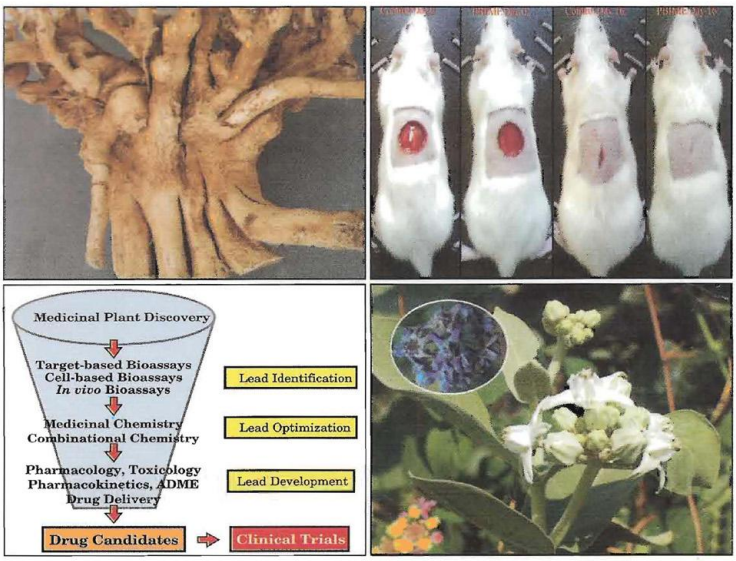
Comprehensive

Bioactive Natural Products

Vol 3

Efficacy, Safety & Clinical Evaluation II



V K Gupta

Studium Press

Comprehensive
Bioactive Natural Products

Volume 3
Efficacy, Safety & Clinical Evaluation II

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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.

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Comprehensive
Bioactive Natural Products

Vol. 3: Efficacy, Safety & Clinical Evaluation II

ISBN : 1-933699-53-1
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.

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(*Multi-Volume Set*)

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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

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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

The natural products have been of great help to mankind in resolving many diseases from earliest time. Primitive people in all ages have had some knowledge of natural medicines. The knowledge of the primitive man has been so modified with the advancement of civilization that know-a-days almost every type of medicine be that of Ayurvedic, Unani or even a number of allopathic medicines are obtained from natural sources. The medicinal value of drug plants is due to the presence of some chemical compounds in the plant tissue that produce a definite physiological action on human body. Early societies learned through trial and error that many plants contained substances with significant curative properties and over the years the natural products became used in more refined ways leading ultimately to the use of pure single component "active ingredient" as drugs.

Nature has continuously provided mankind with a broad and structurally diverse array of pharmacologically active compounds that have proved to be indispensable for the cure of deadly diseases or as lead structures for novel pharmaceuticals. Their dominant role is evident in the approximately 60% of anticancer compounds and 75% of drugs for infectious diseases that are either natural products or natural product derivatives. At present 25% of the modern medicines are developed from plants that were first used traditionally, and many synthetic drugs have also been obtained from natural precursors. Almost, 70% modern medicines in India are derived from natural products. In the USA, the process of synthetic drug discovery and development takes an average of 12 years, and any new drug requires the investment of an average of US$ 230 million. It is seen that natural drugs take a comparatively much less time and expenses than synthetic drugs. Hence natural medicines would be cheaper, unless the market price are inflated by other considerations. Despite this success, during the past couple of decades, research into natural products has experienced a steady global decline. The introduction of high-throughput synthesis and combinatorial chemistry with their promise of a seemingly inexhaustible supply of compound libraries has greatly contributed to this declining interest in the screening of natural products by the pharmaceutical industry.

Resistance of the parasites to existing drugs and their higher cost warrants the search of newer drug molecules. The origin of many effective drugs is found in the traditional medicine practices and in view of this several researchers have undertaken studies to clinically evaluate the medicinal plants for their preclinical safety and efficacy.

The natural product market has been continually growing at an increased rate mainly as a nutraceuticals, dietary supplements, cosmoceuticals, chemopreventives and as herbal remedies. These therapies should emphasis on well-controlled and randomized clinical trials to prove

safety and efficacy and necessary guidelines should be followed in order to harmonies the use of bioactive natural products.

At present, there is limited data on safety, efficacy and clinical trials to prescribe natural products in therapy. Realizing the need to document this knowledge gained through recent researchers, the present volume ***"Efficacy, Safety & Clinical Evaluation-II"*** of the book series **"Comprehensive Bioactive Natural Products"** presents 23 chapters discussing therapeutic potential of a large number of bioactive natural products of plant/animal origin investigated worldwide. Some interesting studies included are: Allylsulfides as bioactive compounds with chemotherapeutics and/or chemopreventive effects; Targeting tumor angiogenesis for preclinical validation of antiangiogenic compounds from medicinal plants; Chemistry and pharmacology of *Shorea robusta*; Safety, efficacy and preclinical evaluation of plant products; *In vitro* and *in vivo* combined anti-influenza virus effects of a plant polyphenol-rich extract and synthetic antiviral drugs; Antihypertensive and hypolipidemic effects of tuber of *Apios americana* Medikus in SHR; Anti-ulcer effects of aqueous extract of *Persea americana* Mill (Avocado) leaves in rats; Anti-asthmatic medicinal plants; Hepatoprotective effects of *Pimpinella anisum* seed extract in rats; Antisickling activity and thermodegradation of an anthocyanin fraction from *Ocimum basilicum* L.; Pilot clinical trial of KJJ® in children with uncomplicated respiratory tract infections; Chemical constituents from anti-inflammatory fraction of *Taraxacum mongolicum* and their inhibition of AChE; Inhibition of hyaluronidase by essential oils and other natural fragrant extracts; Pharmacokinetic studies on hepatic uptake mechanism of tetrodotoxin in the puffer fish *Takifugu rubripes*; Herbal drugs - applications in the post genomic era; Antifungal activity of marine bacterial associates of deep-sea origin against plant pathogens.

The studies included are likely to lead further researches in this direction and it is hoped that this publication would attract world wide audience of researchers and the academicians of allied disciplines engaged in the search of new drug from natural resources.

Jammu, India

V.K. Gupta

ISBN : 1-933699-53-1

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Allylsulfides as Bioactive Compounds with Chemotherapeutic and/or Chemopreventive Effects

C. SCHERER[1,2], C. JACOB[2], M. DICATO[3] AND M. DIEDERICH[1,*]

ABSTRACT

Chemoprevention and chemotherapy are up to date two of the most promising approaches in the prevention and therapy of different human cancers. However there are no drugs on the market combining both, therapeutic effects in patients and preventive effects to avoid either relapses of former patients or indisposition of endangered people. Hence naturally occurring compounds that could be applied as functional food or as drug formulations are becoming more and more interesting for research, since they might combine both of the anticancer properties, and additionally hardly exert any harmful side effects. Potential candidates for this application field could be allylsulfides, e.g. DAS (diallylsulfide), DADS (diallyldisulfide) or DATS (diallyltrisulfide) that are mainly derived from garlic. These compounds were extensively studied in the field of cancer research and were shown to possess both chemopreventive and chemotherapeutic activity-even-in the same molecules. They inhibit growth of cancer cells quite selectively against normal cells as well by block of proliferation as by induction of apoptosis. Furthermore they stimulate immune function, modulate metabolism of carcinogens and reduce formation of metastases. The underlying molecular mechanisms of all these activities contributing to the anticancer effects are multifaceted and not yet fully understood or investigated. However this review focuses on anticancer activities and strongly linked biological effects and the assumed underlying molecular mechanisms of the most common

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allylsulfides. Besides it will provide information about strongly structurally related organic sulfur compounds (OSCs) from garlic, since many of these OSCs are converted into each other by chemical instabilities or by metabolic processes (e.g. allicin decomposes rapidly to allylsulfides). Moreover effects of garlic oil, garlic powder and garlic extracts are discussed, since their main biologically active contents are represented by OSCs.

Key words : Allylsulfides, other OSCs, *Allium* species, chemoprevention, chemotherapy

INTRODUCTION

Diverse human cancers are always very difficult to treat or even incurable. Hence scientific research makes huge efforts in order to find novel or to improve established anticancer approaches to fight the second most common cause of death (after circulatory diseases) among both sexes in industrialized countries. In this context both chemoprevention and chemotherapy are always preferred in most cases to radiation therapy and surgery, since side effects in patients are generally lower and compliance is higher. Nevertheless application of chemical substances to men is always related with adverse effects occurring simultaneously with the main effect, that a medical scientist or doctor wants to cause by such treatments. Hence side effects have to be strongly considered during drug development studies and different approaches are used in order to minimize or to avoid them as far as possible. One of these approaches is to start preclinical research on compounds from natural sources known from epidemiological data for their solely beneficial effects on human health. In this regard manifold laboratory studies were performed on *Allium* species- mainly *Allium sativum* (garlic)- that are not only widely reported for their protective effects on the cardiovascular system, but also for their high efficiency in chemoprevention and therapy of diverse malign and benign tumors (Ariga & Seki, 2006; Herman-Antosiewicz *et al.,* 2007; Shukla & Kalra, 2007). These records could be confirmed by experimental studies both *in vitro* and *in vivo*. Besides modes and mechanisms of action of the plants and/or their constituents could be elucidated to a huge extent, and organic sulfur compounds (OSCs), such as allylsulfides, turned out to be the compound class responsible for most of the biological activities (Ariga & Seki, 2006). In particular, regarding potential of compounds as agents in cancer prevention and therapy, they exert effects on enzymes of the phase I (CYP450)- or phase II- metabolism of (Pinto *et al.,* 1997) carcinogens, they modulate multidrug resistance enzymes (MDR), they change cellular redox status, they inhibit proliferation and induce apoptosis in diverse human cancer cells targeting cellular DNA and protein- level, they stimulate the immune function, and they prevent formation of metastases. In some cases, crosstalk between the different

effects is very strong, in that they are depending on each other (Herman-Antosiewicz *et al.,* 2007; Shukla & Kalra, 2007; Stan *et al.,* 2008; Wu *et al.,* 2005).

Hence, overall information about modes and mechanisms of action of *Allium* species and their constituents provided so far are very favorable, nevertheless ongoing studies are required to fully elucidate modes and mechanisms of action in different models and to investigate ADME-tox-properties of the natural compounds in order to develop functional food or nature-derived drugs in the not too distant future.

POTENTIAL MEDICINAL APPLICATIONS OF *ALLIUM* SPECIES

Plants of *Allium* species, especially garlic, are commonly known for their manifold beneficial effects on human health, they are even reported that Egyptians used them for medicinal applications about 3500 years ago. Besides based on extensive data derived form epidemiological and laboratory studies, the National Cancer Institute (Jastrzebski *et al.,* 2007) of the UK initiated the "Designer Food Program" about one decade ago, setting garlic on top of different vegetables known for their potency in cancer prevention. Moreover *Allium* species and their contents are widely described for a huge amount of other disease-preventive or even therapeutic effects, such as in the therapy of cancers (leukemic, non-leukemic) or benign tumors (*e.g.* BPH), in the prevention of cardiovascular diseases (*e.g.* atherosclerosis, hypertension), in the stimulation of the immune function (*e.g.* macrophages, T-cells), in the attenuation of inflammatory diseases (*e.g.* cancer, atherosclerosis, myocardial infarction, allergy, asthma, Crohn`s disease), or in the protection against diverse infections (*e.g.* bacteria, fungi, viruses, parasites), toxic agents, oxidative stress or γ-irradiation by variable mechanisms (Agarwal *et al.,* 2007; Aggarwal & Shishodia, 2004; Chen *et al.,* 2004; Devrim & Durak, 2007; Galeone *et al.,* 2007; Herman-Antosiewicz & Singh, 2004; Ngo *et al.,* 2007; Nishimura *et al.,* 2006; Oommen *et al.,* 2004; Rahman, 2007; Shukla & Kalra, 2007; Tsao *et al.,* 2007).

Hence biological activity of garlic is multitargeted, what might be an advantage for its potential application as functional food or as green drug, since many patients suffer from multiple diseases (multimorbidity) and some diseases are strongly linked with each other, *e.g.* inflammation with the immune function, with cardiovascular or cancer diseases.

FACTORS INFLUENCING BIOLOGICAL ACTIVITIES OF GARLIC PREPARATIONS

In this context consumers should take care not to destroy active contents of the vegetables by inappropriate cooking or processing (Ariga & Seki,

2006), since bioactivities of garlic preparations differ strongly (Jastrzebski *et al.,* 2007). Especially organic sulfur compounds such as allylsulfides-being one of the most important compound classes of the plant in cancer prevention and therapy (Ariga & Seki, 2006; Jacob, 2006) can be degraded by cooking (Stan *et al.,* 2008). Additionally the age of a garlic preparation strongly affects the quantity of its different constituents, in particular some OSCs are quite instable and are chemically degraded by time. For example allicin is released only after plant tissue disruption (chopping, crushing) by the binary alliin-alliinase system (Ariga & Seki, 2006), hence being the most prevalent OSCs in fresh garlic preparations. This compound is then enzymatically and chemically (*e.g.* redox-reactions) converted to ajoene and allylsufides that are besides SAC more prevalent in aged garlic formulations (Lau *et al.,* 1991). Needless to say that the amount of the contents in the different garlic formulations is also depending on their lipophilicity, in that amino acids are more soluble in water whereas allylsulfides, alkylsulfides or ajoene are more lipophilic being in consequence the main contents of garlic oil and organic garlic extracts.

ANTICANCER ACTIVITIES AND SUPPOSED MECHANISMS OF ACTION OF GARLIC PREPARATIONS: GARLIC OIL, GARLIC EXTRACT, GARLIC POWDER

Though the composition of the three garlic formulations is not well defined and certainly varying, it is assumed that OSCs mainly contribute to their bioactivities. Other contents that can also play a role in this context are phenolic compounds (flavonoids) or glycoproteins present in garlic besides some salts and vitamins (Hassan *et al.,* 2003; Shukla & Kalra, 2007).

Relations to Modulation of Immune Function, Chronic Inflammation, and NFκB

Though not all crosstalks between cancers (including proliferation and formation of metastases), immune function, inflammation and nuclear factor kappa B (NFκB) are understood so far, very tight interactions and overlaps were already elucidated. In this context it is known, that cancers and other diseases, such as allergies that are mainly based on dysfunction of the immune system, are often linked with a chronic inflammation (Aggarwal & Shishodia, 2004; Delhalle *et al.,* 2004). Besides it was observed that the transcription factor NFκB is upregulated in many inflammatory diseases and in many cancers, what makes the factor a suitable target for drug development in the field of apoptosis inducers and/or anti-inflammatory agents (Delhalle *et al.,* 2004; Delhalle *et al.,* 2003; Garg & Aggarwal, 2002). Moreover a strong link to the cellular redox level exists, *e.g.* NFκB can be

activated by an increase of reactive oxygen species (ROS), but this does not force an antiapoptotic effect in every biological system (Delhalle *et al.,* 2004). Overall it might be favorable for cancer therapeutics to stimulate the immune function, simultaneously decreasing inflammation and reducing activity of NFκB. Hence some *Allium*-preparations and constituents might be suitable candidates in this regard.

Garlic oil (GO) showed different activities *in vitro* and *in vivo*: It was able to inhibit proliferation of leukemic HL60 cells by an induction of their differentiation via integrin modulation (Cd11b up regulation) (Ariga & Seki, 2006; Seki *et al.,* 2000). Besides, GO exerted a protective effect against Fe-NTA-induced tissue injury and carcinogenesis in rodents, what was linked with radical scavenging, attenuation of depletion of intracellular GSH-levels and preservation of antioxidant enzymes (Agarwal *et al.,* 2007). This antioxidant effect was also confirmed in diverse cell lines and in further rodent models for GO and garlic extract (GE) by a decrease of oxidized lipodensity proteins (ox-LDL), scavenging of H_2O_2, reduced peroxide or radical release from endothelial cells (EC) or from neutrophils respectively, or by the block of H_2O_2- induced activation of NFκB. These effects were strongly linked with a modulation of phase I and phase II metabolism and with an anti-inflammatory potency (Aggarwal & Shishodia, 2004; Geng *et al.,* 1997; Ho *et al.,* 2001; Ide & Lau, 2001; Keiss *et al.,* 2003; Liu & Xu, 2007; Sankaranarayanan *et al.,* 2007; Tapiero *et al.,* 2004; Wu *et al.,* 2002). In particular aged garlic extract (AGE) was able to stimulate the immune system (increased natural killer cell (NK)-activity, modulated cytokines) what resulted in antiproliferative effects in a mammary carcinoma-mouse model and in leukemic cells (Hassan *et al.,* 2003). Furthermore it acted antimetatstatic and antiangiogenic *in vitro* (Herman-Antosiewicz *et al.,* 2007). A similar activity pattern was described for garlic powder (GP) that reduced diethylnitrosamine (DEN)-induced hepatocarcinogenesis in rats probably by downregulation of phase I metabolism (CYP2E1) thus preventing toxification of the chemical agent (Ariga & Seki, 2006; Park *et al.,* 2002). Moreover it modulated cytokines in human blood, what led to an anti-inflammatory effect, in that pro-inflammatory cytokines (IL-1β, TNFα) and NFκB were downregulated, whereas however anti-inflammatory cytokines (IL-10) remained unaffected (Keiss *et al.,* 2003). This might prevent the inflammation-triggered initiation of cancers, since NFκB is reported to promote the expression of genes involved in inflammatory response (strongest effect in chronic inflammatory diseases, *e.g.* increased expression of Cox-2), angiogenesis and cell adhesion (Aggarwal & Shishodia, 2004; Delhalle *et al.,* 2004). Besides tumor progression might be reduced by enabling cancer cells to undergo apoptosis.

ANTICANCER AND RELATED ACTIVITIES AND SUPPOSED MECHANISMS OF ACTION OF ORGANIC SULFUR COMPOUNDS (OSCs): ALLYLSULFIDES, ALLICIN, AJOENE, *S*-ALLYLCYSTEINE (SAC), *S*-ALLYLMERCAPTOCYSTEINE (SAMC)

As mentioned above organic sulfur compounds (OSCs) were elucidated to be the compound class of *Allium* species responsible for most of the biological activities of the medical plants of the family (Ariga & Seki, 2006). Consequently research made efforts to clarify the biological effects of pure OSCs. Thus it became obvious, that activity of OSCs is not selective for one specific target, but that compounds can undergo a broad range of chemical reactions and interactions, and the variety of possible reactions is generally increasing with the number of sulfur atoms. For example mono- and polysulfides can act via sulfur chain-shortening or elongation, hydrophobic binding or other interactions with cellular constituents (*e.g.* DNA, proteins, lipids), interactions with metal ions (free or enzyme-linked), or sulfur radical chemistry including release of reactive sulfur species increasing or decreasing ROS of cells (Munchberg *et al.,* 2007). Nevertheless reports about physiologically harmful effects of the compounds are quite rare, what is a strong hint that compounds possess certain selectivity for targets (*e.g.* against cancer cells), but the underlying mechanisms are not yet fully understood and remain to be elucidated. Therefore widespread studies are being performed and very promising pharmacological properties of OSCs were investigated in detail. The most important results (chemical structures of OSCs see Figs 1A, 1B, "overview about major targets/modes of activity of OSCs" see Fig 2) will be summarized in the following sections.

Linear allylsufides such as diallylsulfide (DAS), diallyldisulfide (DADS), diallyltrisulfide (DATS) or diallyltetrasulfide (DATTS), alkylsulfides, "mixed" sulfides, allicin, ajoene, SAC or SAMC are generally isolated from *Allium* species, especially garlic and onion. Nevertheless they are also found in other plant families and they can be produced by chemical synthesis (Jacob, 2006). The mentioned allylsulfides represent more than 54% of total sulfides of garlic oil (Tsao & Yin, 2001), and became -due to a very favorable biochemical profile- the most extensively studied compound class of the plant family so far. As well monosulfides as polysulfides exert a variety of anticancer or strongly related effects in diverse *in vitro* and *in vivo* models (overview see Table 1A) without showing harmful effects if applied at adequate doses (Ariga & Seki, 2006; Hassan, 2004; Hassan *et al.,* 2003). The same was found for other important OSCs from garlic (Table 1B).

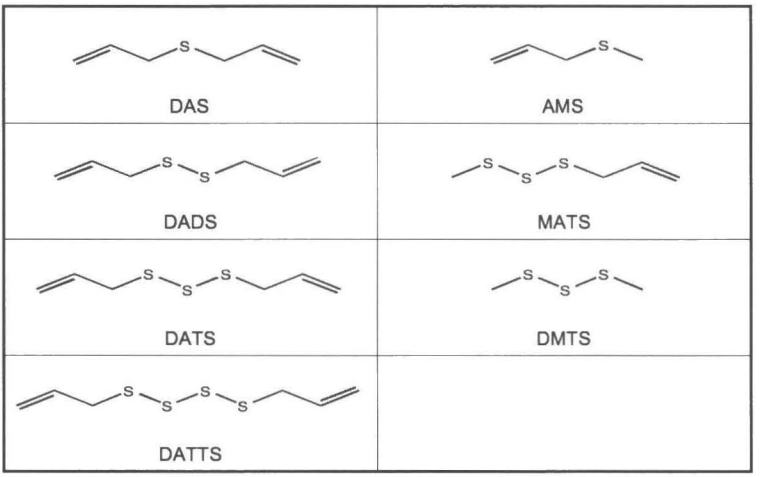

Fig 1A. Chemical structures of selected biologically active OSCs from *Allium*-species: 1A : "Sulfides"

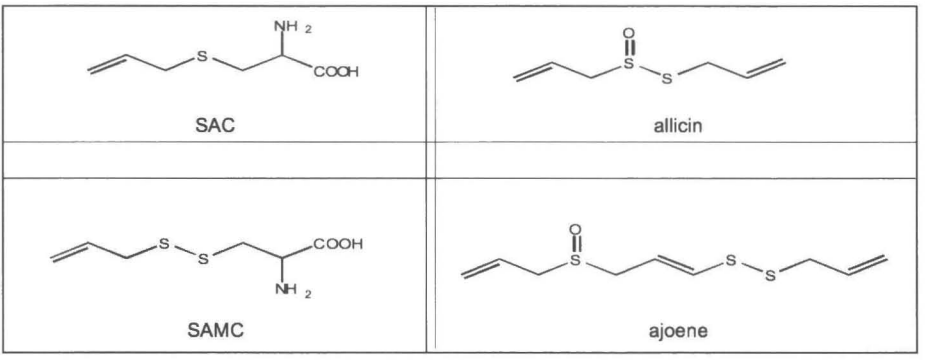

Fig 1B. Amino acids, allicin and ajoene

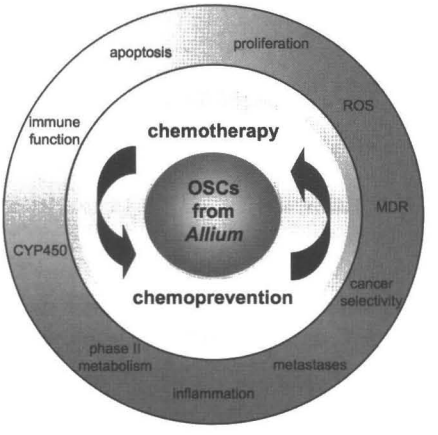

Fig 2. Modes of activity of OSCs

Table 1. Chemopreventive and chemotherapeutic effects and strongly associated biological effects of allylsulfides and other important OSCs from *Allium* species

1A : *Allylsulfides*[*1]

OSC	Modes of action	Supposed mechanisms	Test systems and biological effects	Reference(s)
DAS	Chemoprevention, chemotherapy	Inhibition of carcinogen activation by modulation of phase I-enzymes (CYP450): CYP2E⇓, CYP2B1⇑ Activation of carcinogen detoxification and elimination by phase II-enzymes: GST⇑, QR⇑, CAR⇑, Nrf⇑, hepatic catalase⇑ Modulation of cell growth and induction of apoptosis: cell cycle arrest, activation of mitochondrial pathway of apoptosis	Carcinogenesis in rats/mice⇓, growth/viability of human colon carcinoma cells or human neuroblastoma⇓	(Aggarwal & Shishodia, 2004; Ariga & Seki, 2006; Das *et al.*, 2007; Davenport & Wargovich, 2005; Fisher *et al.*, 2007; Karmakar *et al.*, 2007; Prasad *et al.*, 2008; Sriram *et al.*, 2008; Wu *et al.*, 2002; Yang *et al.*, 2001)
	Anticancer-related effects	Mediation of MDR : P-gp⇓	Growth/viability of human VBL-resistant leukemic cells and hepatocarcinoma in mice⇓	(Arora *et al.*, 2004)
		Modulation of immune function: IL-6⇓, TNFα⇓	MRSA-infection in mice⇓ cells⇑	(TSAO *et al.*, 2004)
		Prooxidative effect: ARE⇑	Cytoprotection in Hep G2 cells⇑	(Chen *et al.*, 2004)

Table 1. *Contd.*

OSC	Modes of action	Supposed mechanisms	Test systems and biological effects	Reference(s)
		Antioxidative effect: ROS⇓	DMBA induced carcinogenesis in mice⇓	(Prasad *et al.*, 2008)
		Antimetastatic effect, antiangiogenic effect: cell motility and invasion⇓, MMP⇓, antiangiogenic factors⇓, IL-2⇑	Mice bearing tumors	(Herman-Antosiewicz *et al.*, 2007a)
DADS	Chemoprevention, chemotherapy	Inhibition of carcinogen activation by modulation of phase I-enzymes (CYP450): CYP2E1⇓ Activation of carcinogen detoxification and elimination by phase II-enzymes: GST⇑, QR⇑, CAR⇑, Nrf2⇑ Modulation of cell growth and induction of apoptosis: cell cycle arrest, activation of mitochondrial pathway of apoptosis; (p53-dependent or independent), HDAC-inhibition	Carcinogenesis in rats/mice⇓, growth/viability of human leukemic cells, neuroblastoma Cells and of human carcinoma cells of colon, lung, breast, prostate⇓, H-ras oncogene transformed xenografts in mice⇓	(Antosiewicz *et al.*, 2006; Ariga & Seki, 2006; Arunkumar *et al.*, 2007; Dashwood & Ho, 2007; Dashwood *et al.*, 2006; Davenport & Wargovich, 2005; Druesne *et al.*, 2004; Fisher *et al.*, 2007; Herman-Antosiewicz & Singh, 2004; Hosono *et al.*, 2005; Jo *et al.*, 2008; Karmakar *et al.*, 2007; Lu *et al.*, 2004; Myzak & Dashwood, 2006; Sakamoto *et al.*, 1997; Singh, 2001; Tsai *et al.*, 2007; Wu *et al.*, 2002; Xiao *et al.*, 2005)
		Inhibition of p21H-ras-membrane association (inhibition of posttranslational modification)	Tumor growth in mice⇓	(Singh, 2001; Singh *et al.*, 1996)

Table 1. *Contd.*

OSC	Modes of action	Supposed mechanisms	Test systems and biological effects	Reference(s)
	Anticancer-related effects	Modulation of immune function: IL-6⇓, IL1β⇓, TNFα⇓, NFKB⇓, Cox-2⇓	Human blood, mice, human embryonal kidney cells	(Elango *et al.*, 2004; Karmakar *et al.*, 2007; Keiss *et al.*, 2003; Lu *et al.*, 2004; Shukla & Kalra, 2007; Tsao *et al.*, 2007)
		Antioxidative effect: NFKB⇓, inhibition of LPS-induced activation of NFκB	Human neuroblastoma, bladder carcinoma, macrophages	(Karmakar *et al.*, 2007; Liu *et al.*, 2006; Lu *et al.*, 2004)
DATS	Chemoprevention, chemotherapy	Activation of carcinogen detoxification and elimination by phase II-enzymes: GST⇑, QR⇑, CAR⇑, Nrf2⇑ Modulation of cell growth and induction of apoptosis: cell cycle arrest, activation of mitochondrial pathway of apoptosis; involvement of ATR/Chk1, involvement of Cdc25C	Carcinogenesis in rats/mice⇓, growth/viability of human leukemic cells and of carcinoma of breast, bladder, colorectum, liver, skin, prostate, nasopharynx⇓, tumors in mice grafted with sarcoma or hepatocarcinoma⇓	(Ariga & Seki, 2006; Hassan, 2004; Herman-Antosiewicz *et al.*, 2007; Herman-Antosiewicz & Singh, 2004; Hosono *et al.*, 2005; Kim *et al.*, 2007; Li *et al.*, 2002; Seki *et al.*, 2008; Xiao *et al.*, 2004; Xiao *et al.*, 2005; Xiao & Singh, 2006)
	Anticancer-related effects	Prooxidative effect: ROS⇑ by degradation of ferritin, oxidative modulation of β-tubulin, destruction and hyperphosphorylation of Cdc25C	Growth/viability of human leukemic cells and of carcinoma of breast, bladder, colorectum, liver, skin, prostate, nasopharynx⇓	(Ariga & Seki, 2006; Hassan, 2004; Herman-Antosiewicz *et al.*, 2007; Herman-Antosiewicz & Singh, 2004; Hosono *et al.*, 2005; Kim *et al.*, 2007; Li *et al.*, 2002; Seki *et al.*, 2008; Xiao *et al.*, 2004; Xiao *et al.*, 2005; Xiao & Singh, 2006)

Table 1. *Contd.*

OSC	Modes of action	Supposed mechanisms	Test systems and biological effects	Reference(s)
		Antioxidative effect: LPS-induced expression of iNOS⇓ linked with inhibition of NFκB	Macrophages	(Liu *et al.*, 2006)
		Modulation of the arachidonic acid cascade: Cox-2⇓, anti-inflammatory, inhibition of platelet aggregation, antithrombotic	Human embryonal kidney cells, rats	(Ariga & Seki, 2006; Elango *et al.*, 2004; Keiss *et al.*, 2003; Shukla & Kalra, 2007; Tsao *et al.*, 2007)
DATTS	Chemoprevention, chemotherapy	Activation of carcinogen detoxification and elimination: GST⇑, QR⇑, GPX⇑, CAR⇑, Nrf2⇑	Carcinogenesis in rats⇓, growth/ viability of human carcinoma of lung and prostate⇓	(Ariga & Seki, 2006; Fukao *et al.*, 2004; Hara *et al.*, 2005; Herman-Antosiewicz & Singh, 2004; Hosono *et al.*, 2005)
		Modulation of cell growth and induction of apoptosis: activation of mitochondrial pathway of apoptosis		
	Anticancer-related effects	Antioxidative effect: Ox-LDL⇓ (cytoprotective, membrane protection)	Cd induced toxicity rats⇓	(Murugavel & Pari, 2007; Murugavel & Pari, 2007)
AMS	Chemoprevention, chemotherapy	Inhibition of carcinogen activation by modulation of phase I-enzymes (CYP450): CYP2E1⇓	Carcinogenesis in rats ⇓	(Davenport & Wargovich, 2005)
MATS	Anticancer-related effects	Modulation of the arachidonic acid cascade: inhibition of platelet aggregation, antithrombotic	Rats	(Ariga & Seki, 2006)

*1 The listed supposed mechanisms and test systems are only exemplary in order to get a general idea about cellular processes involved into the modes of action of the OSCs. Besides all effects are also strongly model-dependent.

Table 1. *Contd.*

1B : *Other important OSCs*

OSC	Modes of action	Supposed mechanisms	Test systems and biological effects	Reference(s)
DMTS	Anticancer-related effects	Modulation of the arachidonic acid cascade: inhibition of platelet aggregation, antithrombotic	Rats	(Ariga & Seki, 2006)
SAC	Chemoprevention, chemotherapy	Modulation of cell growth and induction of apoptosis: activation of caspases	Growth/viability of human carcinoma of colon, lung, skin, neuroblastoma⇓	(Ariga & Seki, 2006; Hosono *et al.*, 2005; Sakamoto *et al.*, 1997)
		Activation of carcinogen detoxification and elimination: GST⇑, NQOR⇑, HO1⇑, Nrf2⇑	Human hepatocarcinoma cells, macrophages, human umbilical vein endothelial cells, cancerogenesis in rats⇓	(Aggarwal & Shishodia, 2004; Chen *et al.*, 2004; Geng *et al.*, 1997; Ho *et al.*, 2001; Ide & Lau, 2001; Keiss *et al.*, 2003; Sundaresan & Subramanian, 2008)
	Anticancer-related effects	Antioxidative effect: Ox-LDL⇓, SOD⇑, catalase⇑, block of NFκB-activation by TNFα or H_2O_2, scavenging of H_2O_2, release of peroxides from EC⇓, intracellular GSH-depletion⇓		
		Prooxidative effect: ARE⇑	Human hepatocarcinoma cells	(Chen *et al.*, 2004)
		antimetastatic effect: E-cadherin⇑ (cell motility⇓), MMP⇓ (cell invasion⇓)	Human breast carcinoma cells	(Gapter *et al.*, 2008)
SAMC	Chemoprevention, chemotherapy	Modulation of cell growth, cell cycle arrest, induction of apoptosis:	Orthotopic prostate cancer and metastases in mice⇓, growth of	(Herman-Antosiewicz *et al.*, 2007; Herman-

Table 1. *Contd.*

OSC	Modes of action	Supposed mechanisms	Test systems and biological effects	Reference(s)
		microtubule depolymerisation, JNK⇑, histone acetlyation (H3, H4)⇑	cells of human breast and colon⇓	Antosiewicz & Singh, 2004; Howard *et al.*, 2008; Howard *et al.*, 2007; Pinto *et al.*, 1997; Pinto *et al.*, 2000; Shirin *et al.*, 2001; Xiao *et al.*, 2005; Xiao *et al.*, 2003)
	Anticancer-related effects	Antimetastatic effect: E-cadherin⇑ (cell motility⇓)		
Allicin	Chemoprevention, chemotherapy	Modulation of cell growth and induction of apoptosis: cell cycle arrest, activation of mitochondrial pathway of apoptosis, activation of PKA, activation of AIF, caspase-dependent or independent apoptosis	Growth/viability of human leukemic cells and of carcinoma of breast, bladder, colorectum, liver, skin, prostate, nasopharynx	(Aggarwal & Shishodia, 2004; Dirsch *et al.*, 2002; Dirsch *et al.*, 1998; Hassan, 2004; Herman-Antosiewicz & Singh, 2004; Li *et al.*, 2002; Miron *et al.*, 2007; Oommen *et al.*, 2004; Park *et al.*, 2005; Zheng *et al.*, 1997)
	Anticancer-related effects	Prooxidative effect : activation of cellular defense, ROS⇑: activation of NFκB	Human non-leukemic cells	(Hassan, 2004)
		Modulation of immune function : stimulation of lymphocytes, ERK1/2 activation,	Human PBMC, human leukemic cells, mice	(Patya *et al.*, 2004)

Table 1. *Contd.*

OSC	Modes of action	Supposed mechanisms	Test systems and biological effects	Reference(s)
		Tissue-protection against γ-irradiation: anti-inflammatory, ICAM1-expression⇓, AP1-activation, c-JNK-phosphorylation⇓	Human umbilical endothelial cells	(Son *et al.*, 2006)
Ajoene (*Z*-isomer or mixture)	Chemoprevention, chemotherapy	Activation of carcinogen detoxification and elimination: NQO1⇑, HO1⇑, Nrf2⇑	Human hepatocarcinoma cells	(Chen, 2004)
		Modulation of cell growth and induction of apoptosis: cell cycle arrest, activation of mitochondrial pathway of apoptosis,microtubule depolymerisation, HDAC-inhibition	Growth/viability of human leukemic cells, primary leukemic cells and of carcinoma of breast, bladder, colorectum, liver, skin skin prostate, nasopharynx⇓, tumors in mice grafted with sarcoma or hepatocarcinoma⇓	(Dashwood, 2007; Herman-Antosiewicz, 2004; Li, 2002) (Herman-Antosiewicz *et al.*, 2007)
	Anticancer-related effects	Antimetastatic/antithrombotic effect: inhibition of platelet aggregation, inhibition of cell adhesion by changes of membrane fluidity (fibrinogen binding, phospholipid-composition)		
		Mediation of MDR: P-gp⇓	Growth/viability of cytarabine & fludarabine-resistant AML	(Hassan, 2004)
		Pro-oxidative effect: activation of cellular defense, ROS⇑: activation of NFκB, activation of ARE	Human hepatocarcinoma cells	(Chen, 2004)

Inhibition of Cell Growth, Induction of Apoptosis, Inhibition of Proliferation, Prevention of Metastases (Adhesion, Invasion and Angiogenesis)

As mentioned above, the two main approaches using drugs to suppress development, growth or proliferation of cancers are chemoprevention and chemotherapy. In particular chemopreventive agents prevent the formation of neoplastic cells or their progeny. They generally aim to reduce the formation of mutagens and carcinogens thereby preventing DNA damage or other cellular damages. Possible mechanisms of action are decrease of cellular ROS level or modulation of metabolism of mutagenic agents (inhibition of toxification by phase I enzymes, induction of detoxification and elimination by phase II enzymes) (Wu *et al.,* 2005). In contrast, the mode of action of chemotherapeutic agents is to induce cell death and to inhibit growth of neoplastic cells. The activity of these agents is mostly based on antiproliferative effects, *e.g.* by cell cycle inhibition or direct antimitotic effects, or the compounds induce programmed cell death (mainly apoptosis) in cancer cells by interactions with several targets. The latter one would be the most favorable mode of action of chemotherapeutic agents, since this would avoid side effects in the normal surrounding tissue (Delhalle *et al.,* 2003).

In this regard DAS was found to inhibit proliferation or to induce apoptosis in a panel of tumor cell lines and in rodents by modulation of cell growth regulatory and apoptotic proteins. Apoptosis mainly occurred via the classical mitochondrial pathway involving calpains, Bcl-2-family-members, caspases, inhibitor of apoptosis proteins (IAPS), mitogen activated protein kinases (MAPKs), extracellular signal-regulated kinases (ERKs) Smac/Diablo or NFκB; besides these events were mostly linked with an cytosolic increase in free Ca^{2+} (Ariga & Seki, 2006; Arora & Shukla, 2003; Das *et al.,* 2007; Karmakar *et al.,* 2007; Prasad *et al.,* 2008; Sriram *et al.,* 2008). Thus DAS exerts both chemotherapeutic and chemopreventive activities. Moreover it was found to act antimetastatic and antiangiogenic *in vitro* and *in vivo* (Herman-Antosiewicz *et al.,* 2007), what is very favorable since many patients suffering from solid cancers die not from the primary tumors but from metastases in other organs.

Similar effects were also observed for DADS, that was able to induce or favor cell death by almost the same pathways. Additionally p53, cell division cycle 25 homolog C (Cdc25C), or cyclin B1 seem to play a role and hyperacetylation of histones was detected in selected models. Furthermore the posttranslational modification of oncogenic Ras was inhibited, thus suppressing growth of tumor xenografts in mice *via* an inhibition of membrane association of p21 (Herman-Antosiewicz *et al.,* 2007; Singh, 2001).

A comparable range of activities was also elucidated for DATS that induced apoptosis and inhibited proliferation in multiple models *in vitro* and *in vivo* by similar pathways as DAS and DADS (Aggarwal & Shishodia, 2004; Hara *et al.,* 2005; Hosono *et al.,* 2005; Sakamoto *et al.,* 1997; Sundaram & Milner, 1996). Moreover the involvement of ataxia-telangiectasia mutated and Rad3 related checkpoint kinase 1 (ATR/Chk1) in prometaphase arrest was elucidated (Herman-Antosiewicz *et al.,* 2007). Besides induction of apoptosis by DATS occurred cell cycle-dependently in some models, what is a hint for a strong cross talk with the antiproliferative triggers (Hosono *et al.,* 2005).

DATTS was elucidated to protect against chemically induced tissue injury and carcinogenesis in rats (Ariga & Seki, 2006; Fukao *et al.,* 2004; Fukao *et al.,* 2004) and to trigger apoptosis in diverse cell lines (Ariga & Seki, 2006; Hara *et al.,* 2005; Herman-Antosiewicz & Singh, 2004; Hosono *et al.,* 2005).

S-allylcysteine (SAC) was demonstrated to exert not only direct anticancer effects against cancer cell lines and primary tumors *in vivo* (Ariga & Seki, 2006; Hosono *et al.,* 2005; Sakamoto *et al.,* 1997), but also antimetastatic effects *in vitro*. The adhesion and invasion of breast cancer cells was inhibited by upregulation of E-cadherin and *via* a downregulation of matrix metalloproteinases (MMPs) (Gapter *et al.,* 2008).

S-allylmercaptocysteine (SAMC) showed almost the same activity profile as SAC and was even able to reduce formation of lung and adrenal metastases in mice bearing orthotopic androgen-independent prostate cancer (Howard *et al.,* 2007). Besides SAMC had-however-a special mode to block the cell cycle directly interacting with microtubules. Nevertheless this effect was different from traditionally used mitosis interrupting agents (MIAs) such as taxol or colchemide (Xiao *et al.,* 2003).

Methylallyltrisulfide (MATS) was found to be one of the most active constituents of garlic oil in selective inhibition of secondary platelet aggregation both *in vitro* and *in vivo* (antithrombotic in rats). It was indicated that the inhibition site of MATS within the arachidonic acid cascade was the reversible block of the transformation from prostaglandin G_2 to prostaglandin H_2. MATS did not inhibit thromboxane synthase, and inhibition platelet aggregation could completely be attenuated by the addition of thromboxane A_2 (Ariga & Seki, 2006). This mode of activity might contribute to an antimetastatic effect by inhibition of cancer cell adhesion to endothelia and by reducing the number of freely circulating cancer cells. Other OSCs with strongly similar chemical structure, such as dimethyltrisulfide or diallyltrisulfide were less potent and less selective in this respect.

The anticancer activity of *Z*-ajoene (or mixtures of both isomers) was shown for a panel of human neoplastic cell lines. Moreover the compound could strongly inhibit tumor growth in mice grafted with sarcoma 180 or with hepatocarcinoma. The underlying mechanisms were a mitotic arrest in early stages caused by microtubule disassembly, induction of apoptosis or histone deacetylase inhibitor (HDAC)-inhibition (Ariga & Seki, 2006; Dashwood & Ho, 2007; Herman-Antosiewicz & Singh, 2004; Li *et al.,* 2002).

The activity profile of allicin was more complex, since the compound was able to induce cell death in different leukemic (even primary ones) and non-leukemic cell lines in a caspase-dependent or independent manner. However apoptosis inducing factor (AIF) and protein kinase A (PKA) were activated, cytochrome C was released from mitochondria, the Bcl2-family was modulated, GSH was depleted, or cell cycle was blocked. Apoptosis could be induced also if NFκB was activated (Aggarwal & Shishodia, 2004; Dirsch *et al.,* 2002; Dirsch *et al.,* 1998; Hassan, 2004; Miron *et al.,* 2007; Oommen *et al.,* 2004; Park *et al.,* 2005; Zheng *et al.,* 1997).

Moreover a cyclic OSC- thiacremonone- was described to induce cell death- mostly apoptosis- in different cell lines by modulation of the caspase family, the Bcl-2 family and the IAP family (Ban *et al.,* 2007).

Resuming the results, antiproliferative activities of OSCs were mostly based on inhibition of cellular cycle or by direct effects on mitosis (interactions with microtubuli). The other main mode of cell death triggered by OSCs was the induction of apoptosis mediated via the classical mitochondrial pathway. In some cases mode of inhibition of cancer cell growth was not elucidated and involvement of other pathways can not be excluded.

Relations to Modulation of Immune Function, Chronic Inflammation, and NFκB

Similiarly to garlic preparations allicin was reported to activate lymphocytes, what was supposed to contribute to the antitumoral effect of the compound in mice bearing melanoma or fibrosarcoma (Patya *et al.,* 2004). At the same time it was postulated that this immunestimulatory effect was mediated by redox-sensitive signaling ($p21^{ras}$).

Furthermore modulation of the immune system/ anti-inflammatory activities were observed for DAS, DADS, DATS, SAC, allicin or thiacremonone, in that inflammation-linked transcription factors, cytokines and enzymes (NFκB, IL-1β, IL-6, TNFα, Cox-2, ICAM-1) were downregulated in different models leading partially to antitumor or cytoprotective (for healthy tissue) effects (Ban *et al.,* 2007; Chen *et al.,* 2004; Elango *et al.,*

2004; Hassan *et al.,* 2003; Karmakar *et al.,* 2007; Keiss *et al.,* 2003; Liu *et al.,* 2006; Patya *et al.,* 2004; Shukla & Kalra, 2007; Son *et al.,* 2006; Tapiero *et al.,* 2004; Tsao *et al.,* 2007).

Modulation of Cellular Redox Status

In general redox balance of cells is a prerequisite for biological homeostasis. If this balance is disturbed by different external effectors, such as reactive oxygen species (ROS), reactive nitrogen species or radicals, cells can be damaged at different levels and become neoplastic, what finally leads to carcinogenesis (Tapiero *et al.,* 2004). Hence compounds preventing or attenuating ROS-induced damages would be suitable chemopreventive agents.

However different garlic compounds were found either to attenuate ROS or to increase ROS in order to activate cellular defense mechanisms against oxidative stress (antioxidative or prooxidative activity).

One part of the antioxidative effects of the sulfur containing garlic compounds is relied on the activation or upregulation of antioxidatively acting enzymes like superoxide dismutase (SOD), glutathione-reductase (GR) or catalase (Aggarwal & Shishodia, 2004), what was shown for SAC (Ariga & Seki, 2006; Balasenthil *et al.,* 2001; Balasenthil *et al.,* 2002). DATS protected against carbon tetrachloride (CCl_4) induced liver injury in rats, what was assumed to be due to the phase II-enzyme induction by the compound somehow compensating physiological radical production (trichloromethyl radicals, trichloro peroxy methyl radicals) by the carcinogen (Ariga & Seki, 2006; Sheweita *et al.,* 2001; Slater *et al.,* 1985). The other part of the antioxidant activity is a direct effect of the compounds based on their chemical structure and reactivity with cellular reactive oxygen species (ROS) leading *e.g.* to radical scavenging. This kind of antioxidative effect is *e.g.* reported for DAS attenuating DMBA-induced carcingenesis in mice (Prasad *et al.,* 2008) or for DATTS reducing production of ox-LDL and protecting several enzymes against Cd-triggered cytotoxicity if applied to rats (Murugavel & Pari, 2007).

In contrast an indirect prooxidative effect is described for DATS by triggering the degradation of the iron storage protein ferritin, what increases free iron (Fe^{2+}) leading among other effects to destruction and hyperphosphorylation of Cdc25C, what finally resulted in death of human prostate cancer cells (Antosiewicz *et al.,* 2006; Kim *et al.,* 2007; Xiao *et al.,* 2004; Xiao *et al.,* 2005). Furthermore SAC, ajoene and allicin were reported to exert prooxdiative effects (release of free radicals) leading to the activation of cellular defense mechanisms (antioxidant response element (ARE), nuclear factor E2-related factor (Nrf2), NFκB) *in vitro* or of lymphocytes *in*

vivo, finally resulting in cancer cell death (Chen *et al.,* 2004; Patya *et al.,* 2004). Nevertheless DATS was additionally shown to act as an antioxidant in macrophages, where it reduced the LPS-induced expression of iNOS and the activation of the redox-sensitive NFκB (Liu *et al.,* 2006). Sulfides from *Allium* species can exert prooxidative effects by GSH-depletion, hydrogen peroxide formation, methemoglobin or sulfhemoglobin formation (Munday *et al.,* 2003).

Furthermore, apart from the listed most commonly described effects of ROS, there is one theory that describes that DADS and DATS were activated by ROS to form epoxides (which was mimicked by addition of DMDO leading to epoxide formations) able to inhibit RNA-synthesis and several harmful interactions of carcinogens with DNA (Yu *et al.,* 2003).

These results make clear, that not only activity profile of the OSCs is very multifaceted, but that effects of the same compound can be opposite in different models, what proofs that reactivity of the redox chameleon sulfur- being the most (redox) active structural feature of OSCs- is strongly depending on the physiological microenvironment (Jacob, 2006).

Moreover different cellular "tools" such as the redox system and the phase II -system are sometimes overlapping very strongly, so that the activity of an OSC can not be clearly attributed to one of them since it is affecting both in parallel. In particular it was found for DATS, that activation of phase II-enzymes was probably mediated by Nrf2 and by ARE upregulation (Chen *et al.,* 2004). Besides three major MAPKs, ERKs, c-Jun-N-terminal protein kinase (JNK), p38, but not upstream PKC were involved in this enzyme regulation (Chen *et al.,* 2004; Yu *et al.,* 2000; Zipper & Mulcahy, 2003). This means that DATS induces oxidative stress in this model (Chen *et al.,* 2004) probably by intracellular glutathione depletion (Munday *et al.,* 2003) leading to the activation of the oxidative stress sensor Nrf2 and consecutively to an upregulation of the cellular defense system against oxidative stress- phase II enzymes (Mcmahon *et al.,* 2003).

Modulation of Phase I (CYP450) and Phase II Metabolism

Most xenobiotics (chemical substances foreign to the biological system) are detoxified/eliminated from the body by two physiological enzyme systems, called phase I and phase II. Phase I reactions are redox reactions mainly carried out by CYP450-enzymes [more than 50 different CYP enzymes in the human genome (Autrup, 2000; Davenport & Wargovich, 2005)] and generally transform chemical compounds to structures that can be conjugated with glucuronic acid, glutathione or some amino acids by phase II enzymes (*e.g.* GST) leading to the compounds' detoxification and elimination from the body. Nevertheless transformations of xenobiotics by

CYP-enzymes can also be disadvantageous if these reactions lead to toxifications, *e.g.* activation of carcinogens (Ariga & Seki, 2006). However both phase I and phase II enzymes have to be considered regarding pharmacokinetic and toxicologic aspects. Particularly interaction with CYP-enzymes can even be a knock-out criterion during drug development processes (Agundez, 2004; Riley & Grime, 2004).

For example DAS was shown to reduce chemically induced carcinogenesis in rodents by inhibition of carcinogen activation via modulation of CYP450 enzymes 2E1 and 2B1 and via activation/upregulation of detoxification enzymes such as GST or QR (Aggarwal & Shishodia, 2004; Ariga & Seki, 2006; Davenport & Wargovich, 2005; Fisher *et al.,* 2007; Wu *et al.,* 2002; Yang *et al.,* 2001). This anticarcinogenic effect of DAS was partially described for several animal models to be due to the formation of the metabolites DASO and DASO2 (Ariga & Seki, 2006; Davenport & Wargovich, 2005; Yang *et al.,* 2001), that are competitive inhibitors of CYP2E1 of rodents as DAS; in addition the latter metabolite is even a suicide inhibitor of the enzyme.

Similar results were found for DADS (Ariga & Seki, 2006; Davenport & Wargovich, 2005; Fisher *et al.,* 2007; Tsai *et al.,* 2007; Wu *et al.,* 2002), AMS (DADS is metabolised to AMS (Davenport & Wargovich, 2005; Rosen *et al.,* 2000)), DATS (Ariga & Seki, 2006; Fisher *et al.,* 2007; Fukao *et al.,* 2004; Fukao *et al.,* 2004; Hara *et al.,* 2005; Hosono *et al.,* 2005; Sheweita *et al.,* 2001; Slater *et al.,* 1985; Tsai *et al.,* 2007; Wu *et al.,* 2002), DATTS (Murugavel & Pari, 2007; Murugavel & Pari, 2007), SAC or ajoene (Chen *et al.,* 2004).

Overall activities of CYP450-enzymes were generally downregulated in different models leading to a prevention of generation of toxic agents such as radicals from applied chemicals, whereas detoxifying phase II enzymes were mostly upregulated and activated resulting in a faster elimination of toxic agents from the body. Nevertheless some CYP enzymes were upregulated after treatment with OSCs (Davenport & Wargovich, 2005), what might be unfavourable. On the other hand, this undesirable effect was only shown for very high doses or multiple applications of OSCs, so it could probably be avoided by adequate application schemes.

Reversal of Multidrug Resistance (MDR) Mediated by P-gp

One major problem in chemotherapy is the fast development of drug resistances by cancers, particularly in leukemias, lymphomas, multiple myeloma, neuroblastoma and soft tissue carcinoma (Malayeri *et al.,* 1996; Ross *et al.,* 1993). This multidrug resistance consists partially in the overexpression/upregulation of cellular efflux pumps, such as the glycoprotein P (P-gp)- family proteins belonging to ATP-binding cassette transporters on the plasma membrane, that actively extrude structurally

diverse amphipathic drugs (Ambudkar *et al.,* 1999; Arora *et al.,* 2004; Sugawara, 1990). Unfortunately all actually available modulators of P-gp exert too unacceptable adverse effects to become adjuvants in chemotherapy (Arora *et al.,* 2004).

Hence selected OSCs form garlic might be better candidates for the modulation of P-gp-expression in cancers, since their side effects seem generally not to be harmful for patients. For example DAS was shown to enhance (at non-toxic DAS-concentrations) cytotoxic effects of several antineoblastic agents, such as vinblastine, vincristine and doxorubicin against vinca-alkaloid-resistant, leukemic K562 cells *in vitro.* Besides it was demonstrated to inhibit vinca-alkaloid induced P-gp-overexpression in mouse hepatocytes *in vivo* (Arora *et al.,* 2004). Furthermore ajoene was similiarly shown to attenuate P-gp-triggered resistance of AML against cytarabine and flutarabine (Hassan, 2004).

Selectivity of OSCs against Normal Cells

Histone deacetylase (HDAC)-inhibition

One major feature of several human cancers is the loss of monoacetylation and trimethylation of histone H4 (Dashwood & Ho, 2007; Fraga, 2005). Hence medicinal research developed histone deacetylase (HDAC) - inhibitors that are clinically used agents for cancer therapy, *e.g.* vorinostat® in the treatment of cutaneous T-cell lymphoma (Marks, 2007). Nevertheless HDAC-inhibitors could also be used in chemoprevention, in particular diallyldisulfide showing weak inhibitory activity *in vitro* in human colon cancer cells (Dashwood *et al.,* 2006; Druesne *et al.,* 2004; Myzak & Dashwood, 2006). The supposed mode of action of HDAC inhibitors is the arrest of the cell cycle in the G2/M-phase and to induce apoptosis, however, with certain selectivity for cancer cells (Dashwood & Ho, 2007; Nebbioso *et al.,* 2005; Ungerstedt *et al.,* 2005). The assumed mechanism is not well understood. Recent studies have implicated thioredoxin and intracellular thiol status, the accumulation of ROS, the induction of TRAIL, DR4 and DR5 (Dashwood & Ho, 2007; Nebbioso *et al.,* 2005; Ungerstedt *et al.,* 2005).

Unknown Modes/Mechanisms of Selectivity

Moreover a certain selectivity of OSCs against cancer cells in comparison with normal cells was found in diverse models, though mechanisms of selectivity are not yet fully understood:

Ajoene was found to selectively induce apoptosis in HL-60 cells and in leukemic blood, but not in healthy PBMCs (Dirsch *et al.,* 2002). Besides it exerted a selective antiproliferative effect against a panel of tumor cell lines in comparison with a normal marsupial kidney cell line to a certain degree (Li *et al.,* 2002). Both, DAS and DADS selectively induced apoptosis

in malignant neuroblastoma cells, but not in primary neurons (Karmakar *et al.,* 2007). DADS seemed even to act selectively *in vivo* in the liver what was assumed after a study in mice (Iciek *et al.,* 2007). DATS selectively induced apoptosis in malignant prostate cells (LNCaP) but not in normal epithelial prostate cells (PrEC) (Kim *et al.,* 2007; Munchberg *et al.,* 2007).

Selectivity Approaches

Nevertheless, there are studies in progress that aim at a cancer cell directed therapy to minimize unfavorable properties of garlic compounds, such as instability (*e.g.* allicin (Fujisawa *et al.,* 2008)) and side effects, since OSCs exert multifaceted medicinal activities. For example researchers developed an antibody directed enzyme prodrug therapy for the application of the binary alliin/alliinase-system in cancer therapy in a nude mouse model (Arditti *et al.,* 2005; Miron *et al.,* 2003; Munchberg *et al.,* 2007). Moreover a prolonged, hepatic-targeted activity of DATS could be reached in a mouse-hepatocarcinoma model by coupling the compound on the surface of nanoparticles (Zhang *et al.,* 2007).

However special approaches for selectivity in chemoprevention and chemotherapy might not be necessary, since garlic and its OSCs exert their anticarcinogenic effects by multiple mechanisms, and since multiple signaling pathways are different/dysfunctional in cancers (Nagini, 2008) leading to a certain selectivity by themselves. In addition, the only reason for selective activity of many clinically used chemotherapeutics is the higher proliferation rate of cancer cells if compared with normal cells. Furthermore adverse effects of garlic and its constituents seem not to be toxic in men at adequate doses proven by the fact that the plant is used since 3500 years for medicinal reasons (Shukla & Kalra, 2007)- so they can probably be neglected during cancer therapy.

Structure Activity Relationships (Hassan et al., 2003)

Though mechanisms and modes of action of OSCs seem not to be directed against specific targets, what means that no real pharmacophor of the compounds can be defined, structure activity studies were performed to assess the general influence of structural features such as sulfur chain length or saturation of carbon chains. Hence several correlations between chemical structures and activities regarding selected modes of action were found.

For example Ariga or Wu and co-workers (Ariga & Seki, 2006; Wu *et al.,* 2002; Wu *et al.,* 2001) confirmed previous studies in that monosulfides are strong activators of phase I enzymes, whereas polysulfides are rather weak in this respect. The potency in activating CYP1A1, CYP2B1 or CYP3A1

was decreasing from the DAS to DADS to DATS. Moreover allylsulfides were much more active than their propylanalogs (Davenport & Wargovich, 2005). However since it is always depending on the test model whether an activation of CYP450s is favourable or not (see section "modulation of phase I (CYP450) and phase II metabolism") one has to choose the appropriate sulfide in this regard. However in most cases an activation is not desired (Davenport & Wargovich, 2005).

On the contrary it was shown in several studies that potency of polysulfides in inducing phase II enzymes is generally stronger than the one of monosulfides, *e.g.* activity ranking of allylsulfides in increasing glutathione *S*-transferase (GST), quinone reductase (QR) or glutathione peroxidase (GPx) was DATS>DADS>DAS (Ariga & Seki, 2006; Fukao *et al.,* 2004; Fukao *et al.,* 2004; Wu *et al.,* 2002; Wu *et al.,* 2001).

The same tendency was seen for cellular growth inhibition (inhibition of proliferation, induction of apoptosis), *e.g.* of prostate cancer cells, since activity was increasing with the number of sulfur atoms in the molecule (Ariga & Seki, 2006; Herman-Antosiewicz & Singh, 2005; Kim *et al.,* 2007; Xiao *et al.,* 2004; Xiao *et al.,* 2005). This increase of activities with the number of sulfur atoms present in a molecule was also found for other modes of action (Knowles & Milner, 2000; Li *et al.,* 2002), such as the inhibitory effect of allylsulfides on lipopolysaccharide (LPS)-inducible nitric oxide synthase (iNOS)-expression. This effect is additionally linked to the antioxidant potential of the compounds to inhibit NFκB-activation (Liu *et al.,* 2006). Surprisingly prooxidative effect of allylsulfides is analogically increasing with the number of sulfur atoms and again allylcompounds were more potent than the propylanalogs (Chen *et al.,* 2004) (reasons for anti- and prooxidative effects see section "Modulation of cellular redox status"). Besides side chains strongly affected the antimetastatic activity of certain sulfides, in that MATS was much more potent than dimethyltrisulfide or diallyltrisulfide (Ariga & Seki, 2006).

Furthermore lipophilic OSCs (DADS, DATS) were more potent than the hydrophilic ones (SAC) in this respect, what might be due to pharmacokinetic reasons (Ariga & Seki, 2006).

In addition to the number of sulfur atoms or the saturation of the carbon side chains, other studies correlated more complex structural parameters of the compounds with biological activities. Hence HDAC-inhibitory potency of DADS was linked to its metabolic conversion to SAMC according to some docking studies (Dashwood & Ho, 2007; Dashwood *et al.,* 2006). Besides the allylthiogroup of allicin and of other organosulfur compounds from garlic was considered to be some kind of pharmacophore in the chemoprevention of hepatocarcinoma (*in vitro, in vivo*) (Kwon *et al.*, 2002).

Furthermore metabolic conversion of allylsulfides into equally potent or even more potent compounds has to be considered. For example, DAS, that is widely reported for its potential to prevent cancer (Ariga & Seki, 2006; Arora *et al.,* 2004), is converted by the phase I enzyme CYP2E1 to diallylsulfoxide (DASO) and to diallylsulfone ($DASO_2$), both reported to reduce a multitude of chemically induced cancers in animals (Ariga & Seki, 2006; Yang *et al.,* 2001).

Studies on Absorption, Distribution, Metabolism, Elimination and Toxicity (ADME-Tox-Studies)

Allicin is able to cross freely through artificial and biological membranes (phospholipid bilayers) both by diffusion and permeation (though it interacts with thiol groups). It was also shown to pass rapidly across human red blood cell membranes without inducing leakage, fusion or aggregation of membranes (Miron *et al.,* 2000). Nevertheless it was also shown that allylsulfides from garlic, such as DADS and DATS interact with the membranes (preferentially on the hydrocarbon cores of the phospholipid bilayers), what was assumed to be one possible mechanistic explanation for the antiproliferative, antiplatelet and antimicrobial effects of the compounds (Tsuchiya & Nagayama, 2008).

Besides it was found that mono-,di-, tri-, and tetrasulfides from *Allium* plants (DATTS> DATS> DADS> DAS> propylanalogs) led to an oxidative damage to erythrocytes and to hemolytic anemia in rat models (Munday *et al.,* 2003). The reasons to perform the latter study were records of toxic effects in domestic and farm animals after consumption of garlic and onions. However this study in rats was performed using very high doses and the toxicity could not be confirmed in human blood applying GPE or DADS (Keiss *et al.,* 2003).

CONCLUSIONS AND OUTLOOK

In summary, the activity range of OSCs in the area of cancer research and related fields is very broad and mostly favorable. It was shown that compounds were very potent in chemoprevention and chemotherapy including primary solid tumors, metastases and leukemia (Herman-Antosiewicz *et al.,* 2007; Shukla & Kalra, 2007; Wu *et al.,* 2005). In this context OSCs protected normal cells from carcinogens by modulation of phase I and phase II metabolism and affected cancer cell growth mainly by cell cycle arrests or by induction of apoptosis via the classical mitochondrial pathway. Both of them seemed often to be modulated by ROS in many cases (for example see "proposed mechanisms to explain DATS-induced cell

cycle arrest or apoptosis in human prostate cancer cells" (Herman-Antosiewicz & Singh, 2004) or "schematic presentation of molecular mechanisms of apoptosis in SH-SY5Y cells" after treatment with DAS or DADS (Karmakar *et al.,* 2007). The most important molecular targets and cellular markers that were shown to play a model-dependent role or to be affected in the research for anticancer agents applying OSCs are listed in the tables. However modes of action especially regarding selectivity are not yet completely understood.

What remains to be done in the near future are ongoing studies elucidating mechanisms of observed modes of action and reasons for model restricted activites. Besides effects of OSC on other molecular markers of apoptosis, such as heat shock proteins or survivin should be investigated (Wu *et al.,* 2005). Moreover the role of the death receptor triggered and of the ER-stress triggered apoptosis (Das *et al.,* 2007; Nakagawa *et al.,* 2000) or the affection of autophagy and of cellular survival pathways in the OSC induced programmed cell death is not clear.

Apart from these studies in the area of molecular biology, it would be important-in order to accelerate development of suitable drug formulations, to perform extended ADME-tox studies, probably even in men (clinical phase I). This should be possible according to epidemiological data, where population and clinical studies were performed without reports about toxic side effects (Devrim & Durak, 2007; Engdal *et al.,* 2008; Galeone *et al.,* 2007; Ngo *et al.,* 2007). By this way adequate application schemes for men could be established, what is always difficult by extrapolation of data from animals to men (dosage, single or multiple application) (Davenport & Wargovich, 2005; Tsao *et al.,* 2007; Yang *et al.,* 2001).

Overall resuming the available information about allylsulfides and related OSCs from *Allium* species, they might be a good alternative to existing chemotherapies, since they do not only exert direct anticancer activity, they also reduce metastases (Howard *et al.,* 2007) and related diseases such as inflammation. Moreover they stimulate self defense of the body (immune function), they reverse drug resistances (P-gp (Hassan, 2004)) and they even protect healthy tissue from damages (*e.g.* irradiation (Son *et al.,* 2006)), probably without exerting harmful/toxic side effects in patients. Hence OSCs might act multitargeted resulting in the successful elimination or prevention of diverse human cancers. Furthermore they could be used as adjuvants in traditional radio-, or chemotherapies, thus attenuating side effects in normal tissue or enhancing activities of traditional chemotherapeutics. Another possible use would be the one as functional food.

ACKNOWLEDGEMENTS

CS was supported by a "Bourse de formation-recherche" of the "Ministère de la Culture, de l'Enseignement supérieur et de la Recherche of Luxembourg". Research was supported in part by Télévie, the "Fondation de Recherche Cancer et Sang" and "Recherches Scientifiques" Luxembourg association. The authors thank "Een Häerz fir Kriibskrank Kanner" association, the Action Lions "Vaincre le Cancer", the Foundation for Scientific Cooperation between Germany and Luxembourg, and the Saarland University for additional support.

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Targeting Tumor Angiogenesis for Preclinical Validation of Antiangiogenic Compounds from Medicinal Plants

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ABSTRACT

Since the early 1970's when Folkman popularized the idea that tumor growth is angiogenesis dependent and that an anti-angiogenic strategy may constitute a novel therapeutic approach for treatment of cancers and other angiogenesis related pathologies like psoriasis, diabetic nephropathy etc., there has been an ever increasing interest in the idea of antiangiogenic therapy. Indeed in the last decade identification of novel therapeutically useful antiangiogenic compounds has become an intense field of research. Angiogenesis is a complex multistep process and as such presents a number of key targets for therapeutic intervention. The purpose of this chapter is to provide the reader with an over-view of literature on tumor angiogenesis and its regulation at molecular level and to describe some selected assay systems to validate the anti-angiogenic potential of compounds that are isolated and characterized from medicinal plants. It is not our intention to review the entire antiangiogenic literature which is very vast. The impact of inhibiting angiogenesis on tumor growth and metastasis has led to the development of specific antiangiogenesis assays in addition to the inhibition of standard tumor growth. Various in vitro assays can asses the impact of a therapeutic agent from medicinal plants, on endothelial cell proliferation,

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migration and cord formation. These assays help delineate the mechanism of action of a potential therapeutic compounds. For in vivo studies many laboratories use a semi quantitative, chicken chorioallatoic membrane assay to assess antiangiogenic agents from plant origin. Matrigel plug assay is an in vivo model described to measure the growth of blood vessels into an exogenously administered matrigel subcutaneously into rodents and either angiogenesis induced by VEGF or antiangioenesis of a test compound can be quantitated by counting the number of blood vessels or estimating hemoglobin content. In corneal angiogenesis assay control-release pellet containing either an angiogenic agent or a putative antiangiogenic molecule from medicinal plant is implanted into corneal micro pockets and blood vessels are quantitated. These approaches have been adopted in case of several antiangiogenic drugs in clinics or in clinical trials are, for example, TNP-70 and thalidomide. Metastasis, the process where tumor cells leave their tissue of origin and colonize in distant tissues has become a target for antiangiogenesis. Metastasis is initiated by invasion of basement membrane by proteolysis and motility. The invadopodia assay demonstrating the process of metastasis is a strong assay for preclinical validation of novel antiangiogenic compounds from medicinal plants. Numerous pharmaceutical companies are currently devoting multi-million dollar budgets for developing novel antiangiogenic drugs. There is thus a growing need to find reliable, cheap and fast assays to screen a large number of antiangiogenic compounds using mechanism based in vivo and in vitro assays.

Key words : Angiogenesis, medicinal plants, assays, validation, antiangiogenic drug

INTRODUCTION

Tumor Angiogenesis

Angiogenesis, the formation of new blood vessels from pre-existing blood vessels, is a fundamental physiological process that occurs during embryonic development, wound healing and the reproductive cycle. Abnormal angiogenesis takes place in pathological conditions, such as in diabetic retinopathy, muscular degeneration, rheumatoid arthritis, and psoriasis (Folkman, 1995). In tumor situation, new vessels are necessary for continued outgrowth of virtually all solid tumors supplying oxygen and nutrients to the entire tumor mass, thus allowing tumor expansion (Hanahan & Folkman, 1996; Bouchk *et al.*, 1996; Risau, 1997). Angiogenesis is a complex multi-step process involving extensive interplay between cells, soluble factors, and extracellular matrix (ECM) components. Recent studies have shown the importance of leucocytes as providers of cytokines, chemokines and enzymes

that are involved in angiogenesis. Angiogenesis stimulators and inhibitors target one or more of these steps. (i) In response to hypoxia, injured or diseased tissues synthesize and release angiogenic factors; (ii) angiogenic factors bind to their receptors on endothelial cells (ECs); (iii) receptor binding leads to EC activation; (iv) proteases are released to dissolve the basement membrane; (v) ECs migrate and proliferate; (vi) adhesion molecules (*e.g.* integrin avb3 and avb5) help to pull the sprouting blood vessel forward; (vii) matrix metalloproteinases (MMPs) are produced to dissolve the extracellular matrix and to initiate remodelling; (viii) angiopoietin–Tie-2 interaction modulates tubule formation; (ix) the EphB–ephrinB system regulates loop formation; (x) pericytes are incorporated to stabilize the newly formed blood vessel (Fig 1).

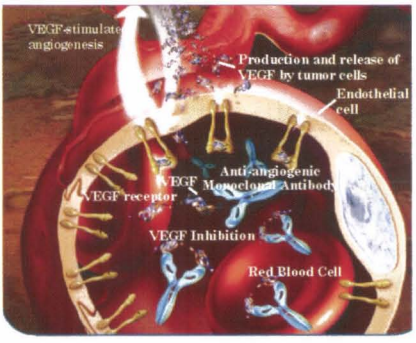

Fig 1. The process of angiogenesis

Angiogenesis is also required for metastatic dissemination, since an increase in vascular density will allow easier access of tumor cells to the circulation. Induction of angiogenesis precedes the formation of malignant tumors, and increased vascularization seems to correlate with the invasive properties of tumors and thus with the malignant tumor phenotype (Cavallaro & Christofori, 2000). We can recognize two distinct phases of tumor progression: the pre-vascular phase and the vascular phase. In the initial avascular phase, tumor growth is sustained by nutrients and oxygen through passive diffusion from the host vasculature; cell proliferation and cell death which are balanced and the size of the tumor does not exceed a few cubic millimeters. When the metabolic demand increases, tumors switch to an angiogenic phenotype and recruit blood vessels from the surrounding stroma. Once neovascularization occurs, the tumor acquires a rapid growth rate and increased metastatic potential (Auguste *et al.*, 2005). Mutations of oncogenes and tumor suppressor genes often lead to transformation of a normal cell to become a malignant cell. However, most malignant cells cannot grow to a clinically detectable tumor mass in the absence of blood vessels. Thus, a clinically manifested large tumor has to switch on an angiogenic phenotype

to support their growth (Cao *et al.,* 2005). The switch of an angiogenic phenotype may represent an imbalanced expression of angiogenic factors and angiogenesis inhibitors.

Regulation of Angiogenesis

Active angiogenesis is an integral part of a vascular tree formation during embryonic development that ceases altogether in mature adult organism. Complete suppression of angiogenesis in normal adult tissues is only lifted in response to a local demand in additional blood flow like wound healing or reproductive events (Bouck *et al.,* 1996), while in pathological situations angiogenesis may be turned on by ischemia, or by a rapid increase in tissue mass, like tumor growth (Folkman & Shing, 1992). This type of dynamics requires molecular switches that could be easily turned on or off. These competing factors fall into two main categories-positive and negative regulators of angiogenesis- inducers and inhibitors. The ratio in favor of antiangiogenic molecules produce the net inhibitory signal and vascular quiescence, while the prevalence of inducers shifts the equilibrium in an opposite direction causing quiescence break and new vessel formation. Both inducers and inhibitors of angiogenesis were found in normal tissues and in secretions of cultured benign cells (Hanahan & Folkman, 1996). In tumors the switch is governed by the same genetic alterations that determine in tumor cell the capacity for unlimited proliferation. Both loss of tumor suppressor genes and activation of oncogenes may lead to the changed inducer/inhibitor ratio and subsequent neovascularization. This dynamic equilibrium might be responsible for the instability and perpetual remodeling of the tumor vasculature and therefore, constantly activated state of tumor endothelium.

The majority of angiogenic factors that appear to play a major role in physiological and pathological angiogenesis are classical peptide growth factors (Gale & Yancopoulos, 1999). New blood vessels can be stimulated to grow when factors that promote angiogenesis are upregulated or those that inhibit angiogenesis are down regulated. The best studied example is the family of vascular endothelial growth factors (VEGF). Other potent angiogenic factors are acidic and basic fibroblast growth factor FGF1 and FGF2 respectively (Christofori, 1997; Vlodavsky & Christofori, 1998). An important family of angiogenic factors named angiopoietins is the ligands for the endothelial cell specific tyrosine kinase receptor Tie-2. Angiopoietins and their receptor also appear to be required for tumor angiogenesis, since the expression of a soluble form of the Tie-2 receptor resulted in the inhibition of tumor growth in tumor transplantation experiments. Other classical growth factors that have been reported to stimulate endothelial cell proliferation are transforming growth factor alpha (TGF α) and platelet derived growth

factor BB (PDGF BB). Protein factors such as pleiotrophin, angiogenin, angiotropin, platelet activating factor (PAF) and the HIV tat gene product are able to induce proliferation. The negative regulators include thrombospondin (Good *et al.*, 1990; DiPietro, 1997) and the 16-kDa N-terminal fragment of prolactin (Ferrara *et al.*, 1991), the plasminogen fragment angiostatin and endostatin, a collagen XVIII fragment (O'Reilly *et al.*, 1997)

VEGF Family Receptors and Cytokines

The vascular endothelial growth factor (VEGF) family of ligands and receptors has been the subject of intense study in the field of vascular biology for more than a decade. VEGF had been characterized as a heparin binding angiogenic growth factor displaying high specificity for endothelial cells (Gospodarawicz *et al.*, 1989; Ferrara & Henzel, 1989). The VEGF family consists of VEGF or VEGF-A, B, C, D, E and placental growth factor (Ferrara *et al.*, 2003). During embryogenesis and throughout adulthood, VEGF is essential for proper vascularization and is expressed in most tissues in a temporally and spatially specific manner (Peters *et al.*, 1993). Given its importance during development and its persistent expression in the adult, it is reasonable to assume that VEGF plays an integral role in vessel growth and maintenance.

The human VEGF gene is organized in eight exons separated by seven introns and is localized in chromosome 6p12. The coding region spans approximately 14kb (Houck *et al.* 1991; Tischer *et al.*, 1991). Alternative exon splicing of a single VEGF gene results in the generation of atleast five different molecular species, having respectively 121, 145, 165, 189 and 206 amino acids, following signal sequence cleavage ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$). $VEGF_{165}$ is the predominant molecular species produced by a variety of normal and transformed cells. Transcripts encoding $VEGF_{121}$ and $VEGF_{189}$ are detected in the majority of cells and tissues expressing the VEGF gene. In contrast, $VEGF_{206}$ is a rare form (Houck *et al.*, 1991). Native VEGF is a basic, heparin-binding, homodimeric glycoprotein of 45 kDa. These properties correspond to those of $VEGF_{165}$, the major isoform. Different isoforms of VEGF proteins have different affinity for heparin and heparin sulfate. Structurally, VEGF-B is closely related to VEGF. VEGF-B has two splice variants: the 167 amino acid form binds to its receptor, while the 186-amino acid form is a freely secreted, soluble product (Makinen *et al.*, 1999). VEGF-C has a central region that is related to other members of the VEGF family (Joukov *et al.*, 1997) that exhibit approximately 30% identity to VEGF and is a fairly selective growth factor for lymphatic vessels (Jeltsch *et al.*, 1997; Oh *et al.*, 1997). VEGF-C is synthesized as a preproprotein, from which a stepwise proteolytic process generates several forms with sequentially increasing binding and activity for its receptors. VEGF-D (also known as c-fos-induced growth factor) is the most recently discovered member of the

mammalian VEGF family. It shares 61% sequence identity with and is proteolytically processed similarity to VEGF-C (Achen *et al.*, 1998). Recently, two polypeptides with significant amino acid sequence similarity to VEGF were identified in the genome of Orf virus, a parapox virus that affects sheep and goats, and occasionally humans, to generate lesions with angiogenesis (Lyttle *et al.*, 1994). The molecules identified – OV-VEGF2 and OV-VEGF 7 share 29 and 23% amino acid identity respectively, with human $VEGF_{121}$ (Wise *et al.*, 1999). PIGF, which was discovered in the human placenta, is around 50% homologous with VEGF. All of the proteins in the VEGF family have a similar molecular structure, but they show distinguishable spectra of function as well as binding specificity towards their shared receptors (Fig 2).

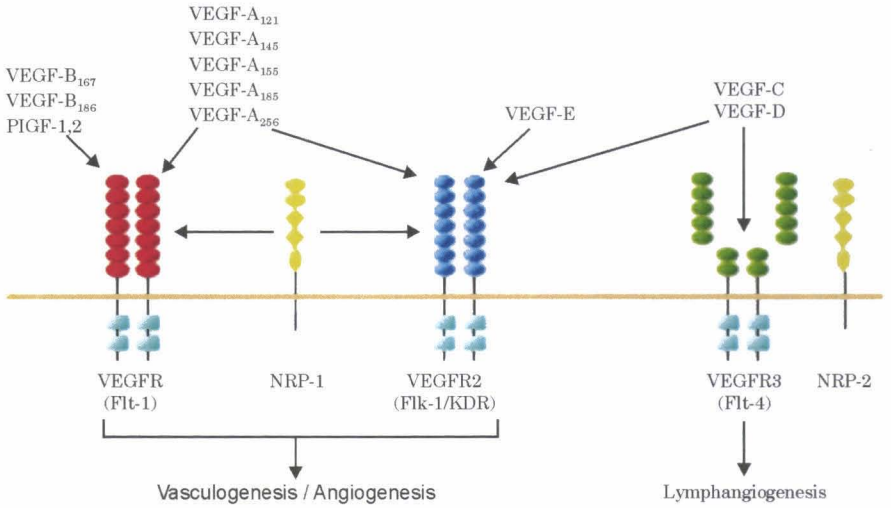

Fig 2. Binding specificity of various vascular endothelial growth factor (VEGF) family members and their receptors (VEGFRs)

Non-classical Roles for VEGF in Tumor Biology-VEGF Signaling to Metastasis

Most cancers are characterized by an ordered progression from premalignant to malignant lesions. The conversion to malignancy can be described at numerous levels. At the molecular level, nuclear translocation and elevated levels of transcription factors such as Twist and Snail are responsible for reprogramming the epithelial cell into a more invasive mesenchyme-like tumor cell (Berx *et al.*, 2007). Concurrent events include the loss of E-cadherin, gain of P, PE, or N-cadherin, increased MT-MMP and MMP production, alterations in integrin expression profiles, and changes in the composition of extracellular matrix molecules. Carcinoma cells undergo a morphological shift that is best illustrated by a switch from apical-basal to

front-back polarity (Radisky *et al.*, 2007). Additional cellular changes include increased lamellipodia, and invadopodia formation, changes in nuclear character, and alterations in cytoskeletal composition and organization. The defining event at the level of the tissue is the invasion into or ablation of the basement membrane that separates epithelial from connective tissue compartments (Sano *et al.*, 2007).

Progression beyond the tissue is influenced by a number of factors including but not limited to the vascularity of the region around the tumor, anatomical site of the tumor, and the location of lymphatics and the direction of their drainage. Increased tumor vascularity is associated with an increased propensity to metastasize to distant sites (Le Tourneau *et al.*, 2008). The location of the original primary tumor often determines what structures are invaded first and the pathway by which the invading cells will be distributed. For example, Head and Neck Squamous Cell Carcinoma (HNSCC) tumors originating in the buccal mucosa typically invade intraorally to regions of the alveolar ridge of the mandible or maxilla, while tumors arising from around the external ear invade the parotid, temporomandibular joint, and the skull base. Like local spread, site of origin significantly determines order of lymph node positivity and spread to adjacent nodes is by lymphatic drainage. Tumors originating from the buccal mucosa tend to spread to the facial and the submandibular nodes, while those from the larynx progress to the jugular chain nodes. Metastasis to distant sites can precede the detection of lymph node involvement, but it is not known whether lymphatics are a required intermediary.

A number of inherent and local "environmental" factors are considered to initiate metastasis, not the least of which are VEGFA and VEGFC. Numerous genetic and epigenetic events are responsible for the "reprogramming" of epithelial cells to more invasive "metastable" cells. Classical carcinogens like tobacco smoke are thought to contribute to the initiation phase of tumor development, but some suggest that other carcinogens like ethanol contribute to the progression to metastatic disease (Sano *et al.*, 2007). More significantly, changes in transcription factors implicated in the process of epithelial to mesenchymal transition (EMT) are thought to be the primary contributors to the change in tumor phenotype (Yanjia *et al.*, 2007). Epithelial to mesenchymal transition is a complex process that involves the reprogramming of epithelial cells into cells with a more mesenchyme like character (Lee *et al.*, 2006). These mesenchymal characteristics imply increased propensity for migration, and invasion. Many initiators have been proposed and include changes in ECM composition, MMP levels, and local gradients of growth factors like TGF, VEGF, EGF, IGF. Each of these initiators is thought to act in concert with one another, and one event is not anticipated to be sufficient for complete EMT. The

process of EMT has primarily been characterized *in vitro*, but histological markers from metastatic tumors like the absence of E-cadherin suggest the relevance of this process *in vivo*. E-cadherin suppression is the current gold-standard of metastatic disease, and is a final outcome of EMT *in vitro*. The process of EMT is thought to be both reversible and irreversible, which has generated controversy within the literature. As a result, some have questioned the relevance of EMT since the complete process has not been observed in metastatic tumors. Those in defense argue that the event is often reversed once a metastatic foci has been reprogrammed by its native environment back into an epithelial cell with heightened tumorigenic activity. Whatever the case, loss of E-cadherin positivity has been significantly correlated with increased refractoriness to treatment, propensity for metastasis, and therefore, poor patient prognosis.

Slomiany *et al.* (2006) have shown that IGF could initiate an IGF-VEGF autocrine/paracrine signaling loop in HNSCC. While VEGF's effects are largely considered to be stromal transformation, some have shown that appears to have actions independent of angiogenesis. Multiple VEGF isoforms appear to be involved in the metastatic process. VEGF-C is of particular interest as it is thought to be the primary cytokine initiating lymphangiogenesis, or the formation of new lymphatic vessels. Lymph nodes are the first site of metastasic foci in many cancers, and therefore VEGF-C dysregulation is thought to be an early event. While VEGFA is well known for its ability to promote angiogenesis, several groups have noted that VEGFA can promote the process of EMT (Yang *et al.*, 2007). Additional studies have shown that VEGFA can promote metastasis through a novel mechanism known as micronodular transformation whereby endothelial cells and tumor cells form a nodular mass that is extravasated into the bloodstream and thus disseminate to distant sites (Kusters *et al.*, 2007). Our studies have sown that VEGF is capable of promoting the formation of specialized cell ventral protrusions for invasions, known as invadopodia. VEGF induces the formation of invadopodia in concert with increased secretion of MMPs and morphological changes that accompany EMT. This implies that VEGFA can both promote the means and the mechanism by which tumor cells reach distant sites.

Invadopodia are thought to be the primary means by which carcinoma cells erode the basement membrane and egress into the stromal compartment (Linder, 2007). The stromal compartment, or dermis in the case of HNSCC, is the gateway to the vaculature and other inflammatory cells which may augment the transformation of the carcinoma cell to a more invasive phenotype. Invadopodia, are primarily composed of actin. Actin dynamics are governed by a complex interaction of Cortactin and the Wasp family members (Gimona, 2008). The polymerized actin pushes specialized membrane microdomains containing invasive machinery like MTMMP's into

the matrix below (Weaver, 2006). Surface transmembrane MMPs degrade the matrix in a polarized fashion, exposing cryptic RGD domains within the matrix proteins. Integrins and possible other transmembrane receptors bind the RGD containing matrix ligands and promote further adhesion and advancement of the invasive cell protrusion (Horenebeck *et al.*, 2003). Stromally secreted VEGF and other growth factors may be chemotactic for invadopodia and may govern their size, rate, and stability. VEGF may also play a role locally by promoting the secretion of MMPs into the local tumor millieu thus facilitating the breakdown of the matrix barrier standing between the tumor cell and vasculature. Taken together, VEGFA may play a prominent role in the promotion of metastasis at multiple levels. VEGFA promotes the transcriptional changes required for transformation into a metastable cell, recruits the vasculature required for dissemination of the metastable cells, and endothelial-tumor cell interactions that are essential for the extravasation of disseminating tumor cells into the bloodstream.

Head and Neck Squamous Cell Carcinoma cells resuspended in heat inactivated serum containing GM6001 were plated onto 4% paraformaldehyde fixed, FITC-gelatin coated coverslips and left to attach in the presence of an general gelatinase inihbitor overnight. Removal of the gelatinase inhibitor was followed by culture in serum containing media. Cells were left to form invadopodia and digest the gelatin beneath for 6-8 h. The cells were harvested by fixing with 4% paraformaldehyde for 10-15 min, permeabilizing with 0.1% triton X–100 and subsequently blocking for immunocytochemistry. The above image is stained for Cortactin (blue), a well-defined component of invadopodia and F-actin with TRITC-Phalloidin (red). Points of digestion are represented by decreases in the FITC-gelatin green fluorescence.

Biological Activities of VEGF

VEGF is a mitogen for vascular endothelial cells derived from arteries, veins, and lymphatics, but it lacks significant mitogenic activity for other cell types (Ferrara & Smith, 1997). Recent studies have indicated that VEGF appears to play a broader role in cancer pathogenesis than previously thought. Overproduction of VEGF may also be related to tumor associated immunosuppression (Ohm *et al.*, 2003). VEGF induces expression of the serine proteases urokinase-type and tissue type plasminogen activators and also plasminogen activator inhibitor 1 in cultured bovine microvascular endothelial cells (Pepper *et al.*, 1991). A major function of VEGF also known as VPF (Vascular Permeability Factor) is the induction of plasma protein leakage. VEGF has been reported to have regulatory effects on blood cells. Broxmeyer *et al.* (1995) have shown that VEGF induces colony formation by mature subsets of granulocyte macrophage progenitor cells. These findings may be explained by the common origin of endothelial cells and

hematopoietic cells and the presence of VEGF receptors in progenitor cells as early as hemangioblasts in blood islands in the yolk sac. VEGF was found to inhibit immature dendritic cells, without having a significant effect on the function of mature cells. These findings led to hypothesis that VEGF facilitate tumor growth also by allowing the tumor to avoid the induction of an immune response. VEGF also has many secondary effects via induction of a number of active substances that have a wide range of actions, including nitric oxide (NO), plasminogen activators, and endothelial cell decay-accelerating factor. These hemodynamic effects, however, are not unique to VEGF since other angiogenic factors such as (a) acidic and basic (b) fibroblast growth factors (FGF) also induce nitric oxide mediated vasodilatation and hypotension (Bouloumie *et al.*, 1999). Although VEGF proteins and blood vessel counts are similar in adenomas and non-metastatic malignancies, their levels are significantly higher in metastatic tumors. Studies have established the role of VEGF in tumor angiogenesis and underscore the importance of identifying the regulatory mechanisms of VEGF expression in tumors. High levels of VEGF are produced by various types of human cancer lines *in vitro* and in surgically resected tumors of the human gastrointestinal tract, ovary, brain and kidney. Elevated serum levels of VEGF have been reported in patients with lung and gynecologic cancers. Clinical significance of VEGF production by tumors has also been suggested by the fact that VEGF producing tumors had significantly lower survival compared with the VEGF negative tumors in squamous cell lung cancer (Volm *et al.*, 1997) and that VEGF expression was independent of prognostic factors for survival. These properties have made the study of the VEGF gene expression relevant and significant for the control of unrestricted growth of tumors.

Regulation of VEGF Expression

Regulation of VEGF expression has been reported to occur at the gene transcription, translation and post translation levels. Transcriptional regulation of VEGF expression has been studied extensively because the impact of most genetic and epigenetic factors on VEGF expression is realized by controlling VEGF gene transcription.

The VEGF gene, which is located on a human chromosome 6 and mouse chromosome 17, is expressed by a wide variety of normal and pathological cell types including tumor cells. Transcriptional regulation of VEGF occurs via both the core promoter and the enhancers or repressors outside of the core promoter. The core promoter of VEGF does not contain typical transcriptional initiation recognition sites such as the TATA box, an initiator element or a TFIIB recognition element. Instead, the VEGF promoter is predicted to be controlled by an Sp1 site that is 50 base pairs upstream of the

transcriptional start site (Loureiro *et al.*, 2005). Basal transcription of VEGF can be enhanced or repressed by the interaction of specific transcription factor with the VEGF promoter, either through or independent of the basal transcription machinery. Transcriptional regulation accounts for much of the up and down regulation of VEGF in tumors. Computer based sequence analysis of the VEGF gene promoter structure has revealed a number of potential binding sites in the 5'-flanking region of the VEGF gene for specific protein-1 (Sp1), hypoxia inducible factor 1 (HIF-1), signal transducer and activator of transcription-3 (Stat 3), activator protein-1 (AP-1), Egr-1, activator protein-2 (AP-2), nuclear factor-IL6, and many others (Lander *et al.*, 1996), indicating the diverse complexity of VEGF transcriptional regulation. Among the many transcription factors, Sp1, HIF-1, Stat 3 and AP-1 appear to be the key factors in the regulation of VEGF expression and have been well characterized. Recently, p73, a new member of the p53 family, has been cloned and mapped to chromosome 1p36, a region that is frequently deleted in a variety of human cancers. p73 can activate p53-responsive promoters and induce apoptosis when overexpressed in certain p53-deficient tumor cells. To evaluate a possible relationship between p73 status and VEGF expression, we have studied the effect of ectopically expressed p73 on the regulation of the VEGF gene. Our results suggest a role for p73 in the inhibition of tumor progression by down-regulation of VEGF expression (Salimath *et al.* 2000).

Hypoxia and Hypoxia Inducible Factor-1

Tissue hypoxia causes a deficiency in the supply of oxygen, and a demand for more oxygen in the tissue. In most malignant tumors, unlimited proliferation induces oxygen deficiency, leading to tissue hypoxia; nevertheless, the tumors continue to grow and invade because of their adaptation to hypoxia through angiogenesis, hyperemia, and glycolysis in the tumor (Semenza, 1998). Hypoxia-inducible factor-1 (HIF-1) is a ubiquitously and constitutively expressed heterodimeric transcription factor composed of a and b subunits. HIF-1 α is involved in the hypoxic response. Under normoxic conditions, HIF-1 α is unstable due to degradation by von-Hippel-Lindau tumor suppressor protein (Maxwell *et al.*, 1999).

In hypoxia, it separates from von- Hippel-Lindau protein and becomes stable and dimerized with HIF-1α, and binds to the hypoxia-response element in the target genes – vascular endothelial growth factor (VEGF), (Iyer *et al.*, 1998) and erythropoietin (EPO), (Beck *et al.*, 1991) and glucose transporters and glycolytic enzymes. These substances are activated to promote angiogenesis, erythropoiesis and glycolysis, respectively. Therefore, malignant tumors are able to survive and proliferate under hypoxic conditions.

VEGF was originally recognized as a vascular permeability factor (VPF) that promoted the influx of serum albumin, leading to ascites in tumor-implanted guinea pigs (Senger *et al.*, 1983). VPF increases the permeability of capillaries, postcapillary venules, and muscular venules by causing fenestration of the endothelium of these vessels. Moreover, VEGF acts as a specific mitogen for endothelial cells, (Keck *et al.*, 1989) induces proliferation of endothelial cells to form vessels and activates chemoattractants for both endothelial cells and nonendothelial cells, monocytes and macrophages. In malignant tumors, VEGF/VPF mRNA is expressed at the boundary of the necrotic area of glioblastoma tumors indicating that hypoxia stimulates the production of VEGF. VEGF has been reported to be expressed in ovarian cancers. It has been shown that inhibition of hypoxia inducible factor-1α (HIF-1α) decreases vascular endothelial growth factor (VEGF) secretion and tumor growth in malignant gliomas (Randy *et al.*, 2006). It is therefore possible that constitutive up-regulation or increased inducibility of HIF-1α may be associated with increased VEGF induction and tumor growth.

Transcription Factor AP-1

Transcription factor AP-1 (activator protein-1) binds to TRE (TPA response element), which is located in the promoter region of many genes responsible for the proliferation and tumor progression of cells. Hypoxia highly induces the binding ability of AP-1 to DNA as well as the transcriptional activation of genes (Hirota *et al.*, 1997). The whole complex of factors (HIF-1, AP-1) contributes to activation and expression of the VEGF gene in hypoxic conditions. However, transcription factor AP-1 is not necessary in the induction of expression of the VEGF gene. The functional role of AP-1 was investigated in hypoxia-induced expression of VEGF by using dexamethasone as an inhibitor of AP-1 activity. Platelet-derived growth factor (PDGF) causes an increase in VEGF mRNA expression, which was strongly suppressed in the presence of dexamethasone, whereas hypoxia-induced VEGF expression was not inhibited by dexamethasone. It was also proved that intracellular calcium (Ca^{2+}) is required for the expression of hypoxia-inducible genes (Salnikow *et al.*, 2002). However, in contrast to hypoxia, the elevation of intracellular Ca^{2+} neither induces the HIF-1α protein nor stimulates HIF-1-dependent transcription. On the contrary, it increases levels of c-Jun protein, causing its phosphorylation. During hypoxia an increase in intracellular Ca^{2+} activates a HIF-1-independent signaling pathway that involves AP-1-dependent transcription, and the cooperation between the HIF-1 and AP-1 pathways allows fine regulation of gene expression during hypoxia (Fig 3).

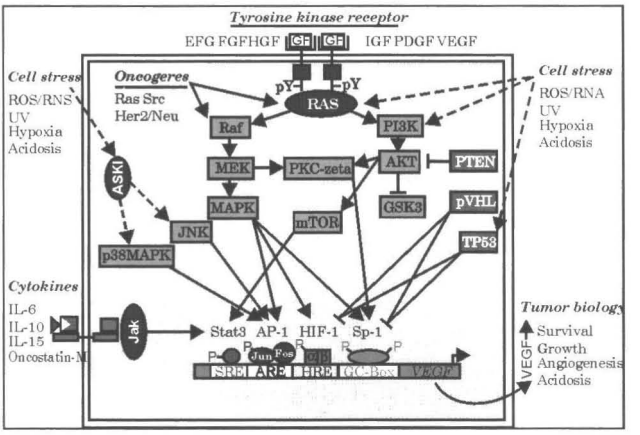

Fig 3. Major signaling-pathways in the regulation of VEGF expression

Transcription Factor Sp1

The transcription factor Sp1 plays a significant role in the constitutive and induced expression of a variety of genes and therefore contributes to the processes of tumorigenesis or the promotion of VEGF gene transcription by interacting directly and specifically with the protein kinase C zeta (PKC zeta) isoform (Pal *et al.*, 1998). The study of human pancreatic cancer cells revealed an elevated steady-state level of VEGF mRNA due to enhanced VEGF gene transcription and increased constitutive VEGF promoter activity that was preceded by the activation of transcription factor Sp1. Therefore it is clear that a constitutive Sp1 activation is essential for the differential overexpression of VEGF, which in turn plays an important role in angiogenesis and the progression of cancer. It was shown that altered expression of transcription factor Sp1 critically impacts the angiogenic phenotype of human gastric cancer. The expression of Sp1 also precedes the increased synthesis of bFGF, PDGF and VEGF during the healing of duodenal ulcers (Szabo *et al.*, 2000). It was shown that Sp1 contributes to accelerated healing without any changes in HCl secretion. In the same way, genetic therapy using the VEGF and angiopoietin genes contributes to a similar healing of gastric ulcers (Jones *et al.*, 2001).

Transcription Factor NF-κB

The transcription factor NF-κB is one of the key regulators of genes involved in the immune/inflammatory response as well as in survival from apoptosis. NF-κB is an inducible transcription factor made up of homo- and heterodimers of p50, p65 (RelA), p52, RelB, and c-rel subunits that interacts with a family of inhibitory κB proteins, of which I-κB is the best characterized. In most cell types, these proteins sequester NF-κB in the cytoplasm by

masking its nuclear localization sequence. Antigen stimulation in T-cells triggers a signaling pathway that results in the phosphorylation, ubiquitination, and subsequent degradation of IκB proteins, resulting in the translocation of NF-κB from the cytoplasm to the nucleus (Karin & Ben-Neriah, 2000). The prototypical, inducible NF-κB complex is a heterodimer containing p50 and p65, and in addition to the control of NF-κB activity exerted at the nuclear translocation level, there is increasing evidence for another complex level of regulation that is mediated by post-translational modifications of both subunits (Garcia-Pineres *et al.*, 2001). It was shown that the NF-κB is also involved in the upregulation of VEGF and its activity in breast cancer cell lines (MDA-MB-231) was associated with the high expression of VEGF mRNA. Advanced glycation end products (AGE), which occur during diabetes, increase the transcriptional activity of NF-κB and therefore upregulate mRNA levels of VEGF. Cervistatin (a hydroxymethylglutaryl CoA reductase inhibitor) completely abolishes this process and might therefore be a promising therapy for patients with proliferative diabetic retinopathy (Okamoto *et al.*, 2002).

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are degradative enzymes that play an important role in all aspects of tumor progression by enhancing tumor-induced angiogenesis and destroying local tissue architecture and basement membranes to allow tumor invasion and metastasis. Efficient breakdown of the ECM surrounding invasive cancer islands involves interplay between tumor cells, stromal cells, and inflammatory cells, all of which express a distinct set of MMPs. Besides the classical role of MMPs in degradation of ECM, MMPs may also indirectly influence the tumor microenvironment through the release of growth factors, cryptic sites or angiogenic factors, or through the generation of matrix fragments that inhibit tumor cell proliferation, migration and angiogenesis, MMP-2 (type IV collagenase/gelatinase A) and MMP-9 (gelatinase B), in particular, are responsible for tumor angiogenesis (Belotti *et al.* 2003).

Inhibition of Angiogenesis

It is now well known that a functioning vascular supply is essential for solid tumor growth and metastasis, and that in the absence of angiogenic growth, tumors are unable to develop beyond a few millimeters and therefore remain dormant. The therapeutic potential of targeting the tumor vascular supply is therefore apparent and rapid developments in this field have resulted in a large number of investigational drugs, many of which are in the clinic or in advanced clinical development. Antiangiogenic drugs are thought to be free of severe side effects that are usually seen with cytotoxic anticancer drugs (Boehm *et al.*, 2003). Besides antiangiogenic therapy, anti neovascular therapy, *i.e.* indirect lethal damage of tumor cells through the damage of

newly formed blood vessels is also promising. Cytotoxic anticancer drugs cause damage to growing neovascular endothelial cells as well as tumor cells. Although tumor cells often acquire drug resistance, neovascular endothelial cells would not be expected to acquire drug resistance. A paramount challenge for antiangiogenic therapy is to design combinational protocols that can counteract the diverse angiogenic stimuli produced by the tumor and its microenvironment. Studies suggest that the addition of antiangiogenic agents to conventional therapeutic strategies *e.g.* chemotherapy, radiation or other tumor targeting agents, will increase clinical efficacy. For relapsed multiple myeloma, the antiangiogenic agent thalidomide has become an important treatment option, and its combination with dexamethasone is now being advocated for newly diagnosed multiple myeloma (Kruse & Joussen, 1998).

Angiogenesis inhibitors have been divided into two classes namely 'direct' and 'indirect'. Direct angiogenesis inhibitors such as endostatin target the microvascular endothelial cells and prevent them from responding to various proangiogenic stimuli. Indirect angiogenesis inhibitors interfere with the proangiogenic communication between the tumor cell and endothelial cell compartments. Antiangiogenic therapy, which targets activated endothelial cells, presents several advantages over therapy directed against tumor cells, as endothelial cells are genetically stable and therefore, less likely to accumulate mutations that would allow them to develop drug resistance.

Agents that Affect Angiogenesis

Insights into the biology of tumor angiogenesis have led to the identification of various molecules that are important for the progression of angiogenesis. Of the many growth factors involved in the angiogenic process, VEGF and FGF-2 are considered the most important mediators of tumor angiogenesis. VEGF is not only crucial for endothelial cell proliferation and blood vessel formation, but also induces significant vascular permeability and plays a key role in endothelial cell survival signaling in newly formed vessels (Ferrara *et al.*, 2003). Several different strategies have been used to inhibit VEGF mediated signals, including anti VEGF antibody, agents that inhibit the VEGF receptor tyrosine kinase, soluble VEGFR-1 that traps VEGF, and so forth. Targeting VEGF or its receptors with monoclonal antibodies (such as Bevacizumab/Avastin) or small molecule inhibitors of VEGFR tyrosine kinase inhibitors (such as ZD6474 and PTK787) has confirmed the anticancer activity of these agents (Sorbera *et al.*, 2002). In recent years, a number of promising new low molecular weight anticancer drugs targeting intracellular pathways or extracellular molecules have been developed such as imatinib, gefitinib, SU-5416, PTK 787, ZK222584, SU6668 and ZD 6474 which are

selective tyrosine kinase inhibitors or non selective multi-target tyrosine kinase inhibitors (Mendel *et al.*, 2000).

Agents that Inhibit MMP Activity

The activity of matrix metalloproteinases (MMPs) is tightly regulated during physiologic tissue remodeling. However, a large body of evidence suggests that this regulation is lost during tumor growth and metastasis. Excessive MMP activity has been detected in colorectal, lung, breast, gastric, cervical, bladder, prostate cancer and malignant glioblastoma. A naturally occurring non-catalytic fragment PEX of MMP-2 was found to prevent binding of the enzyme to the integrin α v β_3 receptor, leading to inhibition of enzymatic activity at the cell surface and block angiogenesis and tumor growth *in vivo*. Marimastat, a broad spectrum MMP inhibitor with nanomolar activity against major enzymatic subtypes (MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9) works via competitive, reversible substrate inhibition and has improved pharmacokinetic properties when compared with its predecessor, Batimastat (BB-94). CGS27023A (MMI270B), a hydroxamic acid derivative showed activity against human xenografts in the nude mouse and inhibited metastatic spread of orthotopically implanted murine tumors (Wood, 1998). A non peptide MMP inhibitor, AG 3340 (Prinomastat) was shown to be active against Lewis Lung tumors in mice, reducing growth of the primary tumor as well as metastatic spread. Bay 129566, a second generation MMP inhibitor was active in preclinical studies, measuring the invasiveness of HT1080 tumor cells in a matrigel invasion assay, and also orthotopic human colon xenografts (HCT 116), where growth inhibition of the primary tumor was seen as well as inhibition of metastatic spread (Flynn, 1998).

Agents that Inhibit Activated Endothelial Cells

Several natural inhibitors of angiogenesis have been detected among which thrombospondin-1 (TSP-1) is considered to be the main physiological inhibitor of angiogenesis, being constitutively produced by normal cells. Its expression is correlated with angiogenesis *i.e.* during tumorigenesis; TSP-1 is downregulated while the angiogenic activity is increased. The most promising tumor shrinking anti angiogenic drugs are derived from an unlikely source: the tumor cells themselves (Yue *et al.*, 1997). Angiostatin and endostatin are examples of endogenous inhibitors that are generated by the proteolysis of inactive circulating precursors. The discovery of angiostatin is based on the observation that some primary tumors, either in a preclinical or clinical setting, appear to inhibit growth of their metastases, removal of the primary tumor then leads to an increase in growth. The specificity of angiostatins for endothelial cells appears attractive, and efficacy studies, using high doses of angiostatin at frequent intervals, have shown good tolerability. On these

grounds, angiostatin has entered phase I clinical trials (mid-2000). Endostatin specifically suppresses endothelial cell proliferation *in vitro* and increases the apoptotic rate in tumors seven fold without affecting the proliferating rate of the tumor cells. *In vivo*, endostatin showed potent inhibitory activity against EOMA, Lewis lung, T241 fibrosarcoma and B16F 10 tumor cell lines. Repeated cycles of systemic endostatin administration in tumor bearing mice caused sustained tumor dormancy in the absence of further treatment (Dhanabal *et al.*, 1999).

Table 1.1. Angiogenesis inhibitors: cellular targets and stage of clinical development

Agent/compound	Target	Mechanism	Clinical trial
Angiostatin	ATP synthatase	Inhibition of endothelial cells	Phase 1
Endostatin	Integrin alpha 5-β 1	Inhibition of endothelial cells	Phase 1 and 2
Vitaxin, humanized	Integrin alpha 5β 3	Inhibition of endothelial cells	Phase 1 and 2
Canstatin	Integrin alpha 5β 3	Inhibition of endothelial cells proliferation and migration	Not yet
Bevacizumab, humanized monoclonal antibody	VEGF	Inhibition of endothelial cell proliferation	Phase 2 and 3
NM-3 isocoumarin	VEGF	Inhibition of endothelial cell proliferation	Phase 1
Gefitinib (ZD 1839), Erlotinib (OSI-774), Cetuximab (C225), CI-1033	VEGF, β FGF, TGF-α	Inhibition of tyrosine kinase activation	Phase 1 or 2
Semaxanib (SU 5416)	VEGFR-1 VEGFR-2	Inhibition of receptor phosphorylation	Withdrawn
SU 668, PTK 787, Zn 4190	VEGFR-1 and/or VEGFR-2	Inhibition of receptor phosphorylation	Phase 2 or 3
Combretastatin	Microtubules	Apoptosis of endothelial cells	Phase 1
2-methoxyestradiol	Microtubules	Apoptosis of endothelial cells	Phase 1 and 2
Thalidomide and analogues	β FGF	Inhibition of endothelial cell proliferation	Phase 1 and 2

NATURAL PRODUCTS IN ANTICANCER THERAPY

The role of natural products as a source for remedies has been recognized since ancient times. With the development of organic chemistry, synthetic products became the mainstream in modern health-care system. Despite major scientific and technological progress in combinatorial chemistry, drugs derived from natural products still make significant contributions to drug discovery today (Farnsworth *et al.*, 1985). An analysis of the number of chemotherapeutic agents and their sources indicates that over 60% of approved drugs are derived from natural compounds. Tremendous chemical diversity makes natural products an attractive source for new therapeutic candidate compounds. Natural products provide greater structural diversity than standard combinatorial chemistry, so they offer major opportunities for finding novel low molecular weight leading structures that are active against a wide range of assay targets. Because less than 10% of the world's biodiversity has been tested for biological activity, many more useful natural leading compounds are awaiting discovery. The chemical novelty associated with natural products is greater than that of any other source: 40% of the chemical scaffolds in a published database of natural products are absent from synthetic chemistry (Harvey, 1999). Additionally, natural products that are biologically active in assays are generally small molecules with drug-like properties. In other words, they are capable of being absorbed and metabolized by the body. Moreover, natural products can be a more economical source of chemical diversity than the synthesis of equivalent numbers of diverse chemicals. The search for novel anti-tumor agents from natural sources continues through the collaboration of scientists worldwide in their search for novel bioactive compounds (Cragg *et al.*, 1997). Experimental agents derived from natural products offer great opportunities to evaluate not only totally new chemical classes of anticancer agents, but also novel and potentially relevant mechanisms of action.

PLANT DERIVED ANTICANCER AGENTS

Plants have been utilized as medicines for thousands of years. These medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations (Samuelsson, 2004). The specific plants to be used and the methods of application for particular ailments were passed down through oral history. Eventually information regarding medicinal plants was recorded in herbals. In more recent history, the use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine from opium in the early 19th century (Kinghorn, 2001). Drug discovery from medicinal plants led to the isolation of early drugs such as cocaine, codeine, digitoxin, and quinine, in addition to morphine, of which some are still in use (Newman *et al.*, 2000). 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. Drug discovery from medicinal plants has evolved to include numerous fields of inquiry and various methods of analysis. The process typically begins with a botanist, ethnobotanist, ethnopharmacologist, or plant ecologist who collects and identifies the plant(s) of interest. Collection may involve species with known biological activity for which active compound(s) have not been isolated (*e.g.* traditionally used herbal remedies) or may involve taxa collected randomly for a large screening program. It is necessary to respect the intellectual property rights of a given country where plant(s) of interest are collected. Phytochemists (natural product chemists) prepare extracts from the plant materials, subject these extracts to biological screening in pharmacologically relevant assays, and commence the process of isolation and characterization of the active compound(s) through bioassay-guided fractionation. Molecular biology has become essential to medicinal plant drug discovery through the determination and implementation of appropriate screening assays directed towards physiologically relevant molecular targets. Pharmacognosy encapsulates all of these fields into a distinct interdisciplinary science. The definition and practice of pharmacognosy have been evolving since the term was first introduced about 200 years ago, as drug use from medicinal plants has progressed from the formulation of crude drugs to the isolation of active compounds in drug discovery. The American Society of Pharmacognosy refers to pharmacognosy as "the study of the physical, chemical, biochemical and biological properties of drugs, drug substances, or potential drugs or drug substances of natural origin as well as the search for new drugs from natural sources". As practiced today, pharmacognosy involves the broad study of natural products from various sources including plants, bacteria, fungi, and marine organisms. Pharmacognosy includes both the study of botanical dietary supplements, including herbal remedies, as well as the search for single compound drug leads that may proceed through further development into Food and Drug Administration (FDA)- approved medicines. Drug discovery from medicinal plants is most frequently associated with the second of these two endeavors. Colleagues in Sweden have suggested a revised definition for pharmacognosy for these types of activities, namely as "a molecular science that explores naturally occurring structure–activity relationships with a drug potential". Numerous methods have been utilized to acquire compounds for drug discovery including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry, and molecular modeling (Ley & Baxendale, 2002). Despite the recent interest in molecular modeling, combinatorial chemistry, and other synthetic chemistry techniques by

pharmaceutical companies and funding organizations, natural products, and particularly medicinal plants, remain an important source of new drugs, new drug leads, and new chemical entities (NCEs). In both 2001 and 2002, approximately one quarter of the best-selling drugs worldwide were natural products or derived from natural products (Butler, 2004). There are also four new medicinal plant-derived drugs that have been recently introduced to the U.S. market.

Natural products discovered from medicinal plants (and derivatives there of) have provided numerous clinically used medicines. Even with all the challenges facing drug discovery from medicinal plants, natural products isolated from medicinal plants can be predicted to remain an essential component in the search for new medicines. Several plant-derived compounds are currently successfully employed in cancer treatment (Table 1.2). One of the most significant examples is the vinca alkaloid family isolated from the periwinkle *Catharanthus roseus*. Another example of a highly active agent derived from a natural product is etoposide, which has produced high cure rates in testicular cancer when used in combination with bleomycin (also derived from a natural product) and cisplatin. Etoposide is a epipodophyllotoxin, derived from the mandrake plant *Podophyllum peltatum* and the wild chervil *Podophyllum emodi*. It has also significant activity against small-cell lung carcinoma (Chabner, 1992). In addition, the camptothecin derivatives irinotecan and topotecan, have shown significant antitumor activity against colorectal and ovarian cancer respectively (Bertino, 1997). These compounds were initially obtained from the bark and wood of Nyssacea *Camptotheca accuminata* and act by inhibiting topoisomerase I.

Table 1.2. Plant derived anticancer agents in clinical trials

Compound	Cancer use	Status
Vincristine	Leukemia, lymphoma, breast, lung, pediatric solid cancers and others	Phase III/IV
Vinblastine	Breast, lymphoma, germ-cell and renal cancer	Phase III/IV
Panclitaxel	Ovary, breast, lung, bladder, and head and neck cancer	Phase III/IV
Docetaxel	Breast and lung cancer	Phase III
Topotecan	Overain, lung and pediatric cancer	Phase II/III
Iriontecan	Colorectal and lung cancer	Phase II/III
Flavopiridol	Experimental	Phase I/II
Acronyciline	Experimental	Preclinical/phase I
Thalicarpin	Experimental	Preclinical/phase I

PLANTS AS A SOURCE OF ANGIOGENESIS-MODULATING COMPOUNDS

During the past decade, major advances have been made in the field of angiogenesis, including the elucidation of the signaling pathways of several angiogenesis factors and the discovery of several natural and synthetic angiogenesis stimulators and inhibitors, leading to the translation of experimental drugs into clinical use. It is clear that plants have the potential to be a rich source of angiogenesis modulators and it is noteworthy that cancer chemotherapeutic strategies commonly require multiple agents. Plants contain many active ingredients. They are complex chemical cocktails with medicinal properties that modern pharmaceuticals cannot reproduce. A wide range of plants contain compounds with angiogenesis modulating properties (Fan *et al.*, 2006).

PURE COMPOUNDS AND EXTRACTS OF PLANTS THAT INHIBIT ENDOTHELIAL CELLS

One strategy for interfering with the process of angiogenesis involves using agents that inhibit the proliferation or response of normal endothelial cells. Targets for this strategy include endothelin, integrins and VEGF receptor complexes. Antiangiogenic studies of medicinal plant extracts and their compounds yielded positive results. Some of the extracts of plants and pure compounds are listed below.

Table 1.3. Plant extracts/compounds that directly inhibit endothelial cells

Pure compound/extract of the plant	Mechanism of action	Reference(s)
Capsicum spp./capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide)	Inhibited vascular endothelial growth factor (VEGF)-induced proliferation, capillary-like tube formation	Min *et al.*, 2004
Cordyceps militaris	Inhibits endothelial cell proliferation	Yoo *et al.*, 2004
Quercetin	Inhibits proliferation, migration, and tube formation in endothelial cells	Tan *et al.*, 2003
Curcuma longa/curcumin	Inhibits endothelial cell proliferation/VEGF and NF-κB signaling pathway	Gururaj *et al.*, 2002; Aggarwal *et al.*, 2005; Choi *et al.*, 2006
Benincasa hispida	Inhibits βFGF-induced endothelial cell proliferation and tube formation	Lee *et al.*, 2006
Atractylodes lancea/	Inhibits VFGF-induced	Tsuneki *et al.*, 2005

Table 1.3. *Contd.*

Table 1.3. *Contd.*

Pure compound/extract of the plant	Mechanism of action	Reference(s)
β-eudesmol	endothelial cell proliferation and tube formation	
Rabdosia rubescens	Inhibits proliferation of endothelial cell, MAP-kinase pathway	Satippour *et al.*, 2005
Morinda citrifolia	Inhibits tube formation in endothelial cells	Hornick *et al.*, 2003
Petasites japonicus/ petasiphenol	Inhibits VFGF-induced endothelial cell proliferation and tube formation	Matsubara *et al.*, 2004
Sinomenium acutum/ sinomenine	Inhibits VFGF-induced endothelial cell proliferation and tube formation	Kok *et al.*, 2005
Urginea undica	Inhibits endothelial proliferation and nuclear translocation of NFκB	Deepak & Salimath, 2005
Glycyrrhiza glabra	Inhibits cell proliferation and VEGF gene expression	Sheela *et al.*, 2006
Curcuma aromatica	Inhibits tumor growth, proliferation and VEGF expression	Thippeswamy & Salimath, 2006
Tinospora cordifolia/ octacosanol	Inhibits endothelial cell proliferation and VEGF gene expression, MMP pathway	Thippeswamy & Salimath, 2008

Table 1.4. Pure compounds and extracts of plants that block activators of angiogenesis

Pure compound/extract of the plant	Mechanism of action	Reference(s)
Gleditsia sinensis	Reduce vascular endothelial growth factor (VEGF) secretion	Chow *et al.*, 2003
Resveratrol	Inhibits hypoxia-inducible factor-1A and VEGF expression MAP kinase pathway	Zhang *et al.*, 2005; Garvin *et al.*, 2006; Brakenhielm *et al.*, 2001
Berberine	Inhibits HIF-1 signalling pathway	Lin *et al.*, 2004
Curcumin	Inhibits VEGF and its recaptor signaling pathway	Gururaj *et al.*, 2002
Nerium oleander/ Olendrin	Suppresses Activation of Nuclear Transcription Factor κB, Activatior Protein-1, and c-Jun NH2-Terminal Kinase	Manna *et al.*, 2000

Table 1.4. *Contd.*

Pure compound/extract of the plant	Mechanism of action	Reference(s)
Benzophoneone and its analogues	Inhibits VEGF via HIF pathway	Prabhakar *et al.*, 2006
Withania somnifera / withaferin A	Inhibits NF-κB singling pathway	Mohan *et al.*, 2004
Pseudolarix kaempferi/ pseudolaric acid B	Inhibits HIF-I signaling pathway	Li *et al.*, 2004
Terminalia catappa/ punicalagin	Inhibits MAP Kinase pathway	Chen & Li, 2006
Piper methysticum/ lactones and halcones/	Inhibitor of NF-κB and MAP kinase pathway	Folmer *et al.*, 2006
Alpinia officinarum/ lesser galangal/	Inhibitor of NF-κB and MAP kinase pathway	Yadave *et al.*, 2003
Dioscoria spp. diosgenin/steroidal saponin,	Inhibitor of NF-κB and MAP kinase pathway	Lager *et al.*, 2006
Butyric acid	Inhibitor of VEGF gene expression	Gururaj *et al.*, 2003

Table 1.5. Pure compounds and extracts of plants that block extracellular matrix

Pure compound/extract of the plant	Mechanism of action	Reference(s)
C. sinensis/epigallocatechin-3-gallate (EGCG)	Inhibited the metalloprotease mediated gelatinolytic activity	Fassina *et al.*, 2004
Grapes and other plants/ resveratrol	Inhibitory effects on MMP-9 protein prodction and MMP-9 mRNA expression	Tang *et al.*, 2003
Camptothecin	Inhibits expression of MMPs	Wang *et al.*, 2003
Hypericum sps./hypericin	Abolish cell migration and adhesion	Lavie *et al.*, 2005
Ecklonia cava	Inhibits matrix metelloproteinases (MMP)	Elkin *et al.*, 1999
Dichroa febrifugal halofuginone	Inhibitor of basement membrane invasion, MMP-2 and MMP-9	Elkin *et al.*, 1999
Momordica cochinchinensis	Inhibits matrix metalloproteinases (MMP)	Tien *et al.*, 2005

ANTICANCER DRUG TARGET IDENTIFICATION AND VALIDATION

The ultimate validation of a drug target rests on the demonstration of a clinically useful therapeutic effect mediated by drug interaction with the target (Fig 4). Thus, pharmacodynamic evaluation of drug effects has become an integral feature of molecular targeted drug development. Gleevec targets the Bcr-Abl kinase uniquely present in chronic myelogenous leukemia and

has been extensively studied from the pharmacodynamic standpoint in preclinical models (Le Coutre *et al.*, 1999). This kinase may currently be viewed as representing the best validated molecular target in the new era of cancer drug discovery and development (Buchdunger *et al.*, 1996). Other targets of potential importance for solid tumor therapy have been extensively studied by the pharmaceutical industry. Ras-mediated cell signaling offers multiple potential targeting points and has yielded drug candidates directed toward farnesylation, geranylgeranylation, and other key pathway points. The Ras-directed farneslyation inhibitors are useful examples of how agents directed at one target may actually exert effects on other targets (farnesylated proteins) (Lebowitz *et al.*, 1995). Thus, a molecular targeting strategy must consider how to focus on the "molecular essence" of a target function to maximize the likelihood that a drug will not have a diverse range of effects. Support for *in vivo* antitumor action at the level of the respective molecular targets is continuing to emerge. Iressa is currently under clinical evaluation as an inhibitor of the epidermal growth factor receptor. Emerging evidence for clinical activity, coupled to target modulation, supports the validity of this target. Evidence of clinical activity of prior therapeutic approaches to this target, notably the development of anti-EGF receptor monoclonal antibodies, further support the validity of this target (Klein *et al.*, 1992). A kinase inhibitor directed towards the vascular endothelial cell growth factor receptor tyrosine kinase has recently been reported as a novel approach to inhibition of tumor angiogenesis. The availability of the human genome, projects such as the Cancer Gene Anatomy Project (Wheeler *et al.*, 2001), and a growing number of computerized approaches to exploitation of this information provides a very large number of potential targets for drug development. In addition to the kinases discussed above, hundreds of additional kinases are coded in the human genome some of which may function as important regulators of signaling pathways potentially important in cancer.

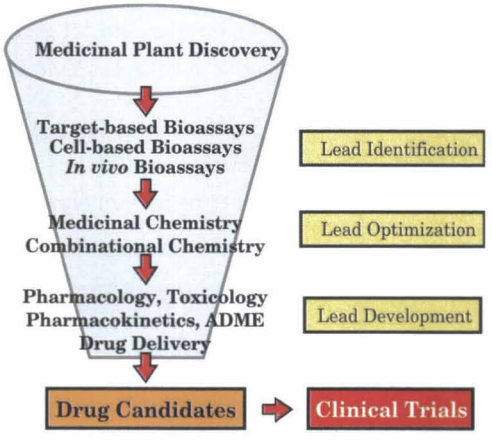

Fig 4. Schematic representation of typical medicinal plant drug discovery and development

In vivo, Cell Based and In vitro Assays for Angiogenesis Inhibitors

Assays that quantify the neovasculature are critical for the development of agents to combat angiogenic diseases like cancer and those that stimulate new vessel growth for the treatment of vascular disease. They are a must to validate data from *in vitro* studies. These assays are based on the principle that new vessels will grow in an area that was previously devoid of blood vessels or has a vascular pattern clearly distinguishable from newly formed capillaries. All the currently available assays have their own unique features and limitations. Some like the chorioallantoic membrane (CAM) assay are suitable for large scale screening of new compounds, while others like the corneal micropocket are technically demanding but have other advantages. *In vivo* angiogenesis assays can be grouped into three main categories: 1. microcirculatory preparations like the rabbit-ear chamber and the CAM assay; 2. vascularisation into biocompatible polymer matrix implants like the sponge models; and 3. excised vascularised animal tissues like tissues from CAM or corneal micropocket assays (Jain, 1997).

Over the last decade, several of the classical angiogenesis assays have been refined and new ones like the hollow fibre-model developed.

Neoangiogenesis is initiated by a vascular sprout arising from a pre-existing vessel. At first basement degradation occurs, followed by the migration and proliferation of endothelial cells that form a solid cord that ultimately becomes canalised and is surrounded by an intact basement membrane. This model of angiogenesis has been demonstrated in various assays like the CAM, corneal micropocket and matrix-implant assays. Both tissue type and stimulus may affect patterns of capillary growth. In a rat mesentery assay, certain stimuli promoted lengthening of existing capillary sprouts, while others promoted new sprouting with more frequent short lateral connections to adjacent capillaries (Hansen-Smith, 2000). More recently, in tissue susceptive microvascular growth has been proposed as an alternative mechanism to sprouting angiogenesis and has been demonstrated in the CAM assay (Patan, 1993; Djonov *et al.*, 2003). A complex capillary network is formed by insertion of a multitude of transcapillary pillars, a process called intussusception. The pillars then increase in diameter and form a capillary mesh. It is important to understand that the initiation of angiogenesis in *in vivo* assay systems is dependent on host growth factors, but this process can be modulated by exogenously added growth factors such as VEGF and βFGF or inhibited by putative antiangiogenic compounds. It is this property of angiogenesis models that enables their use as quantitative screening assays. Some of the commonly used assays are discussed below.

Due to the complex cellular and molecular activities of angiogenic reactions, *in vivo* studies are more informative than *in vitro* studies providing

that the biology of the assay and the experimental design are relevant; *in vitro* studies of ECs are, however, in many instances a necessary complement to *in vivo* experiments. Trauma, either physical or chemical (abnormal osmolarity, altered pO2 tension, changes in pH or toxicity) that leads to cell damage induces an inflammatory reaction. Since several pro-angiogenic cytokines are released during inflammation from tissue-bound and circulating cells, which include platelets, this reduces the sensitivity and specificity of any trauma-based assay. Assays in which the new blood vessels are close to tissue-air interfaces may allow exposure to artificially high concentrations of oxygen, as in the corneal micropocket and the chick CAM assays. It has been proposed that a test material that induces angiogenesis should be designated as being angiogenic only when it is in a non-inflammatory state. Clearly, care should be taken to avoid or reduce to a minimum any inflammatory reaction in the test tissue. The test substance for inducing an angiogenic response should ideally be used at a dose that approximates the physiological dose, whereas the doses used for modulating an angiogenic reaction should ideally be comparable to the range used (or that could be used) in the clinic. In many assays, the best situation is to compare test animals/samples with vehicle-exposed counterparts. However, to allow safe interpretation of the acquired data, one needs to have a good understanding of how the vehicle controls differ from the untreated controls. In this respect, the inability to control the spatial and temporal distributions of test substances *in vivo* has hindered the generation of rigorous and reliable dose-response curves. Since newly formed microvessels are delicate, histological microscopy provides the most detailed information on *in vivo* angiogenesis. Mammalian systems are considered to be more representative of human pathophysiology than, for example, the embryonic avian chorioallantoic membrane (CAM) or embryonic zebrafish assay (Hasan *et al.*, 2004). Considering the heterogeneity of tissues and the molecular and cellular complexities of angiogenic reactions, it is not surprising that a single assay that is optimal for all situations has not yet been described, although ingenious ways have been developed for measuring angiogenic processes. Indeed, many workers with expertise in this area have expressed a certain amount of disillusionment with the available assays. Having used the chick CAM and the rabbit cornea micropocket assays, (Vallee *et al.*, 1985) conclude that "The design and verification of [new] specific, reliable, repeatable and precise methodology to measure angiogenesis is considered an imperative of high priority in the field of angiogenesis research". Auerbach *et al.* (1991) state, "Perhaps the most consistent limitation in all these studies and approaches has been the availability of simple, reliable, reproducible, quantitative assays of the angiogenic response".

The Chick Chorioallantoic Membrane (CAM) Assay

As noted, Folkman and associates introduced this assay in 1974. The CAM assay originally used by embryologists to study the developmental potential of embryonic tissue grafts, the CAM assay has been adapted to study tumour angiogenesis, angiogenic factors and anti-angiogenic compounds (Folkman, 1974; Auerbach, 1974; Ausprunk & Folkman, 1977; Knighton, 1977; Nguyen *et al.*, 1994). Unlike the rabbit-ear chamber preparation, neoangiogenesis in the CAM assay can be assessed over a much shorter time scale making it suitable as a screening assay (Fig 5). The allantois of the chick embryo appears at day-3 of incubation and rapidly enlarges until day-10. The adjacent mesodermal layers of the chorion and the allantois fuse to form the CAM. Within this doublelayered mesoderm a rich capillary network develops. Rapid capillary proliferation continues until day-11, after which the endothelial cell mitotic rate decreases rapidly and the avascular system attains its final set-up on day-18, just before hatching (Ausprunk *et al.*, 1974). CAM angiogenesis undergoes three phases of development involving angiogenesis by sprouting and non-sprouting angiogenesis or intussusceptive microvascular growth (Schlatter, 1997). In an early phase, from day-5 to day-7, the major mechanism of capillary network growth is sprouting. In the intermediate phase, from day-8 to day-12, sprouts are no longer present, replaced by tissue pillars and IMG is predominant with a maximal frequency at day-11. At days 13 and 14, CAM structure undergoes expansion with only a small increase in complexity, the growing pillars increase in size to form intercapillary meshes that are greater than 2.5 mm in diameter. Care must be taken not to misinterpret normal age-dependent alterations of the CAM vascular architecture as specific responses to tested agents. The extracellular matrix of the CAM modifies its composition in terms of expression of fibronectin, laminin, and collagen type IV, and in distribution of specific glycosaminoglycans, favouring the angiogenic process that occurs in the space between the chorionic epithelium and the mesodermal blood vessels. Hyaluronic acid and sulfated GAGs (heparin sulfate, chondroitin sulfate, dermatan sulfate) play a key role in the formation, alignment and migration of the capillary plexus of the CAM and in the differentiation and development of arterial and venous vessels of the chorioallantoic membrane (Ribatti, 1998). Fertilized eggs are incubated at 37°C for 72 h and prepared for grafting by removing enough albumin to minimise adhesion of the shell membrane. Tissue grafts are then placed onto the CAM through a rectangular window made in the eggshell. The test compounds can be prepared as slow-release polymer pellets or air dried on plastic cover slips and implanted onto the CAM (Folkman, 1985). Angiogenesis is quantified three to four days after grafting, a radial (spoke–wheel) arrangement of blood vessels directed towards the graft as a hub or a clear increase of vessels around the graft taken as evidence of neoangiogenesis.

When an angiostatic substance is applied, the vessels become less dense around the implant and eventually disappear. With the goal of finding potent antiangiogenic agents from plants, our group is involved in screening of medicinal plants for antiangiogenic and proapoptotic activity. We have shown that the angiogenic and proliferative effect of the cytokine VEGF is inhibited by the methanolic extract of *G. glabra* (Sheela *et al.* 2006). The antiangiogenic activity of *G. glabra* is well demonstrated as shown by the chick chorioallantoic membrane assay (Fig 5).

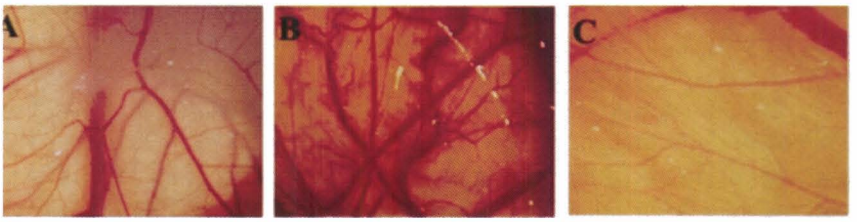

Fig 5. Chorioallantoic membrane assay

A. Control (treated with saline),
B. VEGF induced angiogenesis in CAM,
C. Suppression of VEGF induced angiogenesis by methanolic extract of *G. glabra*.

Curcumin, a major component of turmeric, derived from the rhizome of *Curcuma longa* L., has been shown to display anticarcinogenic properties in animals as indicated by its ability to inhibit both tumor initiation induced by benzyl (α) pyrene and 7, 12-dimethylbenz (α) anthracene (Huang *et al.*, 1992). The intracellular targets of curcumin in inhibiting angiogenesis during growth of ascites tumor and in inducing apoptosis of EAT cells has been demonstrated (Belakavadi *et al.*, 2005). Several methods of quantifying the CAM angiogenesis response have been developed. Quantitation of angiogenesis was initially done by scoring the extent of vascularisation on a graded scale of 0–4. Serial dilution assays were developed to score the number of positives at any particular dilution using four eggs per assay point. With dilution of the test sample and reduction in its concentration, the numbers of positives gradually decrease until an endpoint (0/4) is reached. Use of a numerical grading scale allows calculation of a coefficient of angiogenesis (Form and Auerbach, 1983). Semiautomated image analysis techniques have also been developed (Dusseau *et al.*, 1986). Several variations of the CAM assay have been developed to facilitate the evaluation of potential antiangiogenic compounds. The shell-less CAM assay allows the vascular network to be displayed better. The embryo is transferred to a petri dish at days 3 to 4 of incubation and the CAM develops as a flat membrane, allowing multiple grafts to be analysed concurrently, but suffers from poor embryo viability (Fig 6).

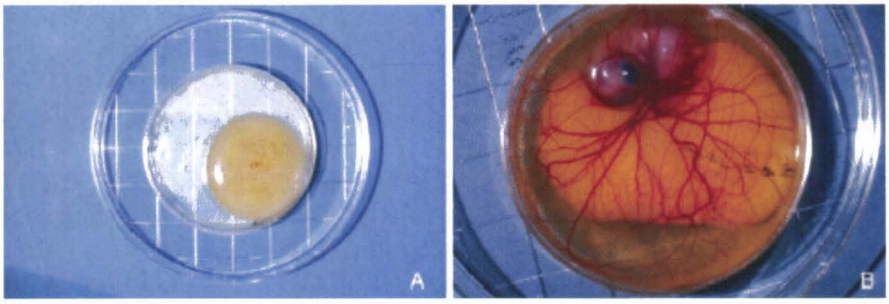

Fig 6. CAM assay

(A) Chick embryo at 72 h of incubation immediately after its placement in a culture dish. (B) Chick embryo after 1 week of additional growth.

Gelatin sponges treated with an angiogenic stimulator or inhibitor can also be implanted on the growing CAM. Blood vessels growing vertically into the sponge and at the boundary between the sponge and the surrounding mesenchyme are counted morphometrically 4 days postimplantation (Ribatti, 1997). Another variant of the CAM assay is based on the vertical growth of new vessels into a collagen gel containing the test sample sandwiched between two parallel nylon meshes. New vessels grow vertically into the gel and the nylon meshes align the capillaries for rapid counting. Quantification of new vessels can be achieved in less than 1 min per embryo. As the CAM can be manipulated outside the eggshell, it is possible to monitor and photograph the angiogenic response on a daily basis. Limitations of the CAM assay include critical timing of grafting and observations, many studies assess neoangiogenesis after 24 h when only vasodilatation is seen, sequential observations are therefore necessary. Real neovascularisation can sometimes be indistinguishable from a falsely increased vascular density due to rearrangement of pre-existing blood vessels that follows contracture of the membrane (Knighton *et al.*, 1991). Non-specific inflammatory reactions due to grafting and reactions induced by carrier materials can induce secondary angiogenesis and confound quantification of the primary response (Rosenbruch, 1989). A non-specific vasoproliferative response is much less likely when the test material is grafted as soon as the CAM begins to develop, while the host's immune system is still immature (Garcia-Pineres *et al.*, 2001). The CAM is a growing and developing structure. It is subject to modification by environmental factors ranging from oxygen tension to pH. Osmolarity and the amount of keratinisation, which has a significant effect on the CAM response to stimulation. This can lead to marked heterogeneity in proximal and distal growth rates of the CAM, size and nature of blood vessels that can affect the reproducibility of the assay system. Drugs that require metabolic activation cannot be assessed in this model and difficulties can arise in discriminating vasodilatation that invariably follows manipulation

of the membrane from neovascularisation. Sometimes an apparent focal 'spoke and wheel' vascular pattern of the CAM may be the effect of local buckling and contraction of the CAM or distortion of the supply vessels due to fibrosis and may not result from a local increase in capillary numbers (Barnhill & Ryanm, 1983). Despite these limitations the CAM assay is widely used and is suitable for the large scale screening of novel compounds. A variety of growth factors such as βFGF, TGF-β and VEGF induce angiogenesis in the CAM assay, although the exact cellular mechanisms have not yet been clearly defined. Angiostatic steroids (Crum *et al.*, 1985), polysaccharides (Tanaka, 1989), vasostatin (Xiao, 2002), and anti-integrin antibodies (Brooks *et al.*, 1994) are some of the putative angiogenesis inhibitors whose activity has been demonstrated in the CAM assay.

Corneal Micropocket Assay

The anterior chamber of the eye is a well-established site for observing the angiogenic response (Gimbrone, 1972; Sholley, 1984). As it is considered immunologically privileged, the anterior chamber has been used as a site for xenogeneic growth of tumours for some time. Of the two-anteriorchamber assays, the corneal micropocket assay and iris implantation assay, the former is the one commonly preferred. The concept that tumours were angiogenesis dependent was established using these systems (Greene, 1943; Gimbrone, 1973). It was shown that tumour spheroids that were freefloating in the anterior chamber remained biologically dormant, while those that came into contact with the iris and became vascularised rapidly grew as a three-dimensional tumour mass. The assay is based on the placement of a pro-angiogenic growth factor or cell suspension into a corneal micropocket in order to evoke a vascular response from the peripherally located limbal vasculature. Micropockets are surgically created in anaesthetised animals at a distance of 2.5–3 mm from the limbus where sustained release pellets incorporating growth factors or cell suspensions can be implanted. Limbal vessels respond to the angiogenic stimulus and infiltrate the corneal stroma. Neoangiogenesis mainly occurs through sprouting. The limbal capillaries form loops and extend into the cornea. Vascular sprouts begin to appear at the apices of the loops and continue their growth towards the implant. Daily observations are performed with a slit lamp stereomicroscope and the degree of angiogenesis, oedema and cellular infiltrate recorded. An angiogenic response is scored positive when budding of vessels from the limbal plexus occurs after 3 to 4 days and capillaries reach the implanted pellet in 7 to 10 days. Implants that fail to produce neovascular growth within 10 days are considered negative and implants showing an inflammatory response are discarded (Ziche *et al.*, 1989). An angiogenic score is calculated by the formula (vessel density) × (distance from limbus) (Ziche, 1994, 1997). The corneas can be removed at predefined endpoints after treatment and assessed

histologically. Microvessel density can be assessed using immunohistochemical techniques with specific endothelial markers. Computerised image analysis systems and high-resolution video technology provide a more objective, non-invasive and continuous assessment of the neovascular response over a period of time (Ziche, 1997). As multiple data points can be generated from the same animal, this technique requires fewer subjects than standard post-mortem quantitation methods. However, this is possible only in rabbit corneal preparations where a slit lamp stereomicroscope can be used to observe neovasculature for periods up to one to two months in unaneasthetised animals. The corneal micropocket assay is considered by many to be the gold standard for the *in vivo* evaluation of angiogenesis. It is reliable and the results easily interpretable. It quantifies neoangiogenesis only as the cornea is initially an avascular structure and virtually eliminates the problem of pre-existing blood vessels, unlike the CAM where the test material is placed on preexisting vessels. Patterns of capillary growth and the development of corneal oedema and inflammation can be assessed in detail (Proia, 1988). The modulation of the angiogenic response by different stimuli can be assessed by the implantation of multiple pellets placed in parallel micropockets in the same cornea. The evolution of the angiogenic response can be studied non-invasively *in vivo* using a slit lamp or stereomicroscope, and at predetermined time points by histological methods. Intravenously administered fluorochrome-labelled high-molecular weight dextran (FITC dextran) is now generally employed for definitive visualisation of corneal neoangiogenesis (Muthukkaruppan & Auerbach, 1979). The assay has been adapted for use in the rodent eye in order to take advantage of inbred strains, transgenic animals and the availability of a number of suitable reagents for these animals (Kenyon, 1996). We have shown that octacosanol, a long-chain aliphatic alcohol from *Tinospora cordifolia* inhibits VEGF-induced angiogenesis *in vivo* by inhibiting VEGF gene expression (Thippeswamy *et al.*, 2008) (Fig 7).

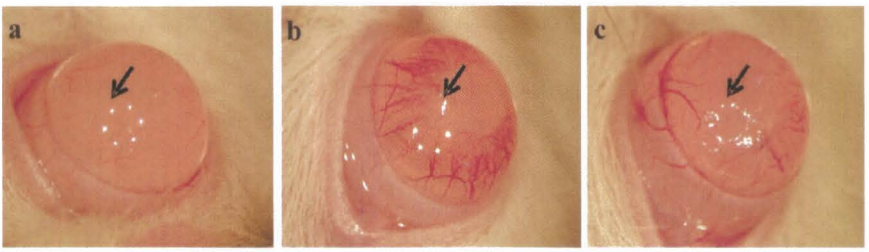

Fig 7. Corneal micropocket assay

(*a*) Control (normal rat cornea)
(*b*) VEGF induced angiogenesis in the rat cornea
(*c*) Inhibition of VEGF induced angiogenesis by octacosanol (*Tinospora cordifolia*)

The low maintenance costs and reduced amounts of space for animal housing are other advantages. The use of nude mice enables the assessment of the angiogenic response in human tumour xenografts implanted subcutaneously. The mouse corneal neovascularisation model was used to detect circulating inhibitors of angiogenesis generated by three human tumours grown in immunodeficient mice (Muthukkaruppan *et al.*, 1982). Rabbits are more amenable to handling and experimentation than rats or mice. However, the assay is less expensive in rodent models and enables smaller quantities of the test agent to be used systemically. The small size of the murine eye makes the procedure even more technically demanding and requires skilled operators to produce reliable results. Since the rat or mouse cornea is thinner than the rabbit's, the three-dimensional growth of vessels is limited in the former (Jain, 1997). The fact that the cornea is an avascular structure makes this assay atypical. Angiogenesis *in vivo* does not normally occur in avascular regions. The corneal pocket itself is exempt from the many blood-borne factors that can influence endothelial cell maintenance and survival. Some of the sustained-release formulations can themselves excite inflammatory reactions that can compromise neoangiogenesis quantification. Also release kinetics within the cornea are different tending to produce an initial burst before release becomes linear (Chen, 1995). When the release periods of 4 different proteins from ethylene vinyl acetate copolymer pellets implanted in the rabbit cornea were studied, it was found that 50% to 60% of the test compounds could be released within the first 1 to 2 weeks. Following that, release rates were more linear over the next 10 to 12 weeks (Auerbach, 2000).

Matrigel Plug Assay

Matrigel, a laminin-rich reconstituted matrix is an extract of the Engelbreth–Holm–Swarm tumour composed of basement membrane components (Langer & Murray, 1983; Kleinman, 1987; Kubota, 1988) The matrigel-plug assay was developed by Passaniti *et al.* (1992). An extract of basement membrane protein was found to reconstitute into a gel when injected subcutaneously into C57/BL mice and to support an intense angiogenic response when supplemented with angiogenic growth factors. New vessels were apparent in the gel after two to three days reaching a maximum after one to three weeks. Matrigel is a liquid at 4°C and forms a gel at 37°C. It is injected subcutaneously alone or in combination with the test agent into the ventral region of mice. The injected matrigel rapidly forms a plug or solid gel trapping the injected compound to allow slow release and prolonged exposure to the surrounding tissues. The matrigel plugs and the surrounding granulation tissue are removed after one to three weeks and angiogenesis quantified by immunohistochemistry on histological sections (Grant, 1993; Prewett, 1999). As the matrigel plug is initially avascular, any vessels found in the plug represent neovascularisation. Assessment of angiogenesis is made by

quantifying the extent of vessel ingrowth into the matrigel plug. Alternatively, total haemoglobin content can be determined by measuring the blood content within the plug. However, this assay cannot differentiate between blood in capillaries and blood in sinuses or larger vessels. As the matrigel is a biological preparation there is some inherent variation between preparations. The matrigel-plug assay provides a more natural microenvironment to assess the angiogenic response, however, it is expensive and a degree of variability in the angiogenic response is seen depending on the characteristics of the animals used and the site of injection. Young mice (6 months old) form less new vessels compared to older ones (12 to 24 months old) and injection on the dorsal surface induces lesser neoangiogenesis compared to ventral injections. It is also difficult to generate identical three-dimensional plugs even though the total matrigel volume is kept constant. The matrigel-plug assay is best used as a rapid screening test for stimulators and inhibitors of angiogenesis. The pro-angiogenic effects of βFGF (Xiao, 2002) and the anti-angiogenic activity of chemotherapeutic agents and VEGF antagonists have been demonstrated in this model (Sweeney, 2001; Hotchkiss, 2002). A modification of the matrigel-plug assay has recently been described which permits more precise visualization of the angiogenic response (Zhang *et al.*, 2002). In this modified sponge/matrigel assay, a sponge or tissue fragment containing the test material is inserted into the matrigel plug and new vessels are quantified after injection of fluorochrome-labelled high molecular weight dextran (FITC dextran). An injection of 200 µl of FITC-dextran is given through the tail vein and the animals sacrificed 3 to 5 min after injection. The matrigel plugs are harvested, fixed in formalin and visualised by phase contrast microscopy. Perfused blood vessels are identified by UV illumination. This assay is, however, more time-consuming than the standard matrigel-plug assay and is limited by the number of animals that can be assayed at a given time (Fig 8).

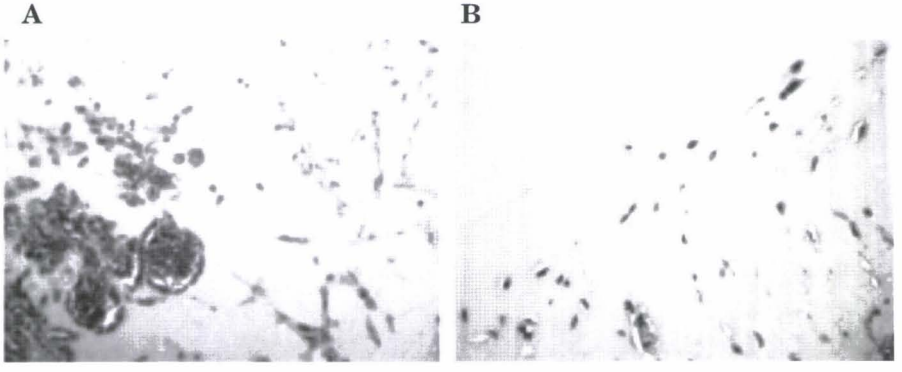

Fig 8. *In vivo* Matrigel plug assay

A. Representative sections from VEGF supplemented Matrigel plugs stained with Masson's trichrome.

B. Effect of test compound on angiogenesis in an Matrigel plug supplemented with VEGF.

Cell-Based Angiogenesis Assays

Cell Proliferation Assay

There are numerous well-established assays for measuring cell proliferation. The most frequently used measure, the thymidine incorporation assay, will serve to introduce several of the key problems of validating *in vitro* angiogenesis assays. The proliferation assay allows determination of the number of cells that are growing in the absence or presence of certain proliferation affecting agents, *e.g.* TNF-α or anti-Fas antibody (IPO-4). A certain number of cells is seeded in the wells of a 12 well plate. The cells are incubated for a certain time (*e.g.* 48 h) at 37°C. Then 3H-thymidine is added to the wells and incubated for another period of time (*e.g.* 48 h). At the same time either a test (proliferation affecting) agent is added or not. Cells incubated without any growth inhibiting agent will grow. During each cell division cells will incorporate 3H-thymidine into their DNA. The more cell divisions (or the higher the proliferation rate) the more radioactivity will be incorporated into DNA. Cells that are incubated in the presence of the growth inhibiting agent (*e.g.* TNF-α) will incorporate less radioactivity. After incubation, the cells are harvested: during harvesting, the cells are washed out of the wells of the 12 well plate with PBS. The cells and organelles are lysed releasing the cell's DNA. The DNA is precipitated using 5–10% TCA and the amount of radioactivity (what corresponds to the number of cells in the well or the number of cell divisions during incubation) is counted in a scintillation counter. To calculate the inhibition of proliferation in presence of a growth inhibitor the counted radioactivity (counts per min=cpm) of cells that were not treated, cpm (untreated), with the cpm in cells that were treated with agent, cpm (treated) is compared. We have shown that the methanol extract of *G. glabra* is antiproliferative to Ehrlich ascites tumor cells (EAT) (Sheela *et al.*, 2006). We have also demonstrated that octacosanol inhibits proliferation of EAT and human umbilical vein endothelial cells in a concentration dependent manner as compared to HEK-293 cells which are untransformed normal cells (Thippeswamy *et al.*, 2008) (Fig 9A, B).

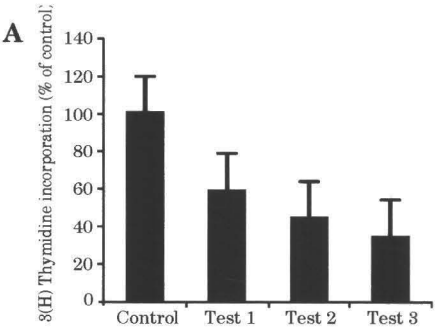

Fig 9A. Antiproliferative effect of different concentrations of *G. glabra* methanol extract on EAT cells monitored by 3[H] thymidine incorporation assay.

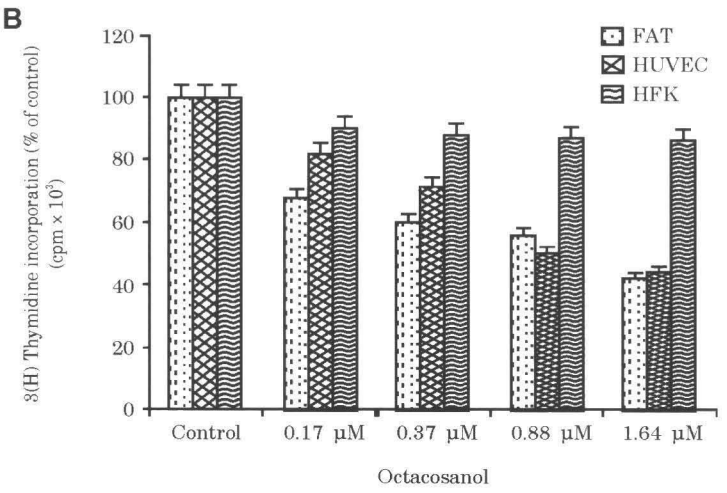

Fig 9B. Octacosanol inhibits VEGF induced proliferation of HUVECs and proliferation of EAT cells

Cell Migration Assay

There are several tests that can be used to determine the migratory response of endothelial cells to angiogenesis inducing or -inhibiting factors (Kolonin *et al.*, 2001). The most frequently used assay is a blind-well chemotaxis chamber [modified Boyden chambers such as those used for classic neutrophil migration (chemotaxis) in which endothelial cells are placed on the upper layer of a cell-permeable filter and permitted to migrate in response to a test factor placed in the medium below the filter. The most accurate measurements require cell enumeration after separation of the retained cells from the cells that have migrated across the filter. The system lends itself to testing concentration gradients and thus may well reflect the conditions that are operative *in vivo*. However, microvascular endothelial cells are more delicate than standard large-vessel endothelial cells or neutrophils, enumeration of traversed cells is tedious, and efforts to adapt the migration chamber for 96-well assays have met with mixed success. A monolayer of 1 micrometre beads is deposited on the bottom of 96-well plates. Endothelial cells (100 cells/well) are then placed in the well, along with test medium. Cell movement is scored after 24 h (phagokinetic track assay), and the assay lends itself to computer-assisted quantification. The assay is equally useful for testing inhibitory factors, such as endostatin or TNP 470, and motility enhancing factors, such as FGF-2 or VEGF, and because of its ease, permits utilization of several different endothelial and non-endothelial cell types to control for specificity of the observed response (Fig 10).

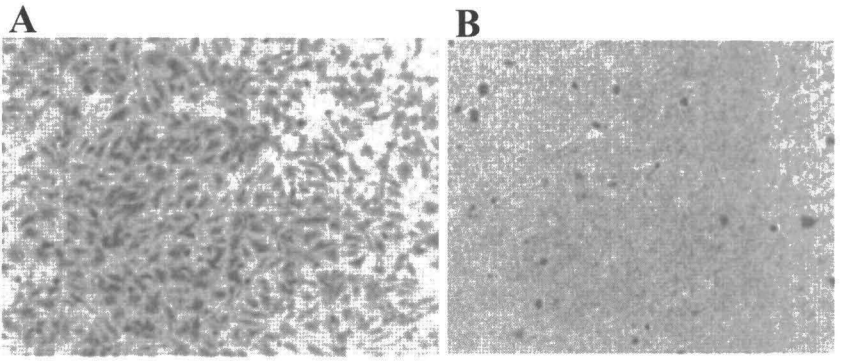

Fig 10. Endothelial cell migration assay

A. VEGF induced migration of HUVECs
B. Inhibition of migration of HUVECs by test compound

Tube Formation Assay

One of the most specific tests for angiogenesis is the measurement of the ability of endothelial cells to form three-dimensional structures (tube formation) (Schor *et al.*, 2001). Endothelial cells of all origins appear able to form tubules spontaneously, given time *in vitro* to lay down appropriate extracellular matrix components. Tube formation can be enhanced by use of collagen or fibrin clots to coat plastic culture dishes. Tube formation on these clots is reasonably faithful to the *in vivo* situation, and the formation of tight junctions can be confirmed by electron microscopy, With the discovery that Matrigel [a matrix-rich product prepared from Engelbreth–Holm–Swarm (EHS) tumor cells whose primary component is laminin (Madri *et al.*, 1988) can evoke endothelial cell tube formation within 24 h, tube formation assays have achieved a prominent place in the array of angiogenesis measures (Grant *et al.*, 1985, 1994). One word of caution, however, is that cultured cells of non-endothelial origin, such as fibroblasts, may also exhibit a response to Matrigel (Grant *et al.*, 1992) and unpublished observations]. It is also critical to control the protein concentration of the Matrigel used, because not all commercial preparations of Matrigel promote tube formation *in vitro*. Tube formation assay of HUVECs was performed as described in earlier report (29). Briefly, a 96-well plate was coated with 50 µl of Matrigel (Becton Dickinson Labware, Bedford, MA), which was allowed to solidify at 37°C for 1 h. HUVECs (5×10^3 cells per well) were seeded on the Matrigel and cultured in EGM medium containing an inhibitor of angiogenesis for 8 h. After incubation at 37°C in 5% CO_2, the enclosed networks of complete tubes from five randomly chosen fields were counted and photographed under an Olympus inverted microscope (CKX40; Olympus, New York, NY) connected to a digital camera at 40x magnification.During the process of investigating

the antiangiogenic effect of *G. glabra* and octacosanol from *T. cordifolia*, we have found that components from both these plants inhibit tube formation on the Matrigel (Fig 11).

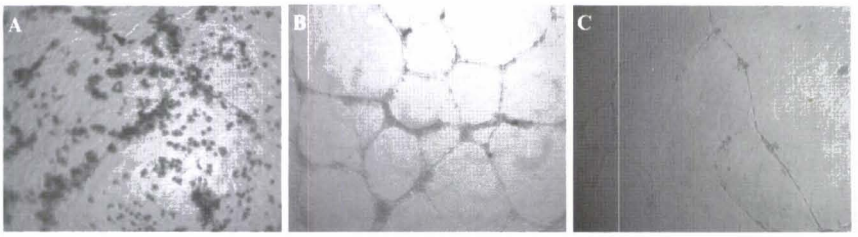

Fig 11. Matrigel tube formation assay of human umbilical vein endothelial cells (HUVECs)

A. Control (Cells with out VEGF)
B. VEGF induced tube formation
C. Inhibition of VEGF induced tube formation by octacosanol

Invadopodia Assay

Carcinoma cells with invasive potential are suspended in heat inactivated serum containing GM6001 (a general gelatinase-inhibitor) and plated onto 4% paraformaldehyde fixed, FITC-gelatin coated coverslips and left to attach overnight. Removal of the gelatinase inhibitor was followed by treating the cells either with an activator or inhibitor(s) of angiogenesis in a serum free media. Cells were left to form invadopodia and digest the gelatin beneath for 6–8 h. The cells were further processed by fixing with 4% paraformaldehyde for 10–15 min, permeabilized with 0.1% triton X-100 and subsequently blocked with bovine serum albumin for immunocytochemistry. The image shown in Fig 12 is immunostained for Cortactin (blue), a well-defined component of invadopodia and F-actin with TRITC-Phalloidin (red). Points of digestion of gelatin indicate active invadopodia formation reflecting the invasive potential of cancer cells and are represented by a decrease in the FITC-gelatin green fluorescence. Inhibition of invadopodia formation by antiangiogenic compounds show either undigested or decreased digestion of gelatin. In Fig 12 is shown a representative image of Head and Neck Squamous Cell forming invadopodia.

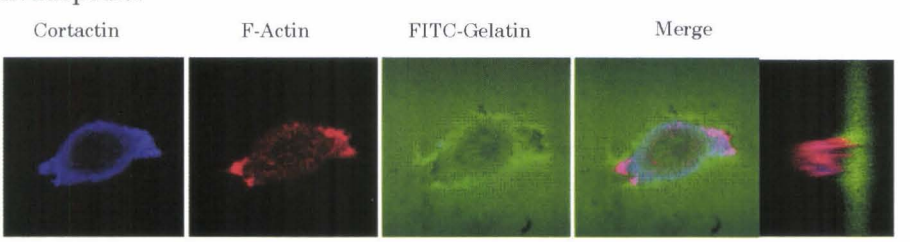

Fig 12. Invadopodia assay: Confocal image of sqamous cell carcinoma cells (FaDu) demonstrating invadopodia formation. Immunocytochemistry showing co-localization of actin, cortactin and gelatinolysis at the site of invadopodia formation

QUANTITATIVE TECHNIQUES

Angiogenesis can be quantified by invasive and noninvasive methods. With excised tissues, techniques employed include measurement of morphometric parameters by light or electron microscopy of vascular casts and histological examination of tissue sections stained with endothelial antibodies or perfused with intravascular markers like colloidal carbon, India ink, radioactively labelled red blood cells and high molecular weight tracers (Hansen-Smith, 2000). The major limitation of histological techniques is that they are invasive and static by design. Neoangiogenesis, on the other hand is a dynamic process that evolves over time. Increasingly non-invasive quantitative techniques like dynamic MR scanning, functional CT and PET scanning are being employed (Obeso *et al.*, 1990). Vascular imaging makes it possible to quantify the number and spacing of blood vessels, measure blood flow and vascular permeability, and analyse cellular and molecular abnormalities in blood vessel walls. These methods allow for continuous and repeated measurements of the angiogenic response. However, a major drawback is the degree of spatial resolution that is significantly lower than that obtained with microscopic methods. Measurement of microvessel density (MVD) in histological sections is still the gold standard for quantitative evaluation of the angiogenic response. MVD gives an estimate of the intercapillary distance and is a well recognized prognostic factor for a number of solid tumours (McDonald & Choyke, 2003). More recently, its utility as a quantitative measure of response to angiogenesis inhibitors has been questioned when tumour mass decreases in parallel with capillary inhibition (Hasan *et al.*, 2002). The appearance of blood vessels in histological sections is greatly influenced by section thickness. Only short segments of vessels are visible in thinner sections, but the vascular network can be seen in thicker sections. Microvessels can be identified by immunostaining of endothelial cells. Two categories of endothelial cell specific antibodies are currently available: the pan-endothelial cell markers and antibodies that bind selectively to activated or proliferating endothelium. The pan-endothelial markers are characterized by equal intensity of staining for small and large vessels and reactivity in both frozen and paraffin-embedded samples. The latter feature is of clinical importance in that it facilitates their use on archival specimens. Generally, anti-CD31 antibody is utilized as the pan-endothelial marker of choice for paraffin sections. Other pan-endothelial markers include vWF, anti- CD34 antibody and thrombomodulin amongst others (Hlatky *et al.*, 2002). The problem of antigen specificity is highlighted by the detection of CD34 antigen on lymphatic vessels, perivascular stromal cells as well as other stromal elements while this is compounded by the absence of FVIII-RA on part of the capillary endothelium in tumour tissue. The disadvantages associated with staining for CD31 antigen include co-staining of inflammatory cells (but these can be distinguished from endothelial cells on the basis of morphological differences), and frequent antigen loss due to fixatives that contain acetic acid (Vermeulen *et al.*, 1996). Microwave antigen retrieval effectively abolishes this problem but in prospective studies a careful selection of the most suitable tissue

fixation procedure should still be performed. The ability to distinguish quantitatively between tumour neovascularisation and pre-existing vessels may be important in the assessment of tumour angiogenesis and could provide more accurate prognostic information. This is now possible using markers for activated endothelium. These markers are suitable for proliferating endothelium giving none or poor staining of lymphatics and normal quiescent blood vessels. They mainly react with fresh or frozen tissues; their activity in paraffin-embedded specimens is fixation dependent. Some of the activation markers currently available include anti-CD105 antibody, LM609, a monoclonal antibody against integrin avb3 and antibodies (3E7, GV39M, 2C3) against VEGF receptors (Brekken *et al.*, 1998; Duff *et al.*, 2003) (Fig 13).

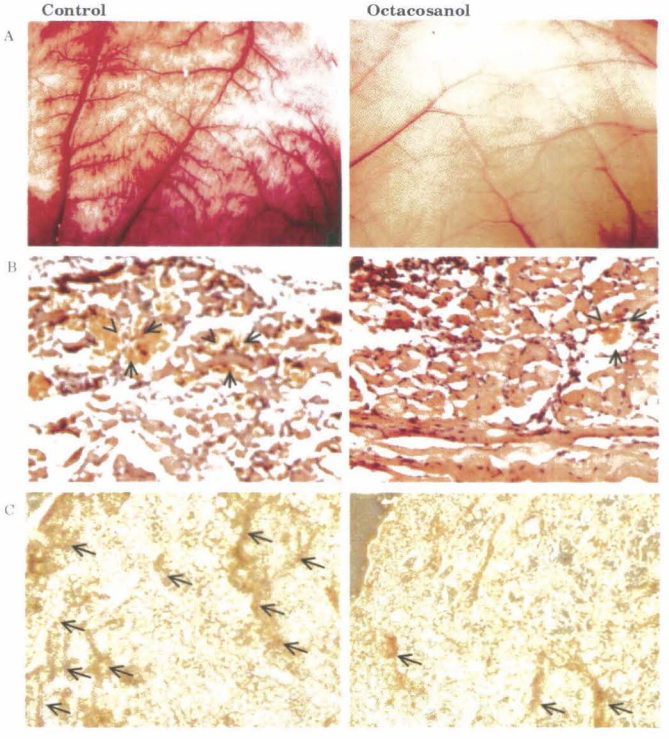

Fig 13. Octacosanol inhibits angiogenesis, microvessel density and activated endothelial cells

A. Inhibition of peritoneal angiogenesis
EAT bearing mice were treated with or without octacosanol for four doses (1.62 µm/dose). The mice were sacrificed and the peritoneal lining was observed for extent of neovascularization. We present representative photograph of peritoneum

B. Reduction in microvessel density (MVD)
The peritoneum of control as well as octacosanol treated EAT bearing mice was embedded in paraffin and 5 µ sections were taken using microtome. The sections were stained with hematoxyline and eosine and observed for microvessel density. Arrows indicate the microvessels

C. Inhibition of activated endothelial cells. Paraffin sections (5 µ) of peritoneum of control and octacosanol were immunostained with anti-CD-31 (PECAM) antibodies. Arrows in dicate the stained activated endothelial cells

VEGF -Promoter-Luciferase-Reporter Analysis

Gene expression is controlled at several steps. Transcription is one of the important steps at which regulation occurs. Tumor suppressors such as p53, which are basically transcription factors, carry out their function primarily by the transcriptional activation of target genes. The promoter region of a gene plays a major role in its transcriptional regulation, so it would be very useful for laboratories involved in the study of the tumor suppressor function to know the techniques used to analyze the regulation of gene promoters, particularly if they encode transcription factors. The most common method of analysis of promoter regulation is the use of promoter–reporter fusions. Typically, one transfects a promoter–reporter fusion construct, wherein a promoter is fused to a reporter gene so that by assaying the reporter gene product one can study the activity of the promoter. The basic requirements are as follows: an easily assayable reporter protein that does not affect any physiologic process inside the cells, an efficient transfection method, and a suitable cell line. Reporter assays could be done both *in vitro* and *in vivo*. *In vitro* assays refer to procedures in which the reporter protein is quantified using the cell lysate or the cultured medium for the secreted reporter proteins. *In vivo* reporter assays are procedures in which the reporter protein is detected in either live or fixed cells or tissues. This method provides information regarding the cell-type specificity of promoters/enhancers as well as the tissue distribution of transcription factors, but it is less quantitative than *in vitro* reporter systems.

Several reporter genes are available for promoter analysis. An ideal reporter should have the following features: the reporter protein should be absent from the host; a simple, rapid, sensitive, and cheap assay method should be available to detect the reporter protein; the assay method should have a broad linear range so that different levels of activation of a promoter can be studied; and the reporter gene expression should not affect the normal physiology of the cells.

B-Galactosidase

The lacZ gene from *Escherichia coli* that encodes β-galactosidase is one of the versatile reporters available. This reporter can be used for both *in vivo* and *in vitro* assay formats with different substrates. The enzyme catalyzes the hydrolysis of various β-galactosides. Apart from its use as a reporter, it is also used as an internal control to normalize the transfection efficiency variability between different samples. Two commonly available assays are the colorimetric assay using *o*-nitrophenyl β-D-galactopyranoside (Mans *et al.*, 2000) and the fluorometric assay using 4-methylumbelliferyl-β-D-galactosidase (Noble, 1990). However, these two methods are less sensitive as compared to the assay using chemiluminescent 1,2-dioxetane, which is 50,000-fold more sensitive (DeVita *et al.*, 1970).

Firefly Luciferase

Luciferase is encoded by the *luc* gene of the firefly *Photonis pyralis* (Williams *et al.*, 1987). It provides a nonisotopic reporter system that is widely used in mammalian cells. Luciferase catalyzes a bioluminescent reaction that requires luciferin (the substrate), coenzyme A, ATP, Mg^{2+} and molecular O2 (Stahelin, 1973). Mixing of luciferin reagent with the cell lysate results in a flash of light, which can be detected using either a luminometer or a liquid scintillation counter. Light intensity is proportional to luciferase concentration in the range 10^{-16}-10^{-8} *M*. The half-life of luciferase enzyme is only 3 h, so it acts as a good candidate for studying inducible systems (Chabner, 1991). Luciferase assay is about 10–1000 times more sensitive and has a broad linear range. The assay system is nonradioactive, relatively inexpensive, and rapid. Minimal endogenous luciferase activity is seen in mammalian cells. Some of the disadvantages are that luciferase lacks "reproducibility" between samples due to rapid emission of light, the half-life of the luciferase in the sample is very short, and the assay requires a luminometer or scintillation counter, which can be a limiting factor.

In order to study the regulation of promoters in the cellular context, it is necessary to have suitable methods to introduce DNA into tissue culture cells. DNA transfection is the process whereby the nucleic acid sequences are introduced into the cells. Biochemical methods of transfection include DEAE-dextran (Miller, 1972), calcium phosphate precipitation (Young *et al.*, 1993), and liposome-mediated transfection methods (Jain & Magrath, 1991). These methods depend on the ability of the formed DNA complex to bind to the negatively charged plasma membrane and subsequently be taken up by endocytosis. Physical transfection methods include direct microinjection and electroporation. While microinjection involves mechanical perforation of the cell membrane, electroporation exposes target cells to brief, defined electrical pulses to create transient pores that allow nucleic acids to cross the cell membrane. The cell type used for the transfection is the major factor, which determines the efficiency of transfection. A suitable method of transfection for a cell type has to be identified by trying different methods Biochemical methods have been used very commonly for the last three decades. The DEAE-dextran method works on the principle that the binding of positively charged DEAE–dextran to the negatively charged phosphate groups of the DNA results in the formation of a complex, which when applied to the cells binds to the negatively charged plasma membrane. Calcium phosphate-mediated transfection is one very commonly used method of transfection. This method requires the formation of an insoluble calcium phosphate–DNA precipitate. The size of these precipitate complexes affects the efficiency of transfection. This method works with wide variety of cell types with varying efficiency. A detailed protocol for the calcium phosphate method of transfection is given below. The liposome method of transfection has become

the most versatile biochemical-based transfection method because of its high efficiency, lower toxicity, and broad host cell range. This method relies on the principle that the positively charged small lipids bind to both phosphate groups of DNA and the negatively charged plasma membrane of the cells. Polycationic lipid reagents yield higher transfection efficiency than monocationic lipids because of the presence of highly positively charged amine head-groups.

For the analysis of gene regulation, the cells transfected with the plasmid containing the promoter gene of interest and a reporter gene are treated with or without the test compound and incubated for 48 h. After incubation, the cell lysates are prepared and assayed for the upregulation or downregulation of the promoter gene activity by the test compound which is reported by the reporter gene. For example, if luciferase reporter gene is coupled to the promoter, it's activity is determined by luciferase assay using a luminometer.

During the course of study of VEGF repression, we have shown that p73 and dietary fiber derived butyrate (Fig 14) down regulates endogenous VEGF gene expression at the mRNA, protein and gene levels. This effect is mediated by transcriptional repression of the VEGF promoter and thus suggests a regulatory role for p73 in tumor angiogenesis (Salimath *et al.*, 2000).

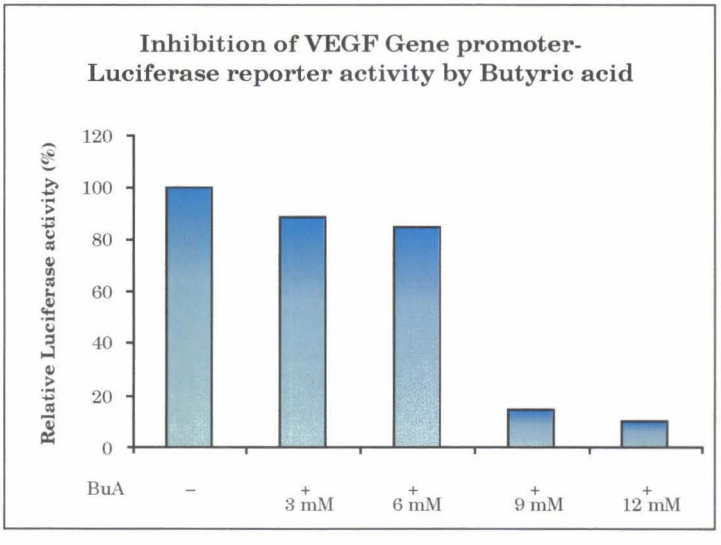

Fig 14. Effect of p73 expression on VEGF promoter activity. Saos-2 cells were transiently transfected with 2 mg of each reporter plasmid, 1 mg of the β-galactosidase expression plasmid pCMV β- gal and 2 mg of the p73 expression plasmid pcDNA3-p73a (+) or 2 mg of the control expression vector pcDNA3 using the calcium phosphate precipitation method. Forty-eight h after transfection, cells were assayed for luciferase activity

Screening of Medicinal Plants

Human use of plants as medicinal agents predates recorded history. Ethno medical plant-use data in many forms has been heavily utilized in the development of formularies and pharmacopoeias, providing a major focus in global health care, as well as contributing substantially to the drug development process. Plant species with a capacity to defend themselves from potential predators and to inhibit other plants competing for space have been selected ('natural selection') (De Wet *et al.*, 1987). In order to survive, plants have developed sophisticated mechanisms including an elaborate chemical arsenal of toxic substances, such as terpenes and alkaloids that inhibit the growth of other plants and make them unattractive to predators. An interesting example is tannin production by certain species of trees. When a predator starts eating a tree in a grove, that tree releases ethylene into the air. These signals to other trees in the grove to increase leaf tannin production, making themselves poisonous and unpalatable to the predator animal. Some plants are also able to produce phenol and tannin when attacked by caterpillars, using a similar signaling process. Several plant-derived compounds are currently successfully employed in cancer treatment. One of the most significant examples is the vinca alkaloid family isolated from the periwinkle *Catharanthus roseus*, which is found in the rain forests of Madagascar (Bronstein *et al.*, 1994). The introduction of the vinca alkaloid vincristine was responsible for an increase in the cure rates for Hodgkin's disease and some forms of leukemia (Thompson *et al.*, 1993). Vincristine inhibits microtubule assembly, inducing tubulin self-association into coiled spiral aggregates. Another example of a highly active agent derived from a natural product is etoposide, which has produced high cure rates in testicular cancer when used in combination with bleomycin (also derived from a natural product) and cisplatin (Vaheri, & Pagano, 1965). Etoposide is a epipodophyllotoxin, derived from the mandrake plant *Podophyllum peltatum* and the wild chervil *Podophyllum emodi* (Graham & van der Eb, 1973). It has also significant activity against small-cell lung carcinoma. Etoposide is a topoisomerase II inhibitor, stabilizing enzyme–DNA cleavable complexes leading to DNA breaks (Felgner *et al.*, 1987). The taxanes paclitaxel and docetaxel, mentioned briefly in the introduction, show impressive antitumor activity against breast, ovarian and other tumor types in the clinic. Paclitaxel stabilizes microtubules, leading to mitotic arrest (Perry, 1992). In addition, the camptothecin derivatives irinotecan and topotecan, have shown significant antitumor activity against colorectal and ovarian cancer respectively (Harvey, 1999; Liu, 1989). These compounds were initially obtained from the bark and wood of Nyssacea *Camptotheca accuminata* and act by inhibiting topoisomerase I (Wani *et al.*, 1971). The taxanes and the camptothecins are presently approved for human use in various countries. Flavopiridol is one of the most exciting plant-based agents currently in development,

representing the first cyclin dependent kinase inhibitor to enter the clinic (Creemers *et al.*, 1996). Flavopiridol is a synthetic flavone derived from the plant alkaloid rohitukine, which was isolated from the leaves and stems of *Amoora rohituka* and later from *Dysoxylum binectariferum* (Maliaceae) (Bertino, 1997; Liu *et al.*, 2000; Kelland, 2000; Harmon *et al.*, 1979). The mechanism of action of flavopiridol involves interfering with the phosphorylation of cyclin-dependent kinases, hampering their activation and blocking cell-cycle progression at growth phase 1 (G1) or G2. In phase I clinical trials with flavopiridol (Cragg & Suffness, 1988; Worland *et al.*, 1973) secretory diarrhoea was found to be the dose-limiting toxicity, and encouraging response rates were noted in a variety of solid and hematological malignancies. These results led to the initiation of phase II trials in patients with colorectal, prostate, renal cell and non-small-cell lung carcinoma, as well non-Hodgkin's lymphoma and chronic lymphocytic leukemia. Based on *in vitro* synergy of flavopiridol with several conventional cytotoxic agents (Losiewicz *et al.*, 1994), phase I combination studies to evaluate flavopiridol with paclitaxel or cisplatin against advanced solid tumors are also ongoing. A number of additional plant-derived agents are currently under investigation. Homoharringtonine, for instance, is an alkaloid isolated from the Chinese tree *Cephalotaxus harringtonia* (Cephalotaxacea) (Senderowicz *et al.*, 1998), and has shown efficacy against various leukemias (Wright *et al.*, 1998). The principal mechanism of action of homoharringtonine is the inhibition of protein synthesis, blocking cell-cycle progression (Kaur *et al.*, 1992), 4-Ipomeanol is a pneumotoxic furan derivative isolated from the sweet potato *Ipomoeca batatas* (Convolvulaceae) (Powell *et al.*, 1970) and has been under clinical evaluation as a lung-cancer- specific antineoplastic agent (Kantarjian *et al.*, 1996). This compound is converted into DNA-binding metabolites upon metabolic activation by cytochrome P450 enzymes that are present in cells of the lung. Finally, β-lapachone is a DNA topoisomerase I inhibitor that induces cell-cycle delay at G1 or S (synthesis) phase before inducing either apoptotic or necrotic cell death in a variety of human carcinoma cells, including ovary, colon, lung, prostate and breast (Zhou *et al.*, 1995).

APPROACHES FOR THE DISCOVERY OF NOVEL PLANT-DERIVED ANTICANCER AGENTS

Various methods may be employed for the discovery of naturally occurring drugs. For example, phytochemical screening or exploitation of chemotaxonomic relationships may be of some practical benefit. In general terms, however, this approach is not directed towards the discovery of new, therapeutically useful substances. Of course serendipity may come into play, and straightforward isolation procedures could lead to the discovery of extremely valuable therapeutic agents. Nonetheless, for most

drug discovery programs it is not acceptable to await the occurrence of a serendipitous series of events that lead to success. In dealing with terrestrial plants, conservative estimates suggest the existence of 250,000 species. With the advent of high throughput screening, it is quite feasible that samples derived from the entire flora covering the face of the earth could be evaluated for potential to mediate specific biologic responses, should they be available. However, the specimens are not available *in toto,* and a formidable task relates to the selection of starting materials. A number of approaches have been devised for the selection process. First, it is comforting when plant materials are reputed to mediate a certain therapeutic effect, in a traditional medicine sense, and scores of manuscripts are currently available describing such ethnomedical usage. Similarly, epidemiological data may come into play that are suggestive of therapeutic efficacy in human populations.

By and large, however, plant materials are procured based on geographical location and various logistical factors often come into play. One key requirement often mandated is that the plant materials are endemic to the site of collection. Once materials are procured, it becomes of the utmost importance to rapidly identify promising leads and to obtain the active chemical principles affiliated with these substances. In this endeavor, recently, a technique in which HPLC/MS is coupled to bioassay procedures was employed (Rehm & Devor, 1993; Rowinsky *et al.*, 1993; Li *et al.*, 1999). Using this approach, it is feasible that active principles contained in crude plant samples can be identified in a very short period of time. Of course, this procedure does not permit identification of structurally novel compounds. Although this aspect is sometimes criticized, the identification of known compounds capable of mediating hitherto unknown biological responses is, in fact, very important. New uses of known compounds is as important as discovering novel compounds with the same activity as the known compound, both in terms of actual benefit to humanity and exclusivity (*i.e.* patent protection). Also, known compounds capable of mediating new activities can enhance our understanding of factors such as molecular recognition sites and structure-activity relationships. Further, such agents could play a role in rationally designed combination chemotherapy regimens, once mechanisms are elucidated. In essence, therefore, novel drug discovery is a biology-driven process.

For situations wherein the biologically active constituent of a plant extract is structurally unique, the approach generally regarded as most practical for drug discovery is referred to as bioassay-directed fractionation.

As illustrated in Fig 15, this entails evaluating plant extracts in a bioassay system, and substances demonstrating a positive response are considered as active leads. After a number of active leads are identified,

decisions are made to fractionate the most promising materials. Each fraction is monitored for potential to mediate a positive response in the bioassay test system, and this process continues until a pure active substance is obtained.

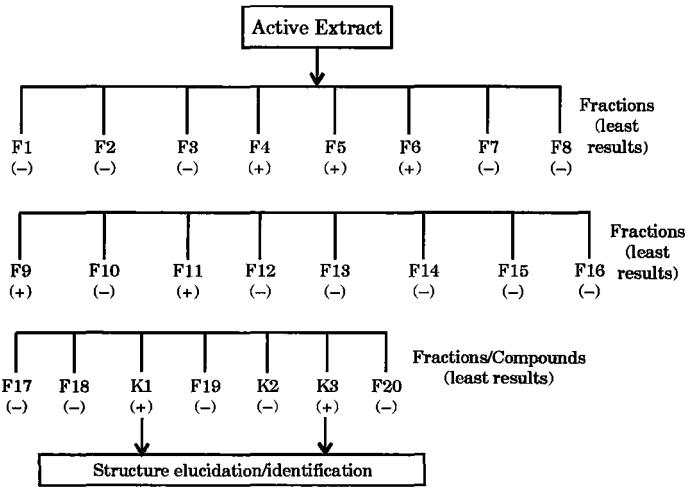

Fig 15. Bioassay-directed fractionation Chromatographic separation of biologically active plant extracts (Active Extract). Each resulting fraction (designated F) is tested in a bioassay system, with the intent of establishing a positive (+) or negative (-) response. Only active fractions are subfractionated, with the objective of ultimately obtaining pure, biologically active compounds (designated K). The active principles are subjected to procedures of structure elucidation/identification

The resulting substance is then subjected to procedures of structure elucidation using the data on HPLC, IR, UV, ^{13}C, ^{1}H NMR, 2D NMR, LC/GC-MS spectra of that compound (Fig 16). Once an active isolate is obtained, more thorough biological evaluation procedures are often performed and, based on the accumulated data, the material is considered as a candidate for more advanced testing and development. A question of paramount importance relates to the bioassay test system. In the area of antitumor drug discovery, a large number of *in vitro* test systems, and the issues that need to be considered when attempting to interrelate *in vitro* test results with *in vivo* efficacy studies, have been described. In general, recent advances in molecular biology have led to a greater understanding of the molecular basis of human disease states. As a correlate, *in vitro* systems that monitor a response that is either closely related to or identical with the molecular event yielding the disease condition can be devised.

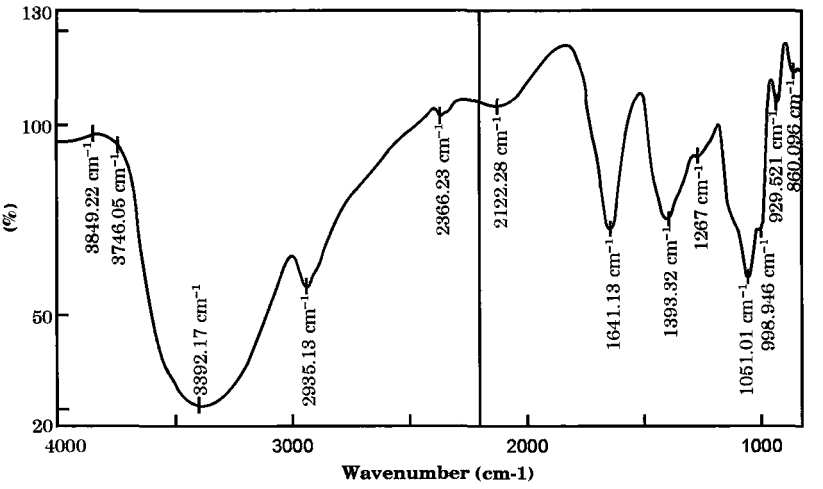

Fig 16. HPLC profile and IR spectra of octacosanol from *T. cordifolia*

CONCLUSIONS

Natural product drug discovery should remain one of the focal points of discovery. As described in this review, natural products continue to represent a rich and largely untapped resource for the discovery of drugs with potential application for the treatment of contemporary diseases that afflict humans. It is solely dependent on our ingenuity to devise and implement cost-effective methods of natural product drug discovery that do not adversely affect the environment. Irrespective of how drugs are discovered, however, one of the great mysteries of our time is why the discovery of drugs useful for the treatment or prevention of human cancer should be limited by fiscal restraints. Another great mystery is the lack of full implementation of cancer prevention strategies, particularly in the area of therapeutic intervention, *i.e.* chemoprevention. As inscribed by Benjamin Franklin in Poor Richard for 1735, "An ounce of prevention is worth a pound of cure". This is well-stated and particularly apropos to the situation of cancer. Perhaps argumentatively, the following may be presented as facts: (a) To an appreciable extent, cancer is a preventable disease, (b) efficacious drugs can be given to humans to augment the prevention of cancer, and (c) this drug-based approach of cancer chemoprevention is not being utilized on a widespread basis. To implement a straightforward concept of "Healthy people through disease prevention," a proposal follows: (a) Assemble "Expert Panels" to design the "best" cancer chemoprevention formulation that is currently

available, (b) study the cost-effectiveness aspect of the proposed cancer chemoprevention formulation and make adjustments as required, and (c) provide the final formulation for wide-scale human consumption. It may be noted that managed health care is a perfect forum for implementing this plan. It is fair to suggest that health care plans should take active and leading roles in the promotion of human disease prevention, including the supply, distribution, or payment of drugs that prevent disease. It is realized that this plan is subject to scrutiny. We can see already the controversy caused by certain clinical trials conducted with cancer chemopreventive agents. As one example, a trial involving β-carotene administration to smokers was terminated recently because no effect was being observed, and there was a "trend" towards a greater incidence of cancer among the group taking the drug (Constant & Beecher, 1995; Constant, 1996; Ziegler *et al.*, 1996). As clinical trials are completed with experimental agents, for example, the panel should reconvene, re-evaluate the verdict (formulation), and make adjustments as necessary. In essence, we can elect to do nothing, or we can do our very best, and we can do it now. It is not necessary to wait for some undefined time in the future. Action can be taken now for the benefit of the general population. The initial formulation may be somewhat "lame," unfortunately, due to gaps in our knowledge and the necessity of remaining conservative. In due course, however, the formulation will undoubtedly improve, in part, simply due to its existence. This may be viewed as a general characteristic of all product development and, more broadly, as a function of human nature. Without a start, there will be no development, and without any development, humanity will not benefit from important knowledge and technology that are currently available. Cancer, estimated to affect one in three during the course of their life, may be considered as an epidemic. It seems quite obvious that now is the best time for large-scale implementation of cancer chemoprevention as a therapeutic strategy.

ACKNOWLEDGEMENTS

The authors thank the Department of Biotechnology (BPS, Overseas DBT-Associateship), Department of Science and Technology (SML) and Department of Atomic Energy, BRNS (BPS), for financial support

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Effects of Hydroalcoholic Extracts of Four Chinese *Hypericum* species on Learning and Memory in Mice

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ABSTRACT

The learning and memory improvement effects of the hydroalcoholic extract of four Chinese Hypericum species, Hypericum perforatum L. (HF), Hypericum erectum subsp. longisepalum (HE), H. hubeiense L.H. Wu et D.P. Yang (HH) and H. seniavinii Maxim (HS), were investigated using the step-down and step-through passive avoidance tasks in mice. The results showed that the impairments of memory acquisition, consolidation and retrieval process were reversed to a different degree after administrating HH, HE and HF at 50, 100 mg/kg orally once a day for seven consecutive days. HS was shown to facilitate the memory acquisition and retrieval, but had little effect on the memory consolidation. These results suggest that the hydroalcoholic extracts of these Hypericum species possess potential action of learning and memory improvement, which was similar to or even better than the positive control drug piracetam.

Key words : *Hypericum*, hypericin, learning, memory

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INTRODUCTION

A number of studies have been reported that learning and memory impairment usually occurred in depressed patients (Brown *et al.*, 1999; Song *et al.*, 2002). Since most of the antidepressant drugs such as amitryptiline and maprotiline used in clinical practice have side effects of memory impairment (Everss *et al.*, 1999; Parra *et al.*, 2000), a good antidepressant drug should have no impact on cognitive capacity, and preferably possess the effect of learning and memory improvement.

Several species of *Hypericum* genus, particularly *H. perforatum* (St. John's wort), have been popularly used for the treatment of mild to moderate depression and other variety of neurological conditions (Hahn, 1992). Kumar *et al.* (2000) reported that 50% ethanolic extract of *H. perforatum* significantly attenuated the impaired retention of active avoidance induced by scopolamine and sodium nitrite. Therefore, the result indicated that *H. perforatum* would likely be clinically effective in memory dysfunction.

There are more than 60 species of *Hypericum* genus in China, and recently six new species of *Hypericum* genus had been reported by our laboratory (Wu *et al.*, 2002, 2004). In this paper, we chose four species or subspecies of *Hypericum* genus to evaluate their action on learning and memory improvement. These four Hypericum plants include two new species found by our lab, *H. erectum* Thunb. ex Murray subsp. *longisepalum* L.H. (HE) and *H. hubeiense* L.H. Wu et D.P. Yang (HH) (Wu *et al.*, 2002); and *Hypericum perforatum* L. (HF), and *H. seniavinii* Maxim (HS). Passive avoidance has been widely used for studying the memory formation and was used in current study to investigate the effects of these four *Hypericum* plants on learning and memory improvement in the behavior paradigms.

MATERIALS AND METHODS

Drug Treatments

The four *Hypericum* plants, HF, HE, HH and HS, were collected in Badong (Hubei province, China) in August 2002. The ethanolic extracts (90% v/v) of the dried aerial parts (leaves, flowers and stem) of these four plants were suspended in 0.5% carboxymethyl cellulose aqueous solution (0.5% CMC), respectively and were orally administrated once a day for 7 consecutive days in doses of 100 and 50 mg/kg. Piracetam (NEPG, China) (500 mg/kg) was used as the standard nootropic agent and administrated in the above protocol to one group of mice. Administration of Vehicle (0.5% CMC) was treated as the control group.

Scopolamine hydrochloride (Scop. Sigma, USA) and sodium nitrite ($NaNO_2$, Shenyang, China) were dissolved in injected water. Scopolamine was intraperitoneally administrated 10 min before training; $NaNO_2$ was subcutaneously administered immediately after the training, and ethanol (EtOH, 30% in saline, v/v) was administered orally (10 mL/kg) 10 min before individual experimental test.

Animals

Male Kunming mice (18–22 g, Medical Animal Center, Sun Yat-sen University, Guangzhou, China) were used and divided randomly into different experimental groups. The mice were fed with the standard laboratory food and tap water, and were housed in the group of 5 in polypropylene cages at ambient temperature of 25 ± 1°C and with a 12 h light/dark cycle. Experiments were conducted between 9:00-15:00 o'clock, and each animal was tested only for one time. All experiments were conducted according to the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No 80–23, revised 1996). The experimental procedures were approved by the local Committee on Animal Care and Use.

One-way Step-down Passive Avoidance Task

Mice were trained in an apparatus previously placed on a rubber platform (4.3 cm *i.d.*) in a lighted box (12 × 10 × 30 cm) with a grid floor through which an electric shock of 3 s, 0.5 mA was delivered. When the mice stepped down the platform, a constant and continuous electric shock was applied. The normal reaction of the mice was to jump back onto the platform. In the first day, the mice were placed in the box for 3 min and then trained for 5 min. After 24 h each mouse was placed on the platform once again and the step-down latency and the number of errors (stepped down the platform) in 5 min were recorded (Masayuki *et al.*, 1999).

One-way Step-through Passive Avoidance Task

A one-trial step-through passive avoidance task was performed by using the light-dark apparatus consisted of two compartments, an illuminated box and a dark box separated by a guillotine door. The size of both boxes was 20 × 10 × 15 cm. During the training, the mouse was placed in the illuminated compartment and was allowed to enter the dark compartment through the door. As soon as the mouse entered the dark compartment, a scrambled foot shock (3 s, 0.5 mA) was delivered through the grid floor. The mouse could escape from the shock only by stepping back into the safe illuminated compartment. Twenty-four hours after the training, the mouse was again placed in the safe illuminated compartment. The response latency to enter the dark compartment and the number of errors (enter the dark compartment) in 5 min were measured. The latency of not entering the dark room during the 5-min observation period was regarded as 300 s (Li *et al.*, 1999).

Statistical Analysis

The one-way analysis of variance (ANOVA) test followed by Duncan's post-hoc multiple group comparisons by SPSS 10.0 software was used to analyze group differences of the data collected during testing. Data are expressed as means ± S.D. The level of statistical significance was set at $p<0.05$.

RESULTS

Locomotor Activity

All of the four extracts, administrated once a day for 7 consecutive days at doses of 50 and 100 mg/kg, showed no significant effect on locomotor activities compared with the control group (data were not shown).

One-way Step-down Passive Avoidance Task in Mice

The effects of the four extracts on memory acquisition, consolidation and retrieval were shown in Figs 1, 2 and 3 respectively. Scopolamine significantly increased the number of errors and reduced the latency in the task (Fig 1). The extracts of HF, HH (100, 50 mg/kg) and HS (100 mg/kg) significantly reduced the number of errors and increased the latency on impairment of memory acquisition induced by scopolamine. HE at 100 mg/kg only significantly reduced the number of errors but did not increase the latency on impairment of memory acquisition induced by scopolamine.

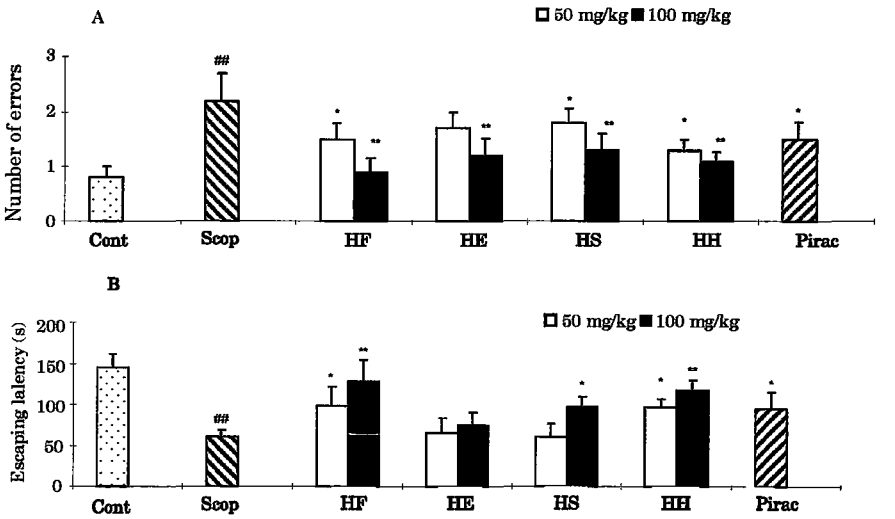


Fig 1. Effects of the hydroalcoholic extracts of four *Hypericum* species on impairment of memory acquisition induced by scopolamine in step-down task in mice (n=10, mean ± S.D.) ## $p<0.01$ vs Cont (Cont=Control); *$p<0.05$, **$p<0.01$ vs Scop (Scop=scopolamine; Pirac=piracetam)

Sodium nitrite could significantly increase the number of errors and reduced the latency in comparison to the control group. The number of errors in the HF, HH and HE treated groups (100 mg/kg, *p.o.*) were significantly reduced when compared with that of sodium nitrite treated group, and were even lower in error numbers or higher in latency than those in the control group (Fig 2). However, the effects were not shown in HS treated groups at both doses.

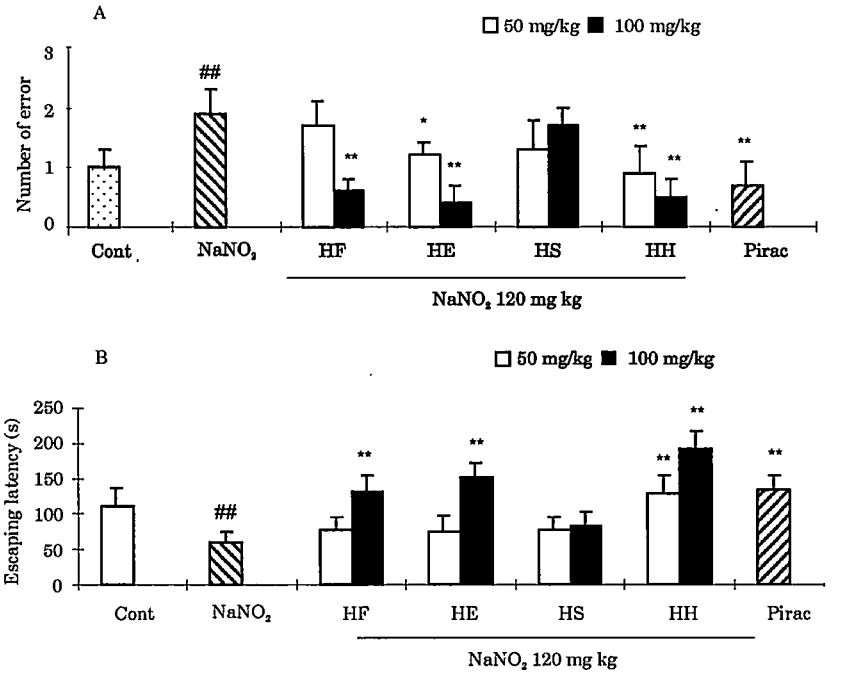
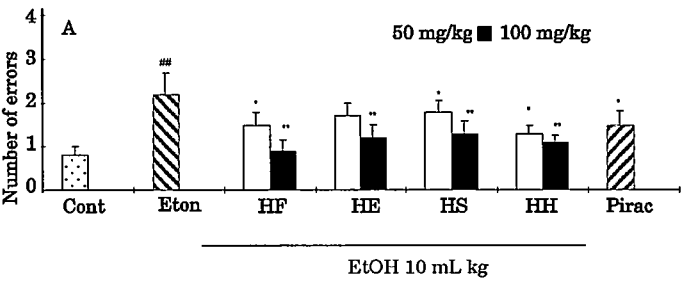


Fig2. Effects of the hydroalcoholic extracts of four *Hypericum* species on impairment of memory consolidation induced by $NaNO_2$ in step-down task in mice (n=10, mean ± S.D.) ## p<0.05 vs Cont (Cont=Control); *p<0.05, **p<0.01 vs $NaNO_2$ (Pirac = piracetam)

All the four extracts at both doses significantly reduced the number of errors induced by 30% ethanol, and significantly increased the latencies in the task (Fig 3).



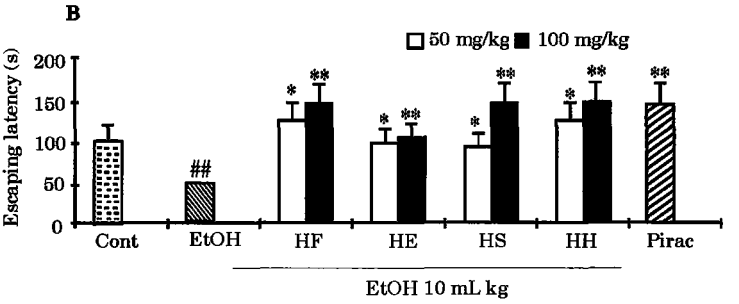


Fig 3. Effects of the hydroalcoholic extracts of four *Hypericum* species on impairment of memory retrieval induced by 30% ethanol in step-down task in mice (n=10, mean ± S.D.) ##p<0.01 vs Cont (Cont=Control); *p<0.05, **p<0.01 vs 30% ethanol, (Pirac=piracetam)

One-way Step-through Passive Avoidance Task in Mice

The results in step-through task (Figs 4, 5 & 6) were similar to those in step-down task in mice. In the trial of memory acquisition, the number of errors were significantly reduced and the latencies were significantly increased in all of these four *Hypericum* extracts treated groups (Fig 4).

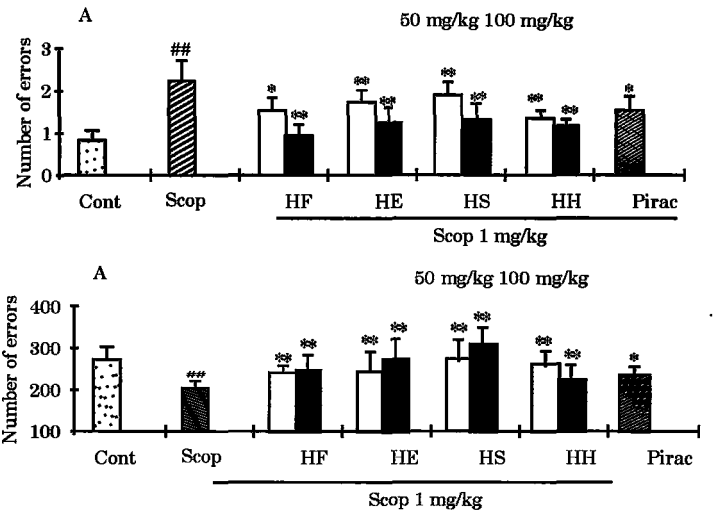


Fig 4. Effects of the hydroalcoholic extracts of four *Hypericum* species on impairment of memory consolidation induced by Scopolamine in step-through tasks in mice (n=10, mean ± S.D.)[##] p<0.01 vs Cont (Cont=Control); *p<0.05, **p<0.01 vs scop (Scop=scopolamine; Pirac=piracetam)

All the four extracts except HS at 50 mg/kg significantly reduced the number of errors induced by sodium nitrite but only HE and HH at 100 mg/kg increased the latency, a trend similar to the step-down task (Fig 5).

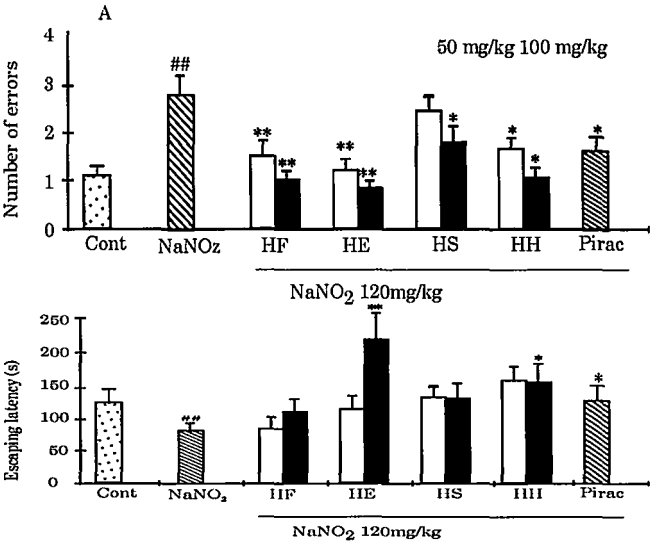


Fig 5. Effects of the hydroalcoholic extracts of four *Hypericum* species on impairment of memory consolidation in step-through tasks in mice induced by $NaNO_2$ (n=10, mean ± S.D.) ##p<0.01 vs Cont (Cont = Control); *p<0.05, **p<0.01 vs $NaNO_2$, (Pirac = piracetam)

All the four extracts at both doses had significant effects on the memory retrieval improvement (Fig 6).

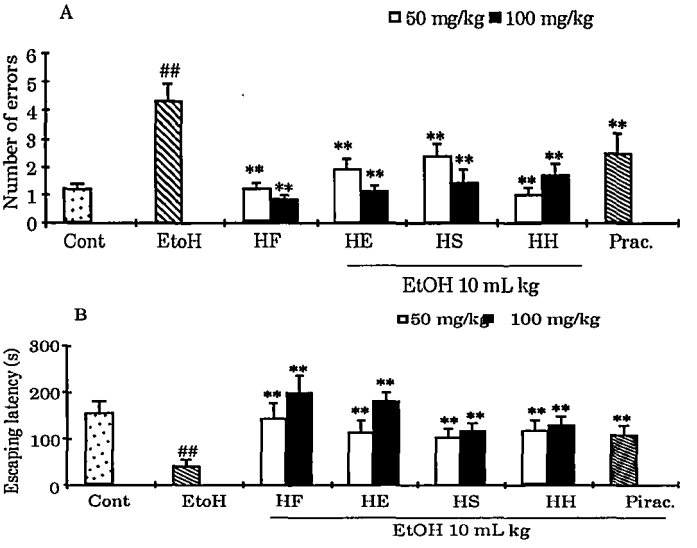


Fig 6. Effect of the hydroalcoholic extracts of four *Hypericum* species on impairment of memory retrieval in step-through tasks in mice induced by 30% ethanol (n=10, mean ± S.D.) ##p<0.01 vs Cont (Cont = Control); *p<0.05, **p<0.01 vs 30% ethanol, (Pirac = piracetam)

DISCUSSION

The results indicated the one-way step-down passive avoidance task and the one-way step-through passive avoidance task gave overall similar patterns for the controls and the drugs. Scopolamine, sodium nitrite and 30% ethanol are commonly used as chemicals for impairing the learning and memory processes in acquisition, consolidation and retrieval stages, respectively. The results obtained in this study demonstrated that the effects of the four extracts are more significant in 30% ethanol model and the scopolamine model and sodium nitrite model which indicates the *Hypericum* extracts improved the impairing the learning and memory processes in acquisition, and retrieval stages more than the consolidation stage.

HH, HE and HF, as well as the control drug piracetam, improved all these processes of memory functions in experimental animals in dose dependent manner. HS has improved the impairments of memory acquisition and retrieval, but had little effect on memory consolidation. The present study also showed that the four *Hypericum* extracts had no significant effects on spontaneous locomotor activities of the animals, which indicated that the effect of extracts increasing the latencies and in decreasing the error numbers in the passive avoidance tasks were devoid of the central sedative or exciting effects on the mice.

It is well known that cholinergic neuronal system plays an important role in acquisition stage of learning and memory and the anti-muscarinic agent, scopolamine, was known to impair learning acquisition (Elrod & Buccafusco, 1988). In the present study, all the four plants could significantly reverse the memory impairment induced by scopolamine, indicating that the improvements might be related to the cholinergic functions in the central nervous system.

In Table 1, some chemical constituents in the extracts of these four *Hypericum* species were identified: hypericin, pseudohypericin and several flavonoids including rutin, hyperoside, quercetin, quercitrin. Hypericin, as an important active constituent of antidepression in *Hypericum* species, has been reported to cause the decrease of serotonin 5-HT_1A receptor in the hippocampus (Butterweck *et al.*, 2001). It was reported that the increase in serotonergic neurotransmission can interfere with learning acquisition and memory consolidation (Jaffard *et al.*, 1989). The hippocampus, a structure critical for proper learning and memory functions, is frequently implicated in aging-related learning deficits. Hypericin was found to be a potential inhibitor of the superoxide radical, and flavonoids (rutin, quercetin, and quercitrin) were also demonstrated to have the free radical scavenging activity in a model of auto-oxidation of rat cerebral membranes (Saija *et al.*, 1995). The antioxidant properties of these constituents in the *Hypericum* species may give an additional benefit on learning and memory improvement processes.

Table 1. Contents of the main constituents in the extracts of HH, HS, HF and HE

Compound	HH (mg/g)	HS (mg/g)	HF (mg/g)	HE (mg/g)
Chlorogenic acid	18.1	16.1	14.3	16.2
Rutin	5.4	2.6	5.0	1.7
Hyperoside	32.7	32.5	18.4	27.0
Quercetin	7.6	13.0	1.6	1.6
Pseudohypericin	1.2	2.6	4.6	4.9
Hypericin	3.7	2.1	3.8	6.0

In conclusion, the hydroalcoholic extracts of the four *Hypericum* species appeared to have significant effects on improving learning and memory performance in passive avoidance tasks in mice. Further work is required to determine the active components and clinical efficacy of these *Hypericum* plants in the treatment of depression, especially those cases accompanying with cognitive impairment.

ACKNOWLEDGEMENTS

The authors thank Dr. Lihong Wu and Dr. Fasong Wang for their contribution in identifying and collecting the plants. We are grateful to Prof. Ruzhu Chen and Ms. Xiaolan Zhu for the pharmacological experimental support. The study was supported and funded by National Natural Science Foundation of China (2004 No. 30470188); and partially supported by the Australia China Special Fund (2007).

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4

Chemistry and Pharmacology of *Shorea robusta*

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ABSTRACT

Shorea robusta is a forest plant of India, the resin of which has been used in the traditional and folkloric medicine. Some interesting chemical molecules especially the ursane derivatives and mono- sesquiterpene derivatives have been isolated from this plant. However, not many pharmacological reports are available on this important forest product. This review gives a detailed account of the chemical constituents and also reports on the antibacterial and wound healing activities of the oil and extracts of S. robusta. The results in the laboratory of authors, clearly demonstrated the utility of S. robusta in healing the wounds, which is evident from biochemical and histopathological studies.

Key words : Dry distillation, phytochemistry, *Shorea robusta,* ursane, derivatives

INTRODUCTION

Shorea robusta Gaertn (Dipterocarpaceae) is one of the dominant tree species in tropical deciduous forests (moist as well as dry types) in India (Champion & Seth, 1968). It is the state tree of Chhattisgarh India.

Vernacular Names: (English): sal tree. (Hindi): borsal, hal, sakhu, sakhwa, sal, shal. (Gujarati): ral. (Bengali): sakhu, sal, shal. (Tamil): kungiliyam (resin). (Urdu): ral. (Trade name): sal. (Nepali): agrakh, sakhua, sakwa, sal. (French): damar de l'Inde. (German): salbaum.

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Taxonomic Hierarchy

Kingdom : Plantae
Phylum : Angiosperms
Class : Magnoliatae
Subclass : Dilleniidae
Order : Theales
Family : Dipterocarpaceae
Genus : *Shorea*
Species : *robusta*

Geographic Distribution

Sal (*Shorea robusta* Gaertn.) occurs gregariously on the southern slopes of the Himalayas and is distributed in Bangladesh, India and Nepal. Its presence is indicated in Bhutan and South China too (Zhao *et al.*, 1994). Broadly, sal's natural range lies between the longitudes of 75° and 95° E and the latitudes of 20° to 32° N. This forest type extends from a few meters to 1500 m above mean sea level. Within this range, the distribution is controlled by climatic and edaphic factors. *S. robusta* is a light-demanding tree that grows to 45 m in height and frequently forms a nearly monospecific canopy (Rautiainen & Suoheimo, 1997). It grows in a broad range of well drained soils (Banerjee *et al.*, 1992; Dinerstein, 1979). Natural zone of sal forest is shown in Fig 1 (Gailaur & Devoe, 2006). Fig 2 shows sal forest at corbelt National Park at Utaranched, India.

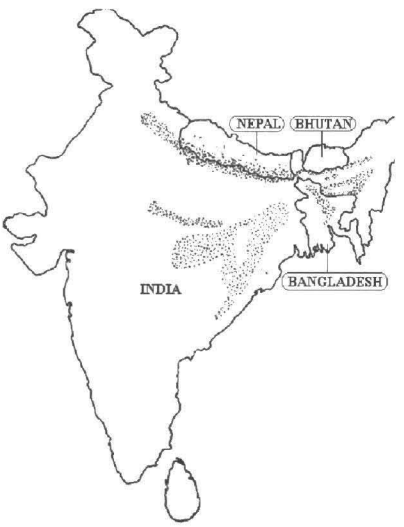


Fig 1. Natural zone of sal forests (shaded dots)

Fig 2. Sal forest

Botanical Description

It is a large, deciduous tree up to 50 m tall and with a dbh of 5 m; these are exceptional sizes, and under normal conditions *S. robusta* trees attain a height of about 18–32 m and girths of 1.5–2 m; bole is clean, straight and cylindrical, but often bearing epicormic branches; crown is spreading and spherical. Bark dark brown and thick, with longitudinal fissures deep in poles, becoming shallow in mature trees; provides effective protection against fire. The tree develops a long taproot at a very young age. Leaves simple, shiny, glabrous, about 10–25 cm long and broadly oval at the base, with the apex tapering into a long point; new leaves reddish, soon becoming delicate green. Flowers yellowish-white, arranged in large terminal or axillary racemose panicles. Fruit at full size about 1.3–1.5 cm long and 1 cm in diameter; it is surrounded by segments of the calyx enlarged into 5 rather unequal wings about 5–7.5 cm long (Sharma *et al.*, 2000). A plate of *S. robusta* is shown in Fig 3 (Brandis, 1874).

Fig 3. Plate of *S. robusta*

Traditional Properties and Uses

Bark, leaves, flowers and fruit: Bark and leaves are astringent, acrid, cooling, anthelmintic, alexeteric, anodyne, constipating, and urinary astringent, depurative (eliminates toxins and purifies the system, especially the blood) and tonic. They are useful in ulcers, wounds, bacterial infections, diarrhoea, dysentery, gonorrhea, leucorrhoea, pruritis, leprosy, cough, hyperhidrosis (condition characterized by abnormally increased perspiration), haemorrhoids and anaemia. Bark is used for the treatment of ulcers, powdered bark of the tree is used for treatment of wounds specially those infected and lacerated

ones forming pus (Warrier *et al.*, 2005). The flowers are good source of honey. The fruits are sweet, astringent, cooling, aphrodisiac, cholagogue, and tonic, and are useful in dyspepsia, burning sensation, dermatopathy diarrhoea, leprosy and gonorrhea.

Resin

It is astringent to bowels, sweet, acrid, anodyne (medicine that relieves or soothes pain), vulnerary (Useful in healing wounds; adapted to the cure of external injuries), antibacterial, deodorant, constipating, detergent, carminative, stomachic, aphrodisiac, expectorant, ophthalmic, lessens perspiration, fever and is a tonic. It is useful in hyperhidrosis, vitiated conditions of pitta, good for wounds, pains ulcers, neuralgia (painful disorder of the nerves), burns, pruritis, fractures, fever, diarrhoea, dysentery, haemorrhoids, gonorrhea, menorrhagia, splenomegaly (enlargement of the spleen), obesity, cephalalgia (headache), odontalgia (toothache), burning of eyes and ophthalmodynia (eye pain). It is good for vaginal discharges, as a collyrium (lotion or liquid wash used as a cleanser for the eyes) good for eyesores. Oil is good for skin diseases, scabies and all kinds of wounds (Kirtikar & Basu, 1975).

Chua oil

From resin is used as a fixative in heavy perfumes, for flavoring, chewing and smoking tobacco and in medicine as an antiseptic for skin diseases and ear troubles. The non-phenolic portion of the oil has a suppressing effect on the central nervous system; the phenolic portion is less effective. The seed oil is used as a good remedy for skin diseases and scabies (Sharma *et al.*, 2000; Kirtikar & Basu, 1975; Chopra *et al.*, 1956).

Other Uses

As timber *S. robusta* as an important commodity, the Indian government saw sal forests more as a timber source rather than for other forest products. A sal tree in addition to timber and fuel wood, produces fodder (Shakya & Bhattarai, 1995; Edwards, 1996); leaves for plates (Rajan, 1995); seed for oil (Sharma, 1981); feed (Rai & Shukla, 1977), resin or latex from heartwood (FRIB, 1947) and tannin and gum from bark (Karnik & Sharma, 1968). It is a multipurpose tree as its bark, resin, leaves and the seeds, available in considerable quantities, are exploited for their varied applications, including medicinal (Anon, 1972).

Traditional Formulations and Their Uses

Compound ointment: Ral (resin), rocksalt, treacle (dark sugar syrup produced during raw sugarcane refining), wax, honey, bdellium (*Commiphora africana*

an aromatic gum), red ochre and clarified butter are taken in equal parts, boiled together and ointment is prepared.

"Murakibhat Ahsani" recommends the following ointment for eczema: *S. robusta*, gum mastiche, each 1 tola, Mom (wax), zard 1.5 tola and mustard oil 4 tola are taken and ointment is prepared. With sugar resin is administered in dysentery, bleeding piles, weak digestion, gonorrhea and as an aphrodisiac. Twenty grains (1300 mg) of pulverized resin mixed with a pint (473 mL) of boiled milk taken every morning is a good aphrodisiac. In dysentery of children, resin is given in doses of about 20 grains (1300) with an equal quantity of sugar treacle. The resin is burnt as an incense of sick rooms for its fragrant smoke (Nadkarni & Nadkarni, 1988).

Ointment used for foetid ulcers: *S. robusta* 5, cinnabar 2, mastiche 3, *Calamus draco* (Dragon's Blood Palm) 3 and ghee 10 parts are taken, mixed and ointment is prepared.

Powder used in diarrhoea: *S. robusta* 4, mocharas (gum is used for treating acute dysentery) 2, dried decorticated mango kernel 5, *Aegle marmelos* 5, and nutmeg 5 parts are taken, mixed and powder is made, dose is 5 grains (325 mg).

Powder used for removal of piles: *S. robusta*, carbonate of iron and lime and cardamoms each 1 part, sugar 10 parts, bark of *Azadirachta indica* and *Ophelia chirata* each of 5 parts are taken, mixed and powder is made. Dose is 15 grains (975 mg) (Nadkarni & Nadkarni, 1988).

Butter used as aphrodisiac: A kind of butter is prepared by frying resin in the ghee and then strained through water. The water is thrown away and thick layer is kept for use as aphrodisiac. The drug is also used in scorpion-sting (Nadkarni & Nadkarni, 1988).

PHYTO-CONSTITUENTS

Seed

The seed contains corilagin **1**, ellagic **2**, chebulinic **3**, and gallic acids **4**. A new phenolic acid shorbic acid **5**, mp. 93° isolated from seed. A new flavonoid, 3, 7-dihydroxy-8-methoxyflavone 7-O-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside **6** is isolated from seeds of S. robusta (Prakash & Rao, 1999).

Sal (Shorea robusta) Seed Fat

Fat from nuts was reported to contain cis-9-10-epoxystearic acid, 9, 10-Dihydroxystearic acid and its triglycerides (DHS-TGs). They were isolated from sal fat by silica gel adsorption and solvent and dry fractionation processes, followed by crystallization (Reddy & Prabhakar, 1987). Sal seeds

also contain palmitic acid (4.5%), stearic and oleic acids (42.2%), linoleic acid (2.8%) and arachidic acids (6.3%) (Hilditch & Zaky, 1942).

Heart Wood and Bark

GC-MS analysis of the essential oil of *Shorea robusta* bast (cambium + secondary phloem) showed it to be a mixture of twenty-eight compounds, of which nine compounds, amounting to 48.79% of the oil were identified. The identified compounds were sesquiterpenes (47.46%), α-terpineol **7** (1.33%), sesquiterpene hydrocarbons characterized as α-copaene **8** (3.79%), β-elemene 9 (1.54%), γ-muurolene **10** (0.45%), viridiflorene **11** (1.62%) and γ-cadinene **12** (2.34%). Oxygenated sesquiterpenes constituted the major portion of the oil (37.72%) and were identified as globulol **13** (4.52%), T-cadinol **14** (16.75%) and α-cadinol (16.45%). T-cadinol is the chief constituent of bast oil (Kaur *et al.*, 2003). A new benzofuran, shoreaphenol **15** was isolated from the bark (Saraswathy *et al.*, 1992.).

Leaves

Terpenoids β-amyrin **16**, friedelin **17** and β-sitosterol **18** were isolated from petroleum ether extract of dry leaves, and α-, β-carotene, lutein 19, phenophytin-a and 7-methoxy-4'-5-dihydroxyisoflavone have been isolated from the acetone extract of the fresh leaves of *S. robusta* (Chauhan, 2002).

Resin

Man-made or natural cuts in the bark of stem or branches lead to the exudation of a resin which is trivially called 'Saal ki raal, oleoresin known as sal dammar, ral or lal dhuma. 3-5 narrow strips of bark are cut, 90-120 cm above the ground. When the tree is blazed the oleoresin oozes out as a whitish liquid and on exposure it hardens quickly and turns brown Fig 4 shows resin exudates from *S. robusta* tree. The cut is freshened by scraping off the hardened resin. In about 12 days the grooves are filled with resin. The grooves are freshened and resin is collected periodically in July, October and January. A good mature tree yields about 5 kg of resin annually Fig 5 shows the dried resin of *S. robusta*. Sal resin, on dry distillation yields an essential oil, known as "chua oil". The yield of the oil varies from 41 to 68% depending upon the source of the oleoresin samples. The oil is light brownish yellow in colour and has an agreeable incense-like odour, with specific gravity 0.9420, acid value 4.42, saponification value 15.72 and saponification value after acetylation 39.49. It consists of 96.0% neutral, 3% and 1% phenolic and acidic fractions respectively. The oil obtained from the dry distillation of resin has been reported to contain several mono-and sesquiterpenoids (Pakniker &

Bhattacharyya, 1961). The presence of some triterpenoids was also reported from the resin of the South Indian sal tree (Purushothaman *et al.*, 1988.). Several ursane derivatives have been isolated from the resin of East Indian sal. They are ursolic acid **20**, 2α, 3β-dihydroxy-urs-12-en-28-oic acid **21**, 2α, 3α-dihydroxy-urs-12-en-28-oic acid, 3β, 23-dihydroxyolean- 12-en-28-oic acid **22**, 2α,3,23-trihydroxy-urs-12-en- 28-oic acid **23** and a new triterpenoid, 2α,3β,23-trihydroxy 11β methoxy-urs-12-en-28-oic acid **24** (Hota & Bapuji, 1993; Hota & Bapuji, 1994). The resin, collected by tapping from the sal forest of the Himalayan foot hills has yielded ursolic acid **20**, α- amyrin **25** and β-amyrin **16**, mangiferonic acid **26**, benthamic acid, asiatic acid **27**, α-amyrenone **28** and uvaol **29** as known compounds, of which only ursolic acid, asiatic acid and Clamyrin have previously been reported from the resin of *S. robusta*. Two new compounds, 3, 25-epoxy-1, 2, 3, 11-tetrahydroxyurs-12-en-28-oic acid **30** and 3, 25-epoxy-1, 2, 3-trihydroxyurs-12-en-28-oic acid **31** have been isolated from resin of *S. robusta* (Mishra & Ahmad, 1997). Recently essential oil (0.12% on moisture free resin) was isolated from petroleum ether extract (8 g) of *S. robusta*. GC-MS analysis of essential oil revealed a total of 37 compounds out of which 17 (78.43) were identified, major compounds were Germacrene-D **32** (29.57%), α-Copaene (16.32%), Glubulol **12** (10.22), δ-Elemene **33** (8.51%), β-Caryophyllene **34** (5.08). α-Copaene, γ-muurolene , γ-cadinene and globulol are the common compounds found in the essential oil of both bast and resin. Oils from heart wood and resin may have very good anti-carcinogenic property due to the presence of sesquiterpenes specially caryophyllenes which are described as potential anti-carcinogenic agents (Kaur *et al.*, 2001). Further ursolic acid and oleanolic acid derivatives have shown potent anti-tumor activities (Hua *et al.*, 2006) one can expect that triterpenoids of *S. robusta* resin can play an important role in anticancer drug discovery.

Fig 4. Resin exudes from the *S. robusta* Tree

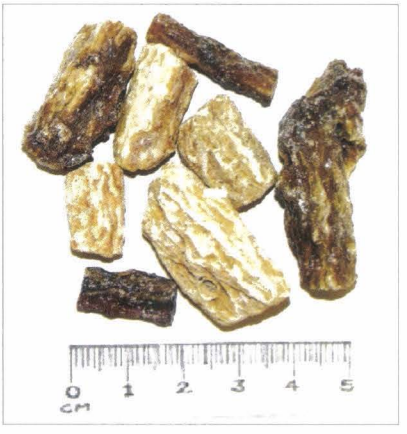
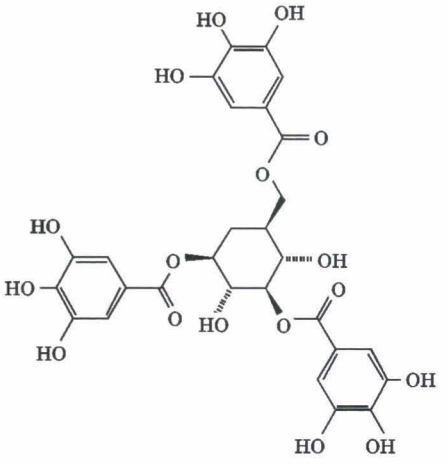
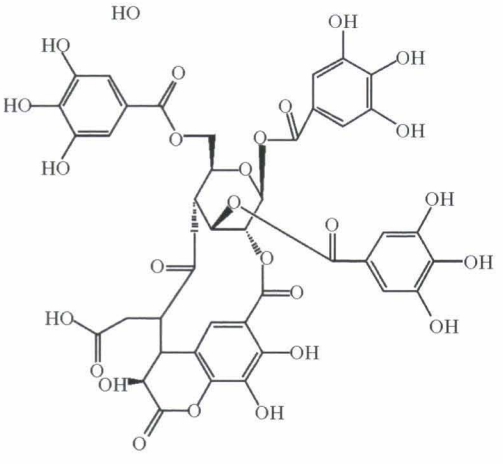


Fig 5. Resin of *Shorea robusta*

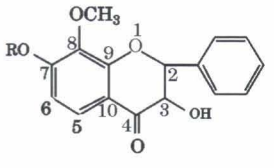
Structure of Isolated Phytoconstituents



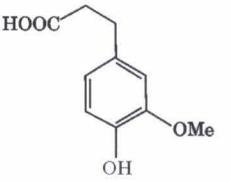
(1) Corilagin



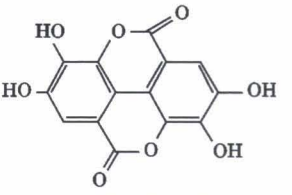
(3) Chebulinic acid



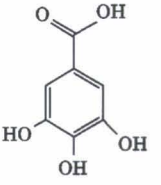
(6) 3, 7-dihydroxy-8-methoxyflavone 7-O-α-L-rhamnopyranosyl-(1→4) -α-L-rhamnopyranosyl- (1→6)-β-D-glucopyranoside R=α-L-rhamnopyranosyl-(1→4) -α-L-rhamnopyranosyl



(2) Shorbic acid

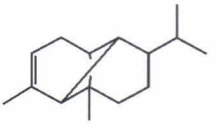


(4) Ellagic acid

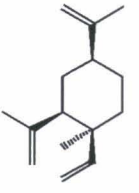


(5) Gallic acid

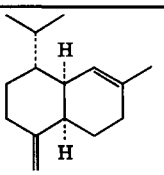
(7) α-terpineol



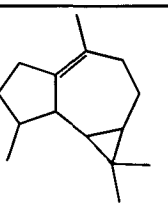
(8) α-copaene



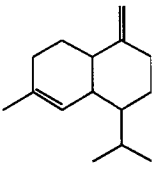
(9) β-elemene



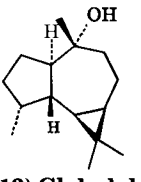
(10) γ-muurolene



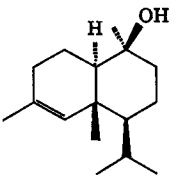
(11) Viridiflorene



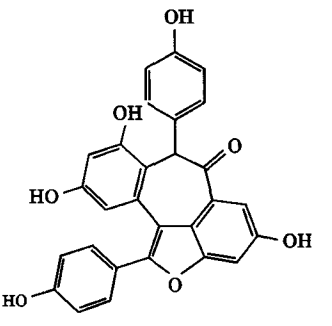
(12) γ-cadinene



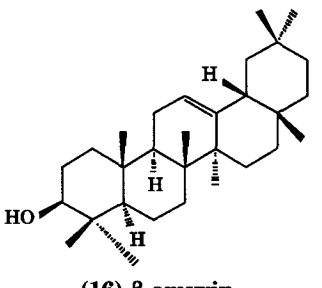
(13) Globulol



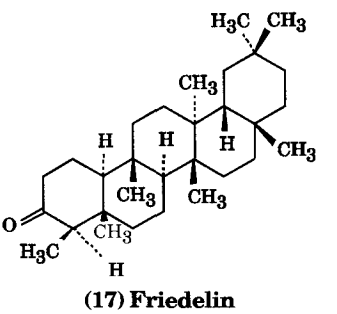
(14) T-Cadinol



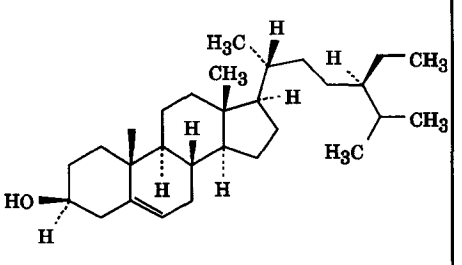
(15) Shoreaphenol



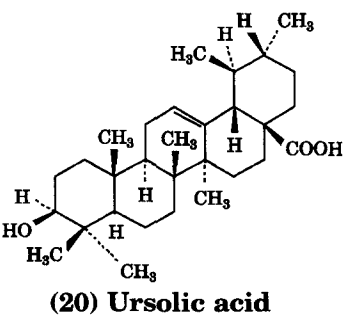
(16) β-amyrin



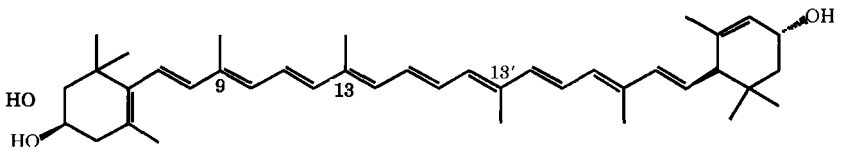
(17) Friedelin



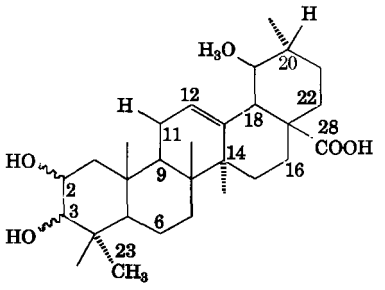
(18) β-sitosterol



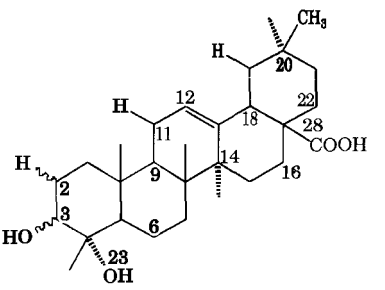
(20) Ursolic acid



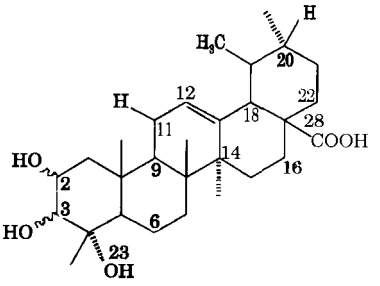
(20) Lutein



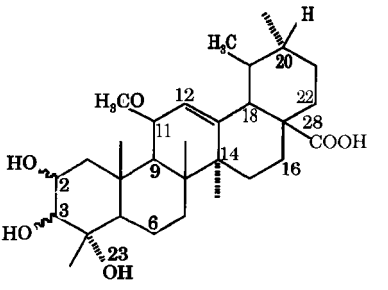
(21) 2α,3β-dihydroxy-urs-12-en -28-oic acid



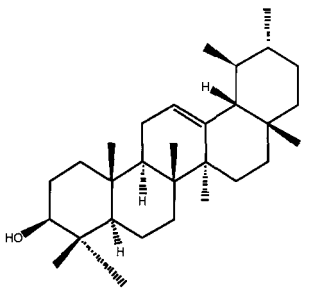
(22) 3β,23-dihydroxyolean -12-en-28-oic acid



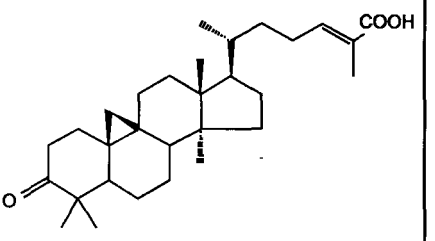
(23) 2α, 3, 23-trihydroxy-urs-12-en - 28-oic acid



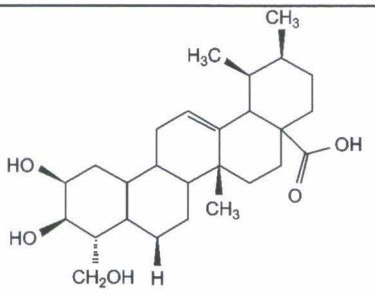
(24) 2α, 3β, 23-trihydroxy 11β methoxy-urs-12-en-28-oic acid



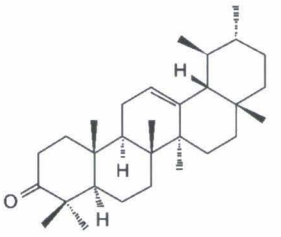
(25) α-Amyrin



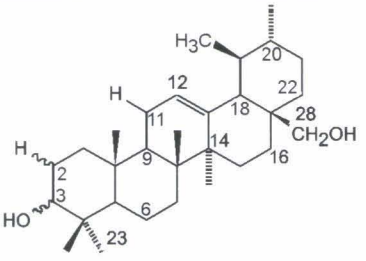
(26) Mangiferonic acid



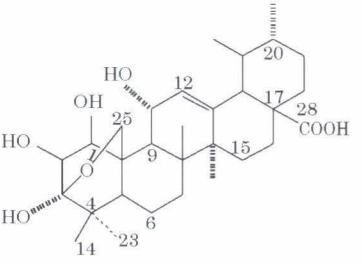
(27) Asiatic acid



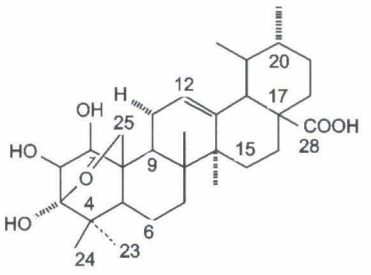
(28) α-Amyrinone



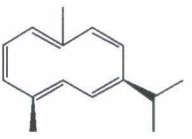
(29) Uvaol



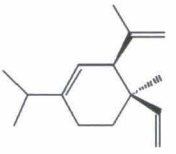
(30) 3, 25-epoxy-1, 2, 3, 11- tetra-hydroxyurs-12-en-28-oic acid



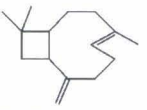
(31) 3, 25-epoxy-1, 2, 3-trihydroxyurs-12-en-28-oic acid



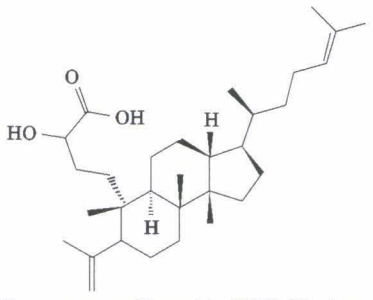
(32) Germacrene-D



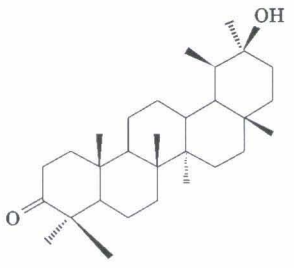
(33) δ-Elemene



(34) β- Caryophyllene



(35) Dammarenolic acid: (20S)-Hydroxy-3, 4-seco-4(28), 24-dammaradien-3-carboxylic acid



(36) epi-ψ-Taraxastanonol

Extraction and Dry Distillation of Resin

We extracted 50 g of *S. robusta* powder with 200 mL of methanol in Soxhlet apparatus to obtain methanol extract (ME). PBIME (Petroleum ether benzene insoluble methanol extract) was obtained from methanol extract by the method described by Hota *et al.* (1993). Essential oil (EO) was obtained by dry distillation of powdered crude drug (100 g) in a Clevenger apparatus.

Qualitative Thin Layer Chromatography

TLC separation of the methanol extract was done on pre-coated TLC Silica gel 60 F254 plates (Merck). Five bands of 5 mm of 2, 4, 6, 8, 10 µl containing 40 µg/µl was placed by HPTLC spotter (Camag Linomat 5) and developed in a solvent system of hexane: ethyl acetate: methanol (7: 2.75: 0.50) in twin trough Camag chambers. Scanning of the developed TLC plate was done at 254 nm on TLC Scanner (Camag). TLC profiling of other extracts and oil is in progress at our laboratory.

TLC of methanol extract at 254 nm and 366 nm showed five bands from 80-400 µg (Fig 6). Fig 7 shows the HPTLC chromatogram of *S. robusta* of all the five bands at 254 nm. Qualitative TLC scanning of 4th track of chromatogram of the methanol extract shows ten peaks at 254 nm at Rf 0.23, 0.31, 0.35, 0.49, 0.54, 0.65, 0.73, 0.79, 0.85, 0.90 for various separated compounds (Fig 8).

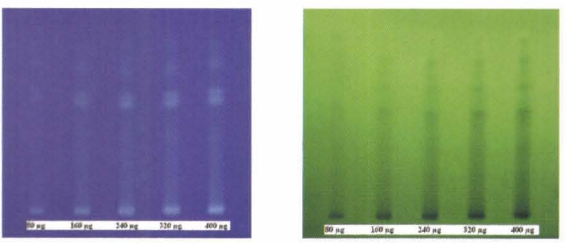


Fig 6. UV image of TLC plate of methanol extract a various concentration

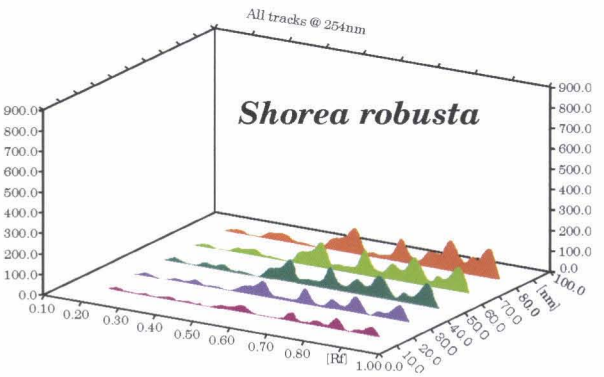


Fig 7. HPTLC scan of *S. robusta* all five tracks

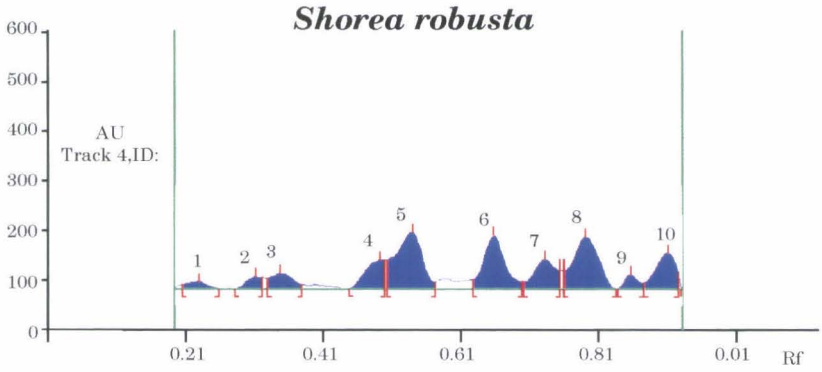


Fig 8. HPTLC scan of *S. robusta* track 4

Physico-Chemical Properties of Starch

Soni *et al.* (1987) isolated starch from the seed of *S. robusta* which has unique characteristics. It was found to be of 3.5 to '12.35 µm in size and round to oval in shape. Gelatinization temperature of sal starch is 85–90°C, which is higher than the reported values of maize, tapioca and other starches. It was also found to contain high nitrogen, phosphorus, lipid, and amylose in comparison to other starches of forest origin.

PHARMACOLOGY

Resin powder of *S. robusta* was tested in Kohl-Chikni-Dawai (KCD) a compound ophthalmic formulation of Unani medicine on naphthalene-induced cataracts in rats. A 3% solution of the drug was reported to be highly effective in inhibiting lens opacification in the naphthalene induced cataract rats. KCD contains *S. roubsta* in the form of dry ash (Siddiqui, 2002). Ambrex, a polyherbal formulation containing 10% *S. robusta* resin was reported to have protective effect in ethanol-induced gastric mucosal lesions in rats. Flavonoids are reported to reduce the histamine release from gastric tissue (Ebadi, 2002). It is presumed flavonoids present in *S. robusta* might be responsible in the reduction of ulcer index by reducing the gastric acid secretion (Narayan *et al.*, 2004). Water extract of stem bark of *S. robusta* was reported to exhibit free radical scavenging activity in an antioxidant assay (ED_{50}: 151.62 ppm), the xanthine oxidase testing produced a 60% inhibition with *S. robusta* extract (Ricardo *et al.*, 2004). Triterpeniods from resin of *S. robusta* like dammarenolic acid **35**, epi-ψ-taraxastanonol **36** and hydroxyhopanone are reported to have antiviral activity against Herpes simplex (Purushothaman *et al.*, 1988).

Anti-Bacterial Activity

We tested the antibacterial activity of *S. robusta* using petroleum ether benzene insoluble methanol extract (PBIME) and essential oils (EO) of the

resin by agar-well diffusion and disc diffusion methods (Sinclair & Dhingra, 1995; Rabe & Van, 1997). Pure cultures of different strains were obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India and from American Type Culture Collection (ATCC), B.V. Patel Pharmaceutical Education, Research and Development Centre, Thaltej, Ahmadabad, India.

PBIME and oils of the resin were used at a concentration of 0.2, 0.4 and 1 mg/mL. 100 µg/mL and 1 mg/mL stock of Gentamycin (GM) as a standard was used for antibacterial assay in agar-well and disc-diffusion method respectively. The diameters of zones of inhibition (in mm) were measured. It was noted that PBIME and essential oils had very prominent activity on all selected bacterial strains *Staphylococcus aureus* (24.5 mm), *Streptococcus pyogenes* (16.9 mm), *Bacillus cereus* (15.9 mm), *Bacillus subtilis* (15.4 mm), *Escherichia coli* (15.6 mm) and *Pseudomonas aeruginosa* (12.6 mm) at 1 mg/mL. PBIME was found to have highest zone of inhibition against *S. aureus*. The oil was also found to be highly effective at 10 µl/well with highest zone of inhibition against *B. subtilis* (22.0 mm). Fig 9 shows zones of inhibition of PBIME against *S. aureus* by disc diffusion method.

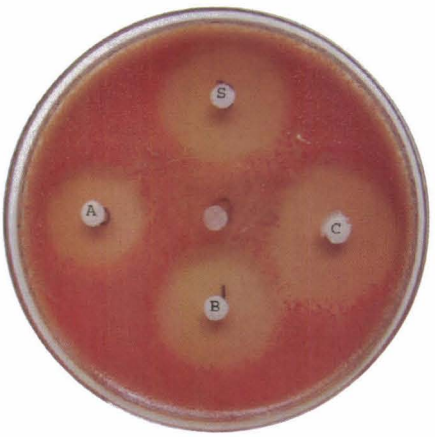


Fig 9. Antibacterial activity of PBIME by disc diffusion on *S. aureus*

Wound Healing Activity of S. Robusta

Wound healing activity of PBIME and EO formulated as 10% ointment were tested in two models. Two, 6-cm long para-vertebral incisions were made by the method of Ehrlich *et al.* (1969). Incision model was used to evaluate tensile strength and histological changes on 10^{th} day. A full thickness of the excision wound (circular area about 300 mm^2 and 2 mm depth) was created and infected with bacterial cultures by the method of Grzybowski *et al.* (1997). Excision model was used to determine wound contraction, period of epithelization and scar area. The granulation tissues formed on 5^{th}, 10^{th} and

15th days were excised to estimate protein, hydroxyproline, hexosamine, lipid per-oxidation and super oxide distmutase (SOD). Histopathological studies of tissues excised on 4th, 8th, 12th and 16th days after wound formation was also done.

A significant increase in the tensile strength of the wounds was observed after topical treatment with PBIME (667.7 g/cm) and EO (602.2 g/cm) of *S. robusta* resin as compared with the control (393.7 g/cm). A significant difference in the wound of control and PBIME can be seen on the 7th day (Fig 10).

Fig 10. Antibacterial activity of PBIME by disc diffusion on *S. aureus*

In the excision wound model significant ($p<0.005$) wound-healing activity was observed in animals treated with the PBIME and EO as compared to control treatments. PBIME and EO were found to be more active than Soframycin (framycetin sulphate). The extract treated animals demonstrated a faster rate of epithelization (PBIME: 16 ± 0.26, EO: 17 ± 0.37, standard: 18 ± 0.26) as compared to control group (23 ± 0.6 days). In *S. robusta* treated groups the scar area (PBIME: 7.67 ± 0.96, EO: 11 ± 1.24, standard: 10 ± 1.28 mm^2) was reduced as compared to control group (21.3 ± 1.3 mm^2).

Multiple cross-sections of excised skin stained with hematoxylin and eosin stained of all the five groups were examined for epithelial regeneration, formation of granulation tissue, and fibrogenesis. It was noted that tissue regeneration was greater in the case of standard, PBIME and oil treated groups as compared to control. Fig 11 shows histopathological comparison of PBIME with control on day 12. In the biochemical study protein and hydroxyproline increased in the granulation tissue of all the groups from days 5–15 and its content was significantly ($p<0.001$) greater in all the treated groups as compared to control. Hexosamine level was found to increase in the initial phase of wound healing from days 5–10 and decreased significantly at the later phase (day 15) of wound healing in the treated group animals whereas in control animals its level was high. Lipid peroxidation products were elevated in all the groups from day 5–10. On day 15, levels of TBARS (MDA) restored to normal in treated group animals, whereas in control animals, the TBARS levels remained at higher level. SOD level was found to decrease in the wound of all groups from day 5–15 and its content was significantly ($p<0.001$) lower in all the treated groups in the respective days when compared to control. The animals in the test group showed a significant

($p<0.001$) reduction in the wound area as compared to the control animals (Fig 12).

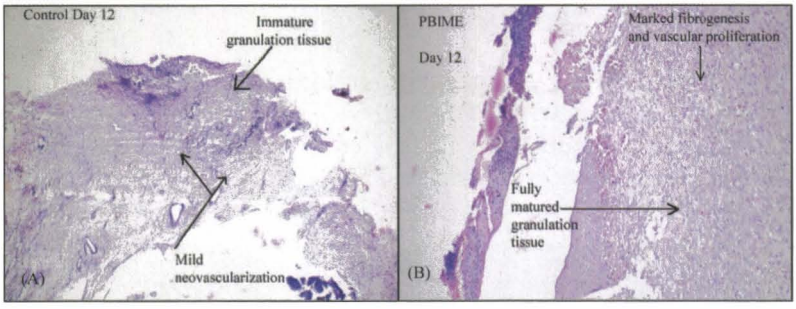


Fig 11. Histopathology of Control and PBIME on Day 12

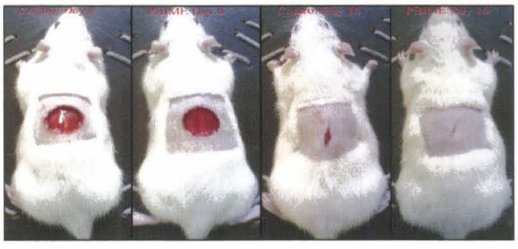


Fig 12. Images of excision wound Model on day zero and sixteen of control and PBIME treated animal

Market Formulations

Herbal Mosquito Repellent

S. robusta is a key ingredient of herbal mosquito repellent; the formulation also contains *Polygonum glaborum* and *Flemigia strobillfera* which are powdered and mixed with binders and fillers to increase the efficiency of the formulation.

Kohl-Chikni Dawa (KCD)

It is a Unani formulation, containing hard soap, copper sulphate and *S. robusta* as its chief ingredient. It is reputed for its beneficial effects in the treatment of premature cataracts.

Herbinol: An Herbal Antiseptic Cream

Herbinol is an antiseptic cream in which *S. robusta* is one of the major ingredients along with *Berberis aristata*, *Leptademia reticulata*, *Bertginia ligulata*, *Mallotus phillippinensis*, *Rubia cordifolia*. The cream is also reported to have prominent bactericidal and bacteriostatic activities against some common microbes causing burn septicemia (Pandya *et al.*, 1989).

Ral Hair Oil (Mayur Pharmacy)

Hair oil containing resin of *S. robusta* is useful for long hairs and preventing hair loss.

Patents

Many cosmetic formulations containing *S. robusta* fat have been patented.

Cosmetic formulations like skin care cream, makeup remover milk, rinse soap cream, sun cream, body milk, anhydrous balm, anhydrous make up remover gel, make up remover cream, body and face oil, hair cream, pre-shampoo oil are reported (Zabotto & Griat, 1985).

Fat is obtained from the stones of the fruit of the tree, fat of *S. robusta* forms aqueous compositions having high water concentrations (greater than 30%) without forming rancid products.

Characteristics features of fat:
Melting point (°C.): 30–38.5
Density (30°C.): 0.860 to 0.900
Index of refraction (40°C.): 1,4560–1,4580
Index of saponification: 180–220
Iodine index: 36–42
Acid index: from 0.1 to 18.9
Non-saponifables (%): 0.73–2.2.
Example of few patented formulations

1. Sun Cream

Oily phase	*Wt.%*
Petrolatum oil	34.0
Fat of *Shorea robusta*	12.0
Beeswax	3.0
Magnesium lanolate	2.4
Lanolin alcohol	0.6
Polyethylene powder	10.0
Perfume	1.0
Water + Preservative	100 q.s

2.0 Anhydrous Balm

Oily phase	*Grams*
Fat of *S. robusta*	60.0
Sesame oil	20.0
Lanolin	15.0
Soy lecithin	4.8
BHA	0.1
BHT	0.1

CONCLUSIONS

The resin of *S. robusta* has tremendous potential, as it contains unique ursane derivatives of very interesting structural features. The diversity of the structures may definitely offer important insights in the discovery of novel drug entities. These compounds offer a promise to be very good anti-bacterial and wound healing agents. Keeping in the view the reports on the anti-diabetic activity of the resin, it may be possible that some of the compounds may prove to be very effective in diabetes and diabetic wounds. Ursane derivatives and related oleanone derivatives are emerging potential candidates for number of activities such as anti-tumor, anti-inflammatory, anti-diabetic, anti-microbial etc. Further work to study the pharmacological profile of each of these compounds from *S. robusta* definitely proves to be rewarding.

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5

Safety, Efficacy and Preclinical Evaluation of Plant Products

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ABSTRACT

Plants have served as traditional herbal medicines for long time, and natural products make excellent leads for new drug development. These products are mostly secondary metabolites, and have become medicines, dietary supplements, and other useful commercial products. These active lead compounds can also be further modified to enhance the biological profiles and developed as clinical trial candidates. The efficacy and safety of any pharmaceutical product is determined by the components (desired and undesired) which it contains. Mixture of compounds produced by plants may provide important combination therapies that simultaneously affect multiple pharmacological targets and provide clinical efficacy beyond the reach of single compound-based drugs. Developing innovative scientific methods for discovery, validation, characterization and standardization of these components is essential to their acceptance into mainstream medicine. In this review, we focus on latest developments in plant products research at global level with special reference to safety, efficacy and preclinical evaluation for various diseases that are reported in different laboratories.

Key words : Herbal medicine, bioactive natural products, safety, efficiency, clinical evaluation, diabetes, cancer, HIV/AIDS, cardiovascular diseases, neurodegenerative diseases, antibacterial, antiviral compounds

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INTRODUCTION

The use of medicinal herbs and herbal medicines is an age-old tradition and the recent progress in modern therapeutics has future increased the use of these products allover the world. In Asia, these therapies include traditional Chinese medicine, Japanese Chinese medicine (kampo), Korean Chinese medicine, jamu (Indonesia), Ayurvedic medicine (India). In Europe, phytotherapy and homeopathy have found medicinal uses. In America, herbal therapies along with several other traditional remedies are classified as "alternative medicines". The combination of alternative medicine, with conventional medicine (Western medicine) is termed as "integrative medicine." The educated public and health professionals have enormous interest in the medicinal uses of herbs but, unfortunately, there is a great deal of confusion about their identification, safety, efficacy, therapeutic doses, toxicity, standardization and regulation. There is lot of scope for research on medicinal plants used in folk medicine, and it is also a good approach for the development of new drugs and improve healthcare planning (Balunas & Kinghorn, 2005; Koehn & Carter, 2005; Brown & Newman, 2006; Jung, 2006; Newman, 2006; Schmidt *et al.*, 2007; WHO, 2008).

Although the use of natural products as herbal drug preparations dates back years ago, their application as isolated and characterized compounds in modern drug discovery and development started only in the 19th century, the dawn of the chemotherapy era. Medicinal chemistry has evolved from the chemistry of bioactive compounds to work at the interface of chemistry and biology and also provides a powerful means in solving problems in clinical trails and marketing the natural products in bulk amounts. It is well documented that natural products play a critical role in modern drug development, especially as antibacterial, antiviral, and antitumor agents (Newman & Cragg, 2007; Mueller, 2007; Itokawa *et al.*, 2008).

PLANT PRODUCT RESEARCH-EFFICACY, SAFETY AND PRECLINICAL EVALUATION

There are many plant products under investigation for their potential for protecting health or preventing diseases. Among the products under investigation include commonly consumed plant products like vegetables, fruits and condiments. Natural products are likely to continue to exist and grow to become even more valuable as sources of new drug lead due to their chemical diversity and novelty of molecular structure. The use of plant-derived products in the field of medicine covers a broad spectrum of activities, which include anticonvulsants, neurotransmission modulators, autonomic activity modulators, anticoagulants, hypolipidemics, antihypertensive agents, cardioprotectants, pulmonary function enhancers, hypoglycemic agents (Jia *et al.*, 2003; Grover & Yadav, 2004), fertility-enhancing agents, wound healing agents, bone healing agents, gastric ulcer protectants (Dahanakur *et al.*,

2000; Schilter *et al.*, 2003), immunomodulators and antiallergens (Mann, 2002), hepato-protective agents (Thyagarajan *et al.*, 2002; Sailaja & Setty, 2006; Muriel & Rivera-Espinoza, 2008), pancreato-protective agents, antineoplastic agents, antimicrobial agents, etc (Lopez *et al.*, 2001; Gurib-Fakim, 2006; Newman, 2006; Rishton, 2008; Itokawa *et al.*, 2008).

The rapid progress in molecular biology, computational chemistry, combinatorial chemistry (combichem), and high throughput screening (HTS) technologies, has begun to reshape the pharmaceutical industry with natural products. On average, any one new drug requires a decade for development and commercialization (McChesney *et al.*, 2007). Examples of commercialized modern drugs from natural products along with their year of introduction, indication, and company are: Orlistat, 1999, obesity, Roche; Miglitol, 1996, antidiabetic (Type II), Bayer; Topotecan, 1996, antineoplastic, SmithKline Beecham; Docetaxel, 1995, antineoplastic, Rhône-Poulenc Rorer; Tacrolimus, 1993, immunosuppressant, Fujisawa; Paclitaxel, 1993, antineoplastic, Bristol-Myers Squibb. Currently, over 500 chemical compounds of plant origin are widely used in several countries (Newman *et al.*, 2003; Spainhour, 2005; Rishton, 2008).

Recent publications have acknowledged herbal medicine's unique position in the growing field of complementary and alternative medicine (CAM) and have provided a context for clinicians to approach patients using herbal medicines (Miller, 1998; Rates, 2001; Briggs, 2002; Battaram *et al.*, 2002; Pribitkin, 2005; Chavez *et al.*, 2006; Giovanni *et al.*, 2006; Rodriguez-Fragoso *et al.*, 2008; Misra *et al.*, 2008). The explosion in sales of plant products used as medicinal agents may have both harmful and beneficial effects in humans, and have not been subjected to the same rigorous standards of efficacy, safety, and purity accorded single chemical entities approved as drugs by regulatory agencies.

Generally the beneficial effects of medicinal plants can be obtained from active constituents present in the whole plant, parts of plant (as flowers, fruits, roots or leaves), or plant materials or combinations thereof, whether in crude or processed state. Many herbalists believe that isolated ingredients have weaker clinical effects than whole plant extracts, a notion that would obviously require proof in each case (Liang & Fang, 2006; Schmidt *et al.*, 2007). Rigorous and systematic pre-clinical studies are necessary to establish the efficacy and safety of plant products and formulas in order to transform traditional herbal practices into evidence-based medicine. Product specifications, composition, characterization, history of use, comparison to existing products, description of the intended use and consequent exposure are the key information on which risk evaluation is based. A decision tree of Schilter *et al.* (2003) will aid in determining the extent of data

requirements and also guide the safety evaluation process. The ultimate safety depends on the establishment of an adequate safety margin between expected exposures and identified potential hazards. In this review, we focus on latest developments in plant product research at global level by highlighting safety, efficacy and preclinical evaluation for various diseases.

Anticancer Plant Products

Recent developments in bioassay systems are aimed at molecular targets in the discovery of potential drug candidates. Important anticancer drug targets include tubulin, DNA topoisomerases I and II (topo I and topo II), cyclin dependent kinases (CDKs), growth and transcription factors, etc (Kruczynski *et al.*, 1998; Itokawa *et al.*, 2006). Regarding source, higher plants have provided many effective, clinically useful anticancer drugs. Compounds that are found to be active agents in the *in vitro* studies on cell lines are then tested for efficacy through *in vivo* xenograft studies. These compounds include Vinca alkaloids, Taxus diterpenes, Camptotheca alkaloids, curcuma, dried roots of Euphorbia and Podophyllum lignans, and modified related compounds (Wall & Wani, 1996; Oberlies & Kroll, 2004; Crag & Newman, 2004; Itakowa *et al.*, 2006). A few of the more promising cancer chemopreventive agents are brusatol of *Brucea javanica*, zapotin of *Casimiroa edulis*, apigenin of *Mezoneuron cacullatum*, deguelin of *Mundelea sericea*, and resveratrol of *Cassia quinquangulata* (Tang *et al.*, 2003; Lee, 2004; Nunomura *et al.*, 2006; Fu *et al.*, 2006; Duarte *et al.*, 2006; Engels *et al.*, 2007; Itakowa *et al.*, 2008).

The Vinca alkaloids (from *Vinca rosea*) vinblastine (A1), vincristine (A2), vinorelbine (A3), and vindesine (A4) are well-known anticancer drugs used in the treatment of Hodgkin's lymphoma, acute childhood lymphoblastic leukemia, lung and advanced breast cancer and malignant melanoma. In the new generation of receptor-specific targeted chemotherapy, EC145 (A5), which is a folic acid conjugate of desacetyl vinblastine monohydrazide, is undergoing Phase I anticancer clinical trials (Vlahov *et al.*, 2005), and vinflunine (A6), a bifluorinated vinolrebine derivative, is in Phase II trials against bladder and kidney cancers (Okouneva *et al.*, 2003). Taxus and Camptotheca alkaloids are using extensively in the patients with advanced and metastatic ovarian and breast tumors (Hennenfent & Govindan, 2006; Clark, 2006; Kingston & Newman, 2007). Podophyllotoxin (D1), isolated from *P. peltatum* rhizomes is having an antineoplastic activity, but failed the NCI's Phase I antitumor drug clinical trials in the 1970s due to the toxicities. However, on chemical modification of D1 led to the successful development of clinically useful anticancer drugs etoposide (D2) and teniposide (D3). These compounds target cellular DNA topo II and are used to treat small cell lung and testicular cancers and lymphomas/leukemia (Gordaliza *et al.*, 2004; Lee & Xiao, 2005).

The bark of indigenous plants from the genus *Cephalotaxus* (Cephalotaxaceae) has long been used for treatment of cancers. Antitumor alkaloids - homoharringtonine (E1) and harringtonine (E2), and E1 has reached Phase I/II clinical trials against myeloid leukemia in US (Kantsrjian & cortis, 2006) and its severe side effects still remain problematic. The tree *Brucea antidysenterica* (Simaroubaceae) is used in Ethiopia to treat cancer, and identified the quassinoid bruceantin (H1) as the active principle which shows activity against various cancer cell types, particularly leukemic cells. However, in subsequent Phase I and II clinical trials, no objective tumor regressions were observed and clinical development was halted (Su *et al.*, 2002; Cuendet & Pezzuto, 2004). *M. illicifolia* is the source of cytotoxic triterpenes used in cancer treatment, including pristimerin (M1) and isotingenone III (M2) , triterpene dimers dihydroisocangorosin A (M3) and cangorosin B (M4), and other new compounds (Shirota *et al.*, 2004). The African plant *M. ovatus* (later renamed *M. serrata*) yielded the antileukemic maytansinoids [*e.g.* maytansine (M5)]. This compound progressed to Phase II clinical trials, but testing was suspended due neurotoxic side effects. Lin *et al.* (2007) investigated the effect of triptolide, derived from the traditional Chinese herb *Tripterygium wilfordii*, on the growth of glioblastoma multiforme (GBM) cells. Treatment of GBM cells with triptolide attenuated both the Ras/ERK and the Ras/Akt signalling pathways and provided a theoretical basis for triptolide treatment in GBM, but further animal studies and clinical research are necessary. Kumagai *et al.* (2007) studied *Scutellaria baicalensis*, a herbal medicine: anti-proliferative and apoptotic activity against acute lymphocytic leukemia, lymphoma and myeloma cell lines and suggested for clinical trials for these hematopoietic malignancies.

Scutellaria barbata D (Lamiaceae) (SB) is a perennial herb, which is natively distributed throughout Korea and southern China. SB has been used as an antiinflammatory and antitumor agent. The SB was found to act as an antimutagen; it mediated antiinflammatory effects; inhibited cyclooxygenase and hydroperoxidase functions (antipromotion activity). In addition, SB inhibited the development of preneoplastic lesions in carcinogen-treated mouse mammary glands in culture and inhibited tumorogenesis in a mouse skin cancer model. When cells were treated with SB, the expression of cyclooxygenase-2 was inhibited. The data suggest that SB needs to be investigated as a potential cancer chemopreventive agent in humans (Suh *et al.*, 2007). Tamayo and Diamond (2007) reviewed the clinical trials evaluating safety and efficacy of milk thistle (*Silybum marianum* [L.] Gaertn). The extracts are used to protect the liver against toxins and to control chronic liver diseases and also have anticancer, antidiabetic, and cardioprotective effects. Promising results have been reported in the protective effect of milk thistle in certain types of cancer; ongoing trials will provide more evidence in the area. Milk thistle extracts are known to

be safe and well tolerated, and toxic or adverse effects observed in the clinical trials seems to be minimum.

Plant Products for Cardiovascular Diseases

Presently, complementary and alternative medicine (CAM) is used globally in therapies and herbal/oral supplements. Few studies have examined the use of specific therapies in cardiac rehabilitation. *Ginkgo biloba*, Crataegus and Garlic are often recommended substances for patients with cardiovascular diseases. Extracts from *Ginkgo biloba* contain several active ingredients, like flavonoids and terpenes, which have antioxidative properties and an inhibitory effect on platelet aggregation by inhibiting platel*et* activation factor (PAF). Ginkgo is mainly used in vascular dementia and peripheral vascular disease. Garlic protects blood vessels against the damage by oxygen radical, and also prevents atherosclerosis due to cholesterol lowering effect. In a few countries, pharmaceutical preparations of garlic are registered medicines for the prevention of arteriosclerosis (Zhou *et al.*, 2004; Arther *et al.*, 2006). Crataegus is often used in patients with heart failure because of its positive inotropic effect (Patwardhan *et al.*, 2005; Itokawa *et al.*, 2008).

The roots and rhizome of *Salvia miltiorrhiza* (called Tanshen) have been widely used to treat cardiac and vascular disorders such as atherosclerosis or blood clotting abnormalities. This plant exhibits hypotensive effects, causes vasodilation of coronary artery, and inhibits platelet aggregation (Zhou *et al.*, 2005; Wang *et al.*, 2007). Clinically available preparations of a *S. miltiorrhiza / Dalbergia* mixture show promise in the treatment of angina (Sugiyama *et al.*, 2002). Although numerous clinical trials have demonstrated that certain Tanshen products in China are effective and safe for the treatment of cardiovascular diseases, most of them lack quality. Therefore, large randomized clinical trials and further scientific research to determine its mechanism of actions will be necessary to ensure the safety, effectiveness, and better understanding of its action (Wu *et al.*, 2008). Xu and Chen (2006) have conducted clinical studies on restenosis after percutaneous coronary intervention (PCI) intervened by TCM. Tongxinluo in angina therapy reduces the risk of subsequent AMI, PTCA or CABG, angina attacks and severity, as well as improving symptoms and ischaemic changes on the electrocardiogram (Wu *et al.*, 2006).

The Ayurvedic treatment consists of the use of herbal preparations, diet, yoga, meditation, and other practices. Based on the review of literature, the evidence is not convincing that any ayurvedic herbal treatment is effective in the treatment of heart disease or hypertension. However, the use of certain spices and herbs such as garlic and turmeric in an overall healthy diet is appropriate. Many herbs used by ayurvedic practitioners

show promise and could be appropriate for larger randomized trials. Yoga, an integral part of Ayurveda, has been shown to be useful to patients with heart disease and hypertension. Yoga reduces anxiety, promotes well-being, and improves quality of life. Its safety profile is excellent. Its use as a complementary therapeutic regimen under medical supervision is appropriate and could be worth considering (Mamtani, 2005). The literature suggests that some CAM approaches may be beneficial as adjuncts to conventional management of cardiovascular disease, but no evidence exists to support their role as primary treatment (Koscielny *et al.*, 1999; Miller *et al.*, 2004).

Antidiabetic Plant Products

Diabetes is a predominant public health concern, and causes substantial morbidity, mortality, and long-term complications and remains an important risk factor for cardiovascular disease. A large number of natural products from 800 to 1200 plants are currently demonstrating hypoglycemic activity. Research and development efforts in this particular area are largely restricted to traditional medicine and in future research may identify a potent antidiabetic agent (Heber, 2003; Bradley *et al.*, 2007; Rodriguez-Fragoso *et al.*, 2008). *Momordica charantia* is most widely studied with regard to its antidiabetic effect and all parts of the plant (fruit pulp, seed, leaves and whole plant) have shown hypoglycemic activity in normal animals (Sarkar *et al.*, 1996; Jayasooriya *et al.*, 2000); and antihyperglycemic activity in alloxan (Pari *et al.*, 2001; Rathi *et al.*, 2002; Kar *et al.*, 2003) or streptozotocin-induced (Ahmed *et al.*, 2001; Sitasawad *et al.*, 2000; Grover *et al.*, 2002) as well as genetic models of diabetes (Miura *et al.*, 2001; Grover & Yadav, 2005). The leaves of *Lagerstroemia speciosa* (Lythraceae), a Southeast Asian tree more commonly known as banaba, have been traditionally consumed in various forms by Philippines for treatment of diabetes (Klein *et al.*, 2007).

A nonrandomized, non-placebo-controlled clinical trial was conducted by Sekhar *et al.* (2002) to evaluate the efficacy of Cogent db (herbal preparation) as an adjuvant in the treatment of patients with type 2 diabetes for 3 months period. At the end of 3 months it was found that there was a significant decrease in the levels of fasting and postprandial blood glucose, cholesterol, triglycerides, glycated hemoglobin and fasting insulin in the treated group compared to the controls. It indicates that Cogent db is safe, reliable, tolerable, and effective to control type 2 diabetes mellitus. Interestingly, a binding resin (Chitosan) of *Garcinia cambogia* is shown to precipitate fat *in vitro* and hence is explored its ability to bind fat in the intestines, so that it can not be absorbed but it failed in *in vivo* model (Haber, 2003). Research on herbal and alternative therapies represents a

potentially important source for new discoveries for prevention and treatment of obesity.

Sadhukhan *et al.* (1994) selected 67 diabetic patients and 12 normal subjects for a clinical study with an indigenous herbal product. In phase I study 25 diabetics (both NIDDM and IDDM) and in phase II 42 NIDDM patients were participated. It was observed that hypoglycemic effect was seen with the test drug containing guar gum, methi, tundika and mesha shringi. The other most studied herbs for diabetes were *G. sylvestre*, *C. indica*, fenugreek, and *Eugenia jambolana*, and their herbal formulas Ayush-82 and D-400 which have hypoglycemic effect. Evidence of effectiveness of several other herbs is less extensive in *C. tamala*, *E. jambolana*, and *Momordica charantia* (Shekelle *et al.*, 2005).

Chinese herbal drugs have served as a major source of medicines for the prevention and treatment of diseases including diabetes mellitus (known as 'Xiao-ke'). It is estimated that more than 200 species of plants exhibit hypoglycaemic properties, including many common plants, such as pumpkin, wheat, celery, wax guard, lotus root and bitter melon. Jia *et al.* (2003) reviewed antidiabetic drugs of plant origin that have been approved by the Chinese health regulatory agency for commercial use in China. It was believed, through pharmacological studies, that medicinal herbs were meticulously organized in these antidiabetic drug formulas such that polysaccharide containing herbs restore the functions of pancreatic tissues and cause an increase in insulin output by the functional beta cells, the other ingredients enhance the microcirculation, increase the availability of insulin and facilitate the metabolism in insulin-dependent reactions.

Plant Products in Neurodegenerative Disorders

Alzheimer's disease (AD) is the most common form of dementia, affecting more than 20 million people worldwide. AD is characterized by an insidious loss of memory, associated functional decline, and behavioral disturbances. There are several studies that are reviewed by Akhondzadeh and Abbasi (2006) which indicate a unique role of herbal medicines in the treatment of AD. These are: (*a*) Galantamine, an alkaloid which inhibit cholinesterase, originally derived from European daffodils or common snowdrops. (*b*) *Ginkgo biloba* is an herbal medicine of China. (*c*) Huperzine A is a chemical derived from a particular type of club moss (*Huperzia serrata*). Like caffeine and cocaine, huperzine A is a medicinally active plant derived alkaloid. It is more a drug than an herb, but is sold as a dietary supplement for memory loss and mental impairment. (*d*) *Melissa officinalis* (lemon balm) improves cognitive function and reduces agitation in patients with mild to moderate AD. Ginseng, the root of *Panax* species, is a well-known herbal medicine. The active ingredients of ginseng are ginsenosides which are also called

ginseng saponins. Recent report (Radad *et al.*, 2006) suggested that some of ginseng's active ingredients exert beneficial effects on aging, central nervous system (CNS) disorders, and neurodegenerative diseases. The antioxidant, anti-inflammatory, anti-apoptotic, and immune-stimulatory activities are important for the possible ginseng-mediated protective mechanisms.

Alzheimer's disease (AD) is a common type of dementia in the ageing population due to a severe loss of cholinergic neurons in selected brain area (Arai *et al.*, 2000). At present, acetylcholinesterase inhibitors (AChEI) are the first group of drugs approved by the FDA to treat mild to moderate Alzheimer's disease. Lin *et al.* (2008) studied the ethanol and aqueous extracts from 26 traditional Chinese medicinal herbs that showed significant inhibition. Dose-dependent inhibitory assays were also performed and indicated that there is a great potential to search for novel molecules in these medicinal herbs for the treatment of AD. Clinical trials were conducted by Lin *et al.* (2003) in 60 AD patients treating with Tiaoxin Recipe (TXR) and Bushen Recipe (BSR) and concluded that both drugs are effective for treatment of AD. Anekonda and Reddy (2005) reviewed cellular mechanisms in the progression of AD and current therapeutic strategies for treating AD, with a focus on the potential efficacy of herbal treatments. Recent advances in molecular, cellular, and animal model studies have revealed that formation of the 4-kDa amyloid beta peptide is a key factor in the development and progression of AD. Several therapeutic strategies have been developed to treat AD, including anti-inflammatory, antioxidant, and anti-amyloid approaches. In animal models and cell models, herbal extracts appear to have fewer adverse effects than beneficial effects on cognitive functions. These extracts have multi-functional properties (pro-cholinergic, anti-oxidant, anti-amyloid, and anti-inflammatory), and their use in the treatment of AD patients look promising (Akhondzadeh & Abbasi, 2006).

Parkinson's disease (PD) is a debilitating degenerative disease resulting from massive degenerative loss of dopamine neurons, particularly in the substantia nigra. The most classic therapy for PD is levodopa administration, but the efficacy of levodopa treatment decreases as the disease progresses. The neuroprotective strategies to rescue nigral dopamine neurons from progressive death are currently being explored, and among them, the Chinese herbs and herbal extracts have shown potential clinical benefit in attenuating the progression of PD in human beings (Houghton & Howes, 2005; Li *et al.*, 2006; Chen *et al.*, 2007). Growing studies indicated a range of Chinese herbs or herbal extracts are able to attenuate degeneration of dopamine neurons and symptoms caused by the neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) *in vitro* and *in vivo* conditions. These are green tea polyphenols or catechins, panax ginseng and ginsenoside, *Ginkgo biloba* and EGb 761, polygonum,

triptolide from *Tripterygium wilfordii*, polysaccharides from the flowers of *Nerium indicum*, oil from *Ganoderma lucidum* and *Verbena officinialis*. In addition, available data have suggested that herbal extracts may promote neuronal survival, growth, and facilitate functional recovery of brain injuries by invoking distinct mechanisms that are related to their neuroprotective roles as the antioxidants, dopamine transporter inhibitor, monoamine oxidase inhibitor, free radical scavengers, chelators of harmful metal ions, modulating cell survival genes and signaling, anti-apoptosis activity, and even improving brain blood circulation (Radad *et al.*, 2006; Lai *et al.*, 2006; Chen *et al.*, 2007; Ramassamy *et al.*, 2007; Wang *et al.*, 2007). New pharmaceutical strategies against PD will hopefully be discovered by understanding the various active entities and valuable combinations that contribute to the biological effects of Chinese herbs and herbal extracts. Chang and So (2008) studied *Lycium barbarum* and its anti-aging properties. Valuable components of *L. barbarum* are not limited to its colored components containing zeaxanthin and carotene, but include the polysaccharides and small molecules such as betaine, cerebroside, beta-sitosterol, p-coumaric, and various vitamins. Despite the fact that *L. barbarum* has been used for centuries, its beneficial effects have not been comprehensively studied with modern technology to unravel its therapeutic effects at the biochemical level. Polysaccharides extracted from *L. barbarum* can protect neurons against β-amyloid peptide toxicity in neuronal cell cultures, and retinal ganglion cells in an experimental model of glaucoma. They have accumulated scientific evidence for its anti-aging effects that should be highlighted for modern preventive medicine and pave a new avenue for the use of Chinese medicine in modern evidence-based medicine. The study of Yu *et al.* (2007) has opened a window for the development of a neuroprotective agent for anti-aging from Chinese medicine. Increasing evidence indicates that neuroglia-derived chronic inflammatory responses play a pathological role in the central nervous system, anti-inflammatory herbal medicine and its constituents are being proved to be a potent neuroprotector against various brain pathologies. Structural diversity of medicinal herbs makes them valuable source of novel lead compounds against therapeutic targets that are newly discovered by genomics, proteomics, and high-throughput screening.

Anti HIV/AIDS Compounds

The acquired immunodeficiency syndrome (AIDS) is a result of human immunodeficiency virus (HIV) infection which subsequently leads to significant suppression of immune functions. AIDS is a significant threat to the health of mankind, and the search for effective therapies to treat AIDS is of paramount importance. Several anti-HIV agents have been developed. However, besides the high cost, there are adverse effects and limitations in

using these anti-HIV agents for the treatment of HIV infection. Plant products, with their broad chemical structural diversity, provide an excellent opportunity to deliver significant therapeutic advances in the treatment of HIV/AIDS. Many plant derived natural products with novel structures have been identified as having anti-HIV activities (Jung *et al.*, 2000; Yang *et al.*, 2001; Li *et al.*, 2003; Yu *et al.*, 2007; Itokawa *et al.*, 2008). Betulinic acid, a triterpenoid isolated from *Syzigium claviflorum*, has been found to contain anti-HIV activity in lymphocytes (Fujioka *et al.*, 1994). The quassinoside glycoside isolated from *Allanthus altissima* has activity to inhibit HIV replication (Sun *et al.*, 1998). A novel phorbol ester isolated from *Excoecaria agallocba* has been reported to be a potent inhibitor of HIV-1 reverse transcriptase (Jung *et al.*, 2000; Cos *et al.*, 2004; Yu *et al.*, 2005). Chen *et al.* (2003) reported Shikonin, a component of Chinese Herbal Medicine, inhibits chemokine receptor action and suppresses HIV-Type1.

Liu *et al.* (2006) summarized in this review the progress of anti-HIV compounds and traditional Chinese medicines. It is a rich source of potentially useful materials for the treatment of HIV infection. Some of them are much more potent in anti-HIV activity. And some components extracted from the herbs are even more tonic than the crude herb medicines. It has been proved that some active components such as alkaloids, proteins, flavonoids, quercetin, terpene, lignanoid are able to work on HIV. One should pay more attention to the study of traditional medicine and the lead compounds for HIV/AIDS. *Lomatium suksdorfii* (Apiaceae) yielded suksdorfin (N1), a dihydroseselin-type angular pyranocoumarin, as a lead anti-HIV natural product. N4, synthetic modification of N1 has been selected as a clinical trial candidate. DCK N6 and DCP N5 compounds inhibit HIV reverse transcriptase (RT). The novel mechanism of action of DCK and DCP compounds (known as strand transfer inhibitors) in comparison to current drugs encourage for further investigation of their possible usefulness in the treatment of AIDS (Yu *et al.*, 2004, 2007). *Syzigium claviflorum*, source of two naturally occurring anti-HIV constituents - betulinic acid (O1) and platanic acid (O2). SAR studies produced dimethyl succinyl betulinic acid (DSB, O3), which has successfully progressed to anti-AIDS clinical trials. To date, O3 has completed seven clinical trials in over 300 patients (noninfected and infected). O3 disrupts the late stage viral maturation processes of HIV, making it unlike any currently approved drug for AIDS. The viral core structure of new HIV particles produced from infected DSB-treated cells is defective and noninfectious. O3 is the first in a new class of drug for AIDS with a novel target of viral maturation. The Phase I and Phase II studies of O3 were completed during 2004. The drug was well tolerated and showed good anti-HIV activity. Beneficial effects were observed in HIV-infected patients and significantly reduced viral load and proved to be very safe with no evidence of organ toxicity or clinical intolerance (Li *et al.*, 2003; Yu *et al.*, 2005).

Wu *et al.* (2001) summarized the reports on herbal medicines that inhibit HIV. They emphasized on Chinese herbal medicine, *Scutellaria baicalensis* Georgi and its identified components (*i.e.* baicalein and baicalin), which have been shown to inhibit infectivity and replication of HIV. In China, medicinal herbs are being used in the treatment of HIV positive subjects and AIDS patients. One example is the traditional Chinese medicinal herb Tian-Hua-Fen (*Trichosanthes kirilowii*), which appears in the classical Chinese medical reference work Trichosanthin (TCS), an active protein component isolated from Tian-Hua-Fen, has been shown to inhibit HIV infection and has been used in the treatment of AIDS. The results of *in vitro* study of anti-HIV effects of JinHuang, a Chinese herbal medicine led to *in vivo* study of safety and efficacy among asymptomatic HIV infected individuals. It was a prospective open study of 21 asymptomatic HIV infected Thai volunteers. No serious adverse event related to JinHuang was detected during study. No significant changes in terms of log viral load and CD4 count were observed after 6-months duration. Most of the patients showed good health (Maekanantawat *et al.*, 2003; Sugimoto *et al.*, 2005).

Hepatoprotective Plant Products

Liver diseases affect over 10% of the world population. This includes chronic hepatitis, alcoholic steatosis, fibrosis, cirrhosis and hepatocellular carcinoma (HCC). Patients with alcoholism or viral hepatitis are much more likely to have liver cell damage and cirrhosis, and in some cases it may lead to HCC, which is often, a fatal malignancy without cure. Alternative medicines - Chinese herbal medicine, Ayurveda of Indian herbal medicine are being actively sought from other sources to control the disease's progression or to achieve tumor regression (Thyagarajan *et al.*, 2002; Wang *et al.*, 2007; Luk *et al.*, 2007; Asl & Hosseinzedeh, 2008; Li *et al.*, 2008; Muriel & Rivera-Espinoza, 2008).

Hepatitis B virus (HBV) infection troubles Asia population and, in Hong Kong, about 10% is Hepatitis B surface antigen carrier. Herbal medicine, KY88 composed of *Fructus schisandrae* possessing immunomodulatory property was adopted by Chinese medicine practitioner for treatment of acute and chronic HBV infection. However, the underlying impact on host immune system is not fully understood (Yip *et al.*, 2007). Pradhan and Girish (2006) discussed in their review about Silymarin, a flavonolignan from 'milk thistle' (*Silybum marianum*) plant which is used for hepatoprotection. It is also discusses about its safety, efficacy and future uses in liver diseases. Silymarin consists of four flavonolignan isomers namely--silybin, isosilybin, silydianin and silychristin. Silymarin is orally absorbed and is excreted mainly through bile as sulphates and conjugates. Silymarin offers good protection in various toxic models of experimental liver diseases in laboratory animals. It acts by antioxidative, anti-lipid peroxidative, antifibrotic, anti-inflammatory,

membrane stabilizing, immunomodulatory and liver regenerating mechanisms. Silymarin may prove to be a useful drug for hepatoprotection in hepatobiliary diseases and in drug induced hepatotoxicity. It has a good safety profile, better tolerability and the price is affordable. New derivatives or new combinations of this drug may prove to be useful in future.

Thyagarajan *et al.* (2002) presented a report on the scientific approaches made to herbal preparations used in Indian systems of medicine for the treatment of liver diseases. In spite of the availability of more than 300 preparations for the treatment of jaundice and chronic liver diseases from more than 87 Indian medicinal plants, only four terrestrial plants have been scientifically elucidated to the internationally acceptable scientific protocols. Studies have proved *Silybum marianum* to be anti-oxidative, antilipidperoxidative, antifibrotic, anti-inflammatory, immunomodulatory and liver regenerative. *Glycyrrhiza glabra* has been shown to be hepatoprotective and capable of inducing an indigenous interferon (Rajesh & Latha, 2004; Fiore *et al.*, 2008). *Picrorhiza kurroa* is proved to be anti-inflammatory, hepatoprotective and immunomodulatory (Thyagarajan *et al.*, 2002). Extensive studies on *Phyllanthus amarus* have confirmed as anti-viral against hepatitis B and C viruses, hepatoprotective and immunomodulating, as well as possessing anti-inflammatory properties. For the first time in the Indian systems of medicine, a chemo-biological fingerprinting methodology for standardization of *P. amarus* preparation has been patented. Licorice root, curcuma species are used mainly for the treatment of peptic ulcer, hepatitis C, and pulmonary and skin diseases, although clinical and experimental studies suggest that it has several other useful pharmacological properties such as antiinflammatory, anti-HIV, antiviral, antimicrobial, antioxidative, anticancer activities, immunomodulatory, hepatoprotective and cardioprotective effects. A large number of components have been isolated from licorice, including triterpene saponins, flavonoids, isoflavonoids and chalcones, with glycyrrhizic acid normally being considered to be the main biologically active component (Asl & Hosseinzedeh, 2008; Itokawa *et al.*, 2008; Fiore *et al.*, 2008).

It was reported earlier from the author's laboratory that mitochondrial dysfunction caused by the administration of alcohol (Sebastian & Setty, 1999) or thioacetamide (Padma & Setty, 1997) or carbon tetrachloride (Padma & Setty, 1999) or allyl alcohol (Sailaja & Setty, 2006) could be prevented by prior administration of an aqueous extract of *Phyllanthus fraternus* and the protective effect was attributed mainly to the antioxidant potential of the extract.

Antibacterial and Antiviral Plant Products

Several bacterial infections are associated with the risk of certain cancer, and viruses are now recognized as the second most important cause of

human cancer. Many chemicals produced in plants are having antimicrobial and antiviral activities, these compounds are being examined for their potential to inhibit human pathogens. A preliminary screening of 35 different Indian spices and herbs indicated that clove, cinnamon, bishop's weed, chilli, horseradish, curcumin, tamarind, black cumin, pomegranate seeds, nutmeg, garlic, onion, tejpat, celery, and cambodge had potent antimicrobial activities against the test organisms, *Bacillus subtilis*, *Escherichia coli*, and *Saccharomyces cerevisiae* (Biradar *et al.*, 2008). Garlic and cloves also possess antimicrobial activity against some human pathogenic bacteria and yeasts *in vitro*; some bacteria that showed resistance to certain antibiotics were sensitive to the extracts of both garlic and clove. *In vitro*, garlic extracts are also potent inhibitors of *Helicobacter pylori*, the bacteria associated with gastric cancer risk. Compounds with antiviral activity include alkaloids, carbohydrates, chromones, coumarins, flavonoids, lignans, phenolics, quinines, xanthones, phenylpropanoids, tannins, terpenes, steroids, iridoids, thiopenes, polyacetylenes, lactones, butenolides, phospholipids, proteins, peptides, and lectins (Martin & Ernst, 2003; Corson *et al.*, 2006; Biradar *et al.*, 2008; Mothana *et al.*, 2008).

The *Echinacea* species-*E. angustifolia*, *E. pallida* and *E. purpurea* have a long history of medicinal use for a variety of conditions, particularly infections, and today echinacea products are among the best-selling herbal preparations in several developed countries. Modern interest in echinacea is focused on its immunomodulatory effects, particularly in the prevention and treatment of upper respiratory tract infections. Evidence from preclinical studies supports some of the traditional and modern uses for echinacea, particularly the reputed immunostimulant properties. Clinical trials of echinacea preparations have reported effects superior to those of placebo in the prevention and treatment of upper respiratory tract infections. However, evidence of efficacy is not definitive as studies have included different patient groups and tested various different preparations and dosage regimens of echinacea. On the basis of the available limited data on safety, echinacea appears to be well tolerated (Barnes *et al.*, 2005).

Popularity of *Momordica charantia* in various systems of traditional medicine for several ailments (antidiabetic, abortifacient, anthelmintic, contraceptive, dysmenorrhea, eczema, emmenagogue, antimalarial, galactagogue, gout, jaundice, abdominal pain, kidney stone, laxative, leprosy, leucorrhea, piles, pneumonia, psoriasis, purgative, rheumatism, fever and scabies) focused the investigator's attention on this plant. Over 100 studies using modern techniques have authenticated its use in diabetes and its complications (nephropathy, cataract, insulin resistance), as antibacterial as well as antiviral agent (including HIV infection), as antihelminthic and abortifacient. Traditionally it has also been used in treating peptic ulcers, and experimental studies have shown its potential against *Helicobacter pylori*

(Newman *et al.*, 2003; Grover & Yadav, 2004). Lopez *et al.* (2001) reported strong antiviral and antimicrobial activities in methanolic extracts from 24 plants used traditional medicine in the treatment of skin infections in Colombia. The most potent extract was obtained from *Byrsonima verbascifolia* (L.). Antimicrobial screening was conducted using the disc diffusion assay against *Klebsiella pneumoniae*, *Escherichia coli*, *Streptococcus faecalis*, *Mycobacterium phlei*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and the human pathogenic yeast, *Candida albicans*.

Antiinflammatory and Immunosuppressant Plant Products

Prostaglandins and other eicosanoids influence carcinogenesis through action on nuclear transcription sites and downstream gene products are important in the control of cell proliferation (Cragg & Newman, 2001). Nonsteroidal antiinflammatory drugs, potent inhibitors of cyclooxygenase (COX), the enzyme responsible for prostaglandin synthesis, are associated with reduced risk of several cancers. Thus, natural products, including spices, have been examined for their capacity to inhibit COX or other parts of the inflammation pathway. Ginger (Ozaki *et al.*, 1991) and curcumin (Ireson *et al.*, 2001; Lampe, 2003) have been reported to interfere with inflammatory processes. Herbal antirheumatics are successfully used in painful inflammatory or degenerative rheumatic diseases. One of these herbal medicines is Phytodolor (STW 1), a fixed combination of extracts from aspen leaves and bark (*Populus tremula*), common ash bark (*Fraxinus excelsior*), and golden rod herb (*Solidago virgaurea*). The effects of the extract and the components have been verified in experimental and human pharmacological investigations. Open clinical studies and randomised, placebo- or verum-controlled double-blind trials, performed in different subtypes of rheumatic diseases, confirm the pharmacological evidence of efficacy and safety (Gundermann & Muller, 2007). Li *et al.* (2007) examined the efficacy of popular Chinese herbs used in a traditional Chinese medicine combination of *Ganoderma lucidum* and San Miao San (SMS), with purported diverse health benefits including antioxidant properties in rheumatoid arthritis (RA).

A wide variety of natural products are claimed to possess immunosuppressant activity, but it is often difficult to separate this activity away from cytotoxicity that is associated with it (Mann, 2002). The survival of individuals with transplants is owed in large part to the discovery of the fungal metabolite cyclosporine A in 1970 and its widespread use starting in 1978. Indeed, cyclosporine A has achieved such success that it is currently being evaluated for value in the treatment of Crohn's disease, systemic lupus erythematosus, and rheumatoid arthritis. Research efforts abound in the area of natural products and immunosuppression. The experimental immunosuppressant (+)-discodermolide isolated from the marine sponge *Discodermia dissoluta* exhibits relatively nonspecific immunosuppression,

causing the cell cycle to arrest during G2 and M phases. The didemnins, cyclic peptides, were first isolated from the marine tunicate *Tridemnum solidum* and exhibit immunosuppressive activity through a generalized cytotoxicity. The trichopolyns I to V from the fungus *Trichoderma polysporum* are lipopeptides that suppress the proliferation of lymphocytes in the murine allogeneic mixed lymphocyte response assay. Triptolide from the plant *Tripterygium winfordii* demonstrates immunosuppressant activity through the inhibition of IL-2 receptor expression and signal transduction. As immunological research progresses, increasingly more potential targets will be elucidated for immunomodulatory therapeutic intervention.

Antimalarial Compounds

Traditional medicines have been used to treat malaria for thousands of years and are the source of the two main groups (artemisinin and quinine derivatives) of modern antimalarial drugs. The dried aerial parts of the herb *Artemisia annua* (Asteraceae) is used to treat fever and malaria. The active principle is artemisinin (P1). This clinically effective antimalarial compound rapidly kills *Plasmodium falciparum*, the malaria parasites, without being harming humans or animals (Li *et al.*, 2006). The synthetic derivatives artemether (P2) and artemether (P3) are widely used in malaria-prone regions, particularly in India (Pareek *et al.*, 2006); World Health Organization (2008) lists artemether and sodium artesunate (a hemisuccinate derivative of dihydroartemisinin) (P4) in its Model List of Essential Medicines. Artemisinin, isolated from *Artemisia anuua*, a sesquiterpene lactone has potent antimalarial activity, and activity against *Pneumocystis carinii* (Yu *et al.*, 2007). Many case studies have reported on herbal antimalarials. Cohort studies mentioning herbal treatments, 17 were for falciparum malaria, 12 for vivax malaria, and 5 for malaria of undefined species (Willcox & Gerard Bodeker, 2004). Gathirwa *et al.* (2008) investigated the toxicity, anti-plasmodial and antimalarial efficacy of several herbal drug combinations. *Lannea schweinfurthii*, *Turraea robusta* and *Sclerocarya birrea*, used by traditional health practitioners in Meru community, were tested for *in vitro* anti-plasmodial and *in vivo* anti-malarial activity singly against *Plasmodium falciparum* and *Plasmodium berghei*, respectively. Methanolic extract of *Turraea robusta* was the most active against *Plasmodium falciparum* D6 strain. Aqueous extracts of *Lannea schweinfurthii* had the highest anti-plamodial and antimalarial activity followed by *Turraea robusta* and *Sclerocarya birrea*. Different combinations of *Turraea robusta* and *Lannea schweinfurthii* exhibited good *in vitro* synergistic interactions. Combinations of *Boscia salicifolia* and *Sclerocarya birrea*; *Rhus natalensis* and *Turraea robusta*; *Rhus natalensis* and *Boscia salicifolia*; *Turraea robusta* and *Sclerocarya birrea*; and *Lannea schweinfurthii* and *Boscia salicifolia* exhibited high malaria parasite suppression (chemosuppression >90%) *in vivo* when tested in mice. The findings are a preliminary demonstration of the

usefulness of combining several plants in herbal drugs, as a normal practice of traditional health practitioners. Many plants sold at markets are known locally as "general tonics," "nerve tonics," or "aphrodisiacs." A well known example of a drug (captopril) extracted from the Brazilian biodiversity is the antihypertensive agent, pilocarpine (*Pilocarpus microphyllus*- anti-glaucoma agent), and quinine (*Cinchona ledgeriana*-antimalarial).

CONCLUSIONS

Plant derived natural products hold great promise for discovery and development of new pharmaceuticals. Careful consideration of the entire process of discovery and development - a "systems" approach - will be required to realize this great promise effectively. The potential benefits of herbal medicines could lie in their high acceptance by patients, efficacy, relative safety, and affordable costs. Patients worldwide seem to have adopted herbal medicines in a major way. The efficacy of herbal medicines has been tested in hundreds of clinical trials, and it is not correct to say that they are all of inferior methodological quality. But this volume of data is still small considering the multitude of herbal medicines several thousand different plants in worldwide are being used for medicinal purposes. Clinical observations on traditional remedies are feasible and useful. Some herbal remedies may be safe and effective for the treatment of diseases, as shown in the review. Nevertheless, better evidence from randomised clinical trials is needed before these medium are recommended on a large scale. As such trials are expensive and time consuming, so it is important to prioritise remedies for clinical investigation according to existing data from sociological, ethnobotanical, pharmacological, and preliminary clinical observations.

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6

In vitro and *In vivo* Combined Anti-Influenza Virus Effects of a Plant Polyphenol-Rich Extract and Synthetic Antiviral Drugs

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ABSTRACT

Influenza is a highly contagious, acute respiratory disease that affects all age groups, can occur repeatedly in any particular individual and remains one of the most serious problems of public health. Despite the achievements of antiviral chemotherapy, the need of new potent antiviral agents for influenza virus infection continues to exist alongside with the necessity of novel strategies for its control. The mode of the anti-influenza virus activity of the semi-standardized plant polyphenol extract, isolated from the medicinal plant Geranium sanguineum L. (PC) has been studied intensively (Serkedjieva & Hay, 1998). It was shown that its in vitro virus-inhibitory effect was strain-dependent, consistent with a selective antiviral action. PC affected the synthetic stages of replication; virus-specific RNA and protein synthesis were selectively inhibited. We have demonstrated also that the plant preparation markedly protected mice from mortality in the experimental influenza virus infection (EIVI, Serkedjieva & Manolova, 1992). Rimantadine hydrochloride (Rim), zanamivir (Zan) and ribavirin (Rib) are approved selective anti-influenza virus drugs. The combined application of PC with any of them resulted in marked enhancement of the inhibitory effect of PC on the reproduction of a range of influenza viruses in MDCK cells. The antiviral activity was determined by the difference in the infectious titers of control and treated viruses and the combined effect was defined on the base of infectious viral yields. The combined effects ranged from indifferent to synergistic. The most pronounced enhancement was achieved by the combinations of PC and Rib. In the EIVI the combinations varied from antagonistic to synergistic and the most effective were the combinations of

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PC with Zan. The present results clearly indicate that combination therapy provides an advantage over single-agent therapy and suggests that the application of natural and synthetic viral inhibitors in appropriate combinations also offers possibilities with this respect.

Key words : Combined antiviral effect, influenza virus infection, polyphenol-rich extract, ribavirin, rimantadine, viral inhibition, zanamavir

INTRODUCTION

Influenza is a major public health problem because of its wide spread, high morbidity rate and considerable social and economic implications. Despite the considerable achievements of antiviral chemotherapy, the options for control and treatment of the disease are limited and an obvious need continues to exist for effective therapies. While the need for novel potent antiviral agents continues to exist, the strategy of combined antiviral therapy with available antiviral drugs has demonstrated its usefulness and proved to be a key strategy in the control of numerous viral infections. The combined use of antiviral agents helps the enhancement of viral inhibition, the reduction of toxicity and the prevention of antiviral resistance. It provides an advantage over single-agent therapy and suggests that the combined treatment may offer an effective basis for prophylactics and therapy of influenza virus infection. The data on the combined inhibitory activity of natural and synthetic antiviral agents though scarce suggest that this could be a promising approach in the control of viral infections (Barquero & Villamil, 1997; Corina *et al.*, 1999; Kahlon *et al.*, 1991; Kurokawa *et al.*, 1995; Musci *et al.*, 1992; Weaver & Arou, 1998), may be used successfully to enhance the antiviral efficacy of the plant preparations and may enable dose reduction of their toxic components. Our group has a vast experience in this field of research (Dzeguze *et al.*, 1982; Gegova *et al.*, 1993; Serkedjieva, 2000; Serkedjieva & Ivanova, 1997; Serkedjieva & Zgorniak-Nowosielska, 1993; Serkedjieva *et al.*, 1986, 2003, 2005; Uzunov *et al.*, 1991).

The mode of the anti-influenza virus activity of the semi-standardized plant polyphenol extract, isolated from the medicinal plant *Geranium sanguineum* L. (PC) has been studied intensively. It was shown that its *in vitro* virus-inhibitory effect was strain-dependent, consistent with a selective antiviral action. PC affected the synthetic stages of replication; virus-specific RNA and protein synthesis were selectively inhibited (Serkedjieva & Hay, 1998). We have also demonstrated that the plant preparation markedly protected mice from mortality in the experimental influenza virus infection (EIVI, Serkedjieva & Manolova, 1992).

The licensed anti-influenza drugs (the M2 ion channel blockers, amantadine and rimantadine, and the neuraminidase inhibitors, oseltamivir and zanamivir) are beneficial for uncomplicated seasonal influenza, but

appropriate dosing regimens for severe seasonal or H5N1 viral infections have not been defined. Treatment options may be limited by the rapid emergence of drug-resistant viruses. Ribavirin has also been used to a limited extent to treat influenza.

The present investigation focused on the combined anti-influenza virus effects of PC and three approved synthetic antiviral drugs - rimantadine hydrochloride, zanamavir and ribavirin – in cell cultures and in the murine EIVI.

MATERIALS AND METHODS

Substances. PC was prepared as described previously (Serkedjieva & Manolova, 1992) and kindly provided by Dr. S. Ivancheva, Institute of Botany, Bulgarian Academy of Sciences. Rimantadine hydrochloride (Rim) was purchased from Hoffman-La Roche Inc., Nutley, NJ, USA. Zanamavir (Zan) was kindly provided by GlaxoSmithKline, UK. Ribavirin (Rib) was obtained from Sigma Aldrich GmbH, Germany.

Cells and Viruses. MDCK cells and CAM tissue cultures, media and influenza viruses A/chicken/Germany/34, strain Rostock (H7N1) (A/Rostock), A/PR/8/34 (H1N1), A/Hong Kong/1/68 (H3N2), A/Aichi/2/68 (H3N2) (A/Aichi) and A/Aichi/2/68 (H3N2), adapted to mice lungs (A/Aichi-a) were as in (Serkedjieva & Hay, 1998).

Mice. Male and female (16–18 g) ICR mice were obtained from the Experimental Animal Station of the Bulgarian Academy of Sciences, Slivnitza. They were quarantined for 24 h before use and maintained on standard laboratory chow and tap water *ad libitum* for the duration of the studies.

Cellular toxicity was monitored as described in (Serkedjieva & Hay, 1998). The 50% toxic concentrations, the doses, causing visible changes in 50% of intact cultures (TC_{50}) were determined.

Antiviral assays. The antiviral effect was studied in multicycle experiments of viral growth. The virus-induced cytopathogenic effect (CPE) and the production of infectious virus were used as measures of viral growth. *CPE-reduction assay* was as in (Serkedjieva & Hay, 1998). The concentration reducing CPE by 50% (EC_{50}) with respect to virus control was estimated from graphic plots. The selectivity index (SI) was determined from the ratio TC_{50}/EC_{50}. *Infectious virus yield (IVY) reduction assay* was as in (Serkedjieva & Hay, 1998). Virus titers were determined by endpoint titration (Reed & Muench, 1938) and expressed accordingly in HA units (HAU) and 50% tissue culture infectious doses ($TCID_{50}$/mL). The concentrations that reduced virus infectivity by 90% (1 $\log_{10}TCID_{50}$/mL) were determined (EC_{90}).

PC (0.15–2.5 μg/mL), Rim (0.15–2.5 μg/mL), Zan (0.15–2.5 μg/mL) and Rib (0.31–5.0 μg/mL) were inoculated simultaneously with viral infection. The type of the *combined antiviral effect* was determined according to the method of Schinazi *et al.*, (1982), based on virus yields. The fractional yield of the compound A (Y_A) was defined as the viral titer in the presence of the compound divided by the titer obtained in the absence of the compound. The same was done for the second compound (Y_B) and the combination (Y_{AB}). Then Y_C was calculated according to the formulae $Y_C=Y_A x Y_B$, if $Y_C>Y_{AB}$ - the effect was synergistic; if $Y_C=Y_{AB}$ - the effect was indifferent; if $Y_C<Y_{AB}$ - the effect was antagonistic.

Experimental influenza virus infection (EIVI) was induced under light ether anesthesia by the intranasal inoculation of A/Aichi-a in mice, which causes hemorrhagic pneumonia. To produce lethal infection mice were infected with 10 LD_{50}. PC was applied by intranasal instillation 3 h before the infection in the doses 1.25–5 mg/kg in the volume of 0.05 mL PBS. Rim and Rib were administered orally -24, -2, +24, +48, +72 h with respect to infection in the dose of 10 mg/kg in the volume of 0.2 mL PBS; Zan was applied nasally -24, -2, +24, +48, +72 h with respect to infection in the dose 2.5 mg/kg in the volume of 0.05 mL PBS. Experimental groups were of 12 animals each. To determine infectious lung parameters additional groups of 3 animals from each experimental group were sacrificed on day 6 p.i., lungs were removed aseptically, lung consolidation (score) was scored and lung suspensions were assayed for infectivity in MDCK cells as in (Serkedjieva & Ivanova, 1997). Virus-induced CPE was used as a measure of viral replication. The *protective effect* was estimated by the reduction of the rate of mortality, the increase of the protective index (PI) and prolongation of mean survival time (MST) as in Serkedjieva & Ivanova, 1997. PI was determined from the equation (PR-1)/PRx100, where PR (protection ratio) was $M_{control}/M_{experiment}$ and M was mortality. Mice were observed for death daily for 14 days. After the end of the experiments surviving animals were sacrificed by cervical dislocation. Toxicity controls (4 mice per combination) were run in parallel. The results are the mean of 3 experiments.

The combined effect was evaluated according to (Webb, 1966). The effect of the combination ($E_{1,2}=PI_{1,2}/100$) and the effects of the individual substances ($E_1=PI_1/100$ and $E_2=PI_2/100$) are related in the equation $E_{1,2}=E_1+E_2-E_1 x\ E_2$; the combined effect is synergistic if $E_{1,2}$ is >, indifferent if $E_{1,2}$ is = and antagonistic if $E_{1,2}$ is $<E_1+E_2-E_1 x\ E_2$.

Statistical Methods

Results are given as arithmetic mean values. Student's *t*-test was used to evaluate differences in virus titres and *in vivo* experiments to analyze differences in lung scores and lung virus titres. $p<0.05$ was accepted for statistical significance.

RESULTS AND DISCUSSION

Influenza is an acute respiratory disease that affects all age groups, can occur repeatedly in any particular individual and remains one of the most serious problems of public health. The infectious agent - influenza virus - is highly contagious and causes seasonal epidemics, which affect 10 to 20% of the population. Furthermore, the WHO has recently decreed that "another influenza pandemic is inevitable and possibly imminent", an alarming prospect considering the devastation and fatalities experienced in the previous pandemic of recent times. Influenza infection is usually self-limiting, culminating in a local and systemic reaction. However, there remains a significant proportion of patients who develop severe illness and complications, such as the elderly, the very young and the immunocompromised. Even with the development of killed virus vaccines, illness due to influenza virus infection continues to be a major health problem throughout the world. Currently available antiviral therapies were developed with the aim of reducing the impact of seasonal influenza, rather than to treat life-threatening disease, but the emergence of the virulent H5N1 virus has forced the community to take another look at influenza and begin to approach it with the seriousness it deserves. Thus new and better antiviral therapies are needed for the better achievement of the goals of antiviral treatment for influenza, namely to decrease symptoms and functional disability and, more important, to decrease associated complications, hospitalizations and mortality. One of the possible ways of enhancing the effect of antiviral substances is to use them in appropriate combinations. This provides a prospect to potentiate the inhibitory effect of the substances, to reduce their toxic action and to prevent the appearance of resistant virus mutants. The data on the combined inhibitory activity of natural and synthetic antiviral agents, though scarce, suggest that this could be a promising approach in the control of viral infections. Plant extracts, decoctions and infusions have been used traditionally for the treatment of various human diseases. Our data together with the evidence provided by (Barquero & Villamil, 1997; Corina *et al.*, 1999; Kahlon *et al.*, 1991; Kurokawa *et al.*, 1995; Musci *et al.*, 1992; Weaver & Arou, 1998) suggest that the combined application of natural and synthetic viral inhibitors may be used successfully to potentate the antiviral efficacy of the plant preparations and may enable dose reduction of their toxic components.

We have studied intensively the mode of the anti-influenza virus activity of the partially standardized semi-standardized polyphenol-rich extract, isolated from *Geranium sanguineum* L., designated as polyphenolic complex (PC) (Serkedjieva & Hay, 1998). It was shown that its *in vitro* virus-inhibitory effect was strain-dependent, consistent with a selective antiviral action. In one-cycle experiments of viral growth with A/Rostock in chicken embryonic fibroblast cells, PC was most effective applied 1 to 3 h after virus infection

(synthetic stages of viral replication). Virus-specific protein synthesis as well as virus-specific RNA synthesis in rabbit kidney cells, infected with A/WSN/33 (H1N1) were selectively inhibited. In addition the selectivity of inhibition was confirmed by the generation of PC-resistant mutants of A/Rostock (Serkedjieva, 2003). However the investigations showed that the *in vitro* virus-inhibitory activity was fairly modest and this was in contrast with the significant protection *in vivo*. Thus the therapeutic effect of PC needed explanation. We presumed that it might be attributed to a combination of more than one biological activity - selective antiviral effect, non-selective immunomodulating activity and some non-specific biological and pharmacological interactions known for natural polyphenols, such as protein binding, radical scavenging and antioxidant activities (Serkedjieva, 2008). Following this line of investigation we established that in model systems the plant preparation possessed a number of biological activities: a stimulating effect on cell type immune response, induction of serum interferon after intraperitoneal application (Toshkova *et al.*, 2004), O_2^- radical scavenging activity (Sokmen *et al.*, 2005) and protease-inhibitory effect (Antonova-Nikolova *et al.*, 2002). The immunomodulatory action, the effect on the lung protease activity and the antioxidant capacity of the extract were confirmed *in vivo* (Ivanova *et al.*, 2005; Serkedjieva *et al.*, 2007; Murzahmetova *et al.*, 2008). The variety of biological activities of the plant extract was related to the presence of large quantities of polyphenolic compounds.

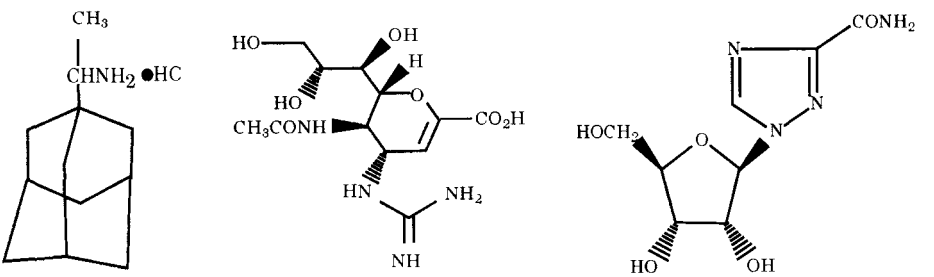
To provide evidence how a maximum therapeutic advantage can be derived of the extract, PC was inoculated by 6 different routes according to 29 schedules of treatment (Serkedjieva *et al.*, 2002). The nasal and the aerosol application proved to be highly effective. This might be due to an increased amount of the extract reaching the viral-targeted tissues (Serkedjieva *et al.*, 2007).

Phytochemical analysis of PC showed that the total polyphenolic content of the extract was 167.8 μg/mL; the extract contained tannins (34%), flavonoids (0.17%), catechins and proanthocyanidines (2 mg/kg). The identification of individual compounds showed that flavonoids - aglycones and glycosides (quercetin, quercetin 3-O-galactoside, morin, myricetin, kaempferol, rhamnasin, retusin, apigenin), phenolic acids (caffeic, ellagic, quinic, chlorogenic), gallotannins and catechins were present. The chemical composition was confirmed by HPLC analysis (Pantev *et al.*, 2006). We presume that the antiviral effect of the preparation could not be attributed to one or few separate ingredients; the presence of a variety of biologically active compounds as well as the possible synergistic interactions between the constituents seemed to be more significant for the overall virus-inhibitory effect.

For infections with the influenza virus, the M2 channel inhibitors (amantadine and rimantadine) and the neuraminidase inhibitors (oseltamivir

and zanamivir) have been formally licensed. The first synthetic compound shown to inhibit influenza replication was amantadine. This compound blocks the migration of H+ ions via the viral M2 ion channel into the interior of the virus particles residing in the endosomes (Hay, 1989). Acidification of the interior of the viral particle results in destabilization of the capsid and leads to uncoating. Rimantadine hydrochloride (Fig 1A), an analogue of amantadine hydrochloride has well documented prophylactic (Dolin *et al.*, 1982) and therapeutic (Van Voris *et al.*, 1981) activity in uncomplicated influenza A virus infection after oral administration. It specifically inhibits the replication of influenza A viruses by interfering with the uncoating process of the virus (Bukrinskaya *et al.*, 1982). For certain influenza H7 infections inhibition takes place at a later stage during replication and prevents virus release by a specific interaction with the viral M2 protein (Hay, 1989). The drug is effective against all influenza A subtypes that have previously caused disease in humans (H1N1, H2N2 and H3N2), but not against influenza B virus, because the M2 protein is unique to influenza A viruses. Rimantadine has no marked antiviral activity or therapeutic effectiveness in established influenza. Some adverse effects of rimantadine has also been reported. (Herrmann *et al.*, 1990) found that following rimantadine treatment some depression of cytotoxic T- lymphocyte response and antibody response in a mouse model occurred. In addition development of viral resistance to rimantadine has been identified as a problem in the use of this drug (Hayden, 2006). In recent years, there has been a dramatic increase in the prevalence of Rim-resistant influenza virus strains (both seasonal influenza and H5N1 avian influenza). The extensive use of amantadine in poultry farms in Asia may be one of reasons of the high amantadine-resistance incidence (for review see Lleyssen *et al.*, 2008 and references cited in).

There is abundant evidence for the mechanism by which influenza virus is released from the host cell by the viral neuraminidase. After budding from the host cell, the viral haemagglutinin interacts with the host cell receptor, bearing N-acetylneuraminic acid (NANA). The neuraminidase cleaves off NANA from the cell-surface glycoprotein at a specific bond, the sialic acid linked to galactose by an alfa 2,3 or alfa 2,6 linkage. This enables the progeny virus to leave the infected cells and to spread to other host cells.



A *B* *C*

Fig 1. Structure of rimantadine hydrochloride (A), zanamivir (B) and ribavirin (C).

Inhibition of the neuