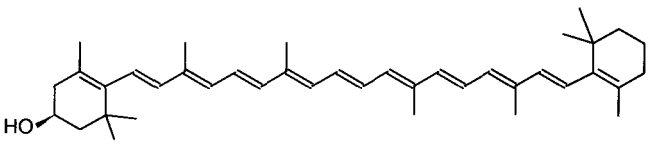
Vitisin A

Xanthophylls (E161)

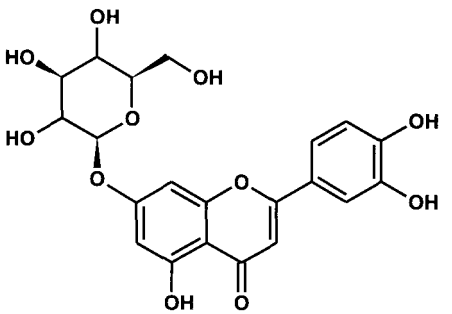
These are the hydroxylated derivatives of carotenoids

Colourant	Shade
Flavoxanthin	Yellow
Cryptoxanthin	Yellow
Rubiaxanthin	Yellow
Violaxanthin	Yellow
Rhodohanthin	Yellow
Canthaxanthin	Orange

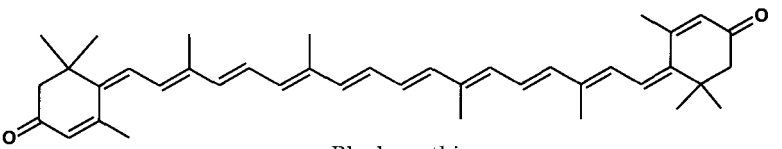
Being xanthophyll derivatives most of them are consumed as a part of normal diet, in confectionery, sugar syrup, wines, beer and malt beverages and in sauce.



Cryptoxanthine



Flavoxanthine



Rhodoxanthin

Calcium Carbonate ($CaCO_3$)

It is used as fairming agent for preserved fruit and vegetables.

Gold (E175)

It is used in food to give a metallic surface colour. Typical products include sugar coated flour confectioneries (Food Additives Guide, 2008).

Silver (E174)

It is used in food to give a metallic surface colour. However, prolonged consumption may lead to argyria, a blue-grey skin. Typical products include sugar coated flour confectioneries (Food Additives Guide, 2008).

Aluminium (E173)

It is used in food as a metallic surface coating. Typical products include silver coated tablets (Food Additives Guide, 2008).

Tables below illustrate the colours permitted for use in food and drinks in different countries.

Table 2. Natural colours permitted in food and drinks in the USA by the Food and Drug Administration (FDA) and exempt from certification (Mortensen, 2006)

Annatto extract
Beet powder
Canthaxanthin
Caramel
Carrot oil
Grape colour extract
Paprika and paprika oleoresin
Saffron
Turmeric and turmeric oleoresin

Table 3. Colours of natural origin listed as permitted for foods by the European Community (Mortensen, 2006)

E100 Curcumin
E101 Riboflavin
E120 Cochineal/carminic acid/carmines
E140 Chlorophyll
E141 Copper complexes of chlorophyll and chlorophyllins
E150 Caramel
E160
a. Annatto extracts, Bixins, Norbixin
b. Paprika extracts, Capsantin, Capsorubin
c. Lycopene
E161
a. Flavoxanthin
b. Lutein
c. Cryptoxanthin
d. Rubixanthin
e. Violaxanthin
f. Rhodoxanthin
g. Canthaxanthin
E162 Beetroot red, betanin
E163 Anthocyanins

Table 4. Recognized ingredient classification in the European Community for natural materials and extracts with colouring power but not presently approved for the "E" list of natural colours

Product	Category
Santalin (red sandal)	
Spice extract blends	**Spice extracts**
Alfalfa	Natural (Vegetable extracts)
Marigold	
Crocin	
Saffron	
Safflower	
Hibiscus	

CONCLUSIONS

As it has been proved that synthetic colourants can cause cancer and various other disorders, the food and pharma sector is experiencing a backtrend towards natural colourants. This change has not been driven by the industry but by the consumers who are concerned over possible health risks associated with synthetic colourants. As a result, the search is in progress for newer natural colourants.

REFERENCES

Commission Directive 95/45/EC of 26th July 1995 laying down specific purity criteria concerning colours for use in food stuff. *Off. J. Eur. Commun.* (1995) **L 226**: 1.

Commission Directive 1999/75/EC of 22nd July 1999 ammending commission directive 95/45/EC laying down specific purity criteria concerning colours for use in food stuff. *Off. J. Eur. Commun.*, (1999) **L 206**: 19-21.

Commission Directive 2001/50/EC of 3rd July 2001 ammending directive 95/45/EC laying down specific purity criteria concerning colours for use in food stuff. *Off. J. Eur. Commun.*, (2001) **L 190**: 14-17.

Bosland, P.W. (1996). Capsicums: Innovative Uses of an Ancient Crop. *In*: Janick, J. (*ed*), Progress in new crops. ASHS Press, Arlinngton, VA, pp. 479-487.

Code of Federal Regulations, (2002)-Title 21. Part 73-Listing of color additives exempt from certification.

Dweck, A.C. (2002). Natural ingredients for colouring hair. *Cosmetic Science*, **24**: 287-302.

Erdogrul, O. and Sebile, Azirak (2004). Review of the studies on the red yeast rice (*Monascus purpureus*). *Turkish Electronic Journal of Biotechnology*, **2**: 37-49.

Evans, W.C. (1996). Trease and Evans Pharmacognosy. WB saunders Company Ltd, London.

Farrell, K.T. (1985). Spices Condiments and Seasoning. Van Nostrand Reinhold Company, New York.

Food Additives Guide, (2008). Colours. South Australia. 100-181. Available from http://mbm.net.au/health/100-181.htm.

GNT-Healthy colours for healthy life. Available from *www.gnt-group.com/uploads/media/Press_Exb_CP_english.pdf.*

Mortensen, A. (2006). Carotenoids and other pigments as natural colourants. *Pure Appl. Chem,* **78**: 1477-1498.

Saraswathy, S., Azhakia Manavalan, R.S. and Subramaniam, S. (2004). Studies on various extraction methods on dye yield in annatto (*Bixa orellana* L). *Madras Agric. J.*, **91**: 524-526.

Spyros, A., Assimopoulou, A.N. and Papageorgiou, V.P. (2005). Structure determination of oligomeric alkannin and shikonin derivatives. *Biomedical Chromatography*. **19**: 498-505.
Stevens, C.V. and Verhe, R. (2004). Renewable Bioresouce: Scope and modification for non food applications. John Willey & Sons Ltd.
www.agsci.ubc.ca.
www.chm.bris.ac.uk.
www.molinterv.aspetjournals.com.

Appendix

Table of Contents of Other Volumes (2 to 8)

ISBN : 1-933699-52-3

VOLUME 2: EFFICACY, SAFETY & CLINICAL EVALUATION I

ISBN : 1-933699-53-1

VOLUME 3: EFFICACY, SAFETY & CLINICAL EVALUATION II

ISBN : 1-933699-54-X

VOLUME 4: ANTIOXIDANTS & NUTRACEUTICALS

ISBN: 1-933699-55-8

VOLUME 5: IMMUNE-MODULATION & VACCINE ADJUVANTS

ISBN : 1-933699-56-6

VOLUME 6: EXTRACTION, ISOLATION & CHARACTERIZATION

ISBN : 1-933699-57-4

VOLUME 7: STRUCTURAL MODIFICATIONS & DRUG DEVELOPMENT

ISBN: 1-933699-58-2

VOLUME 8: QUALITY CONTROL & STANDARDIZATION

Index

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z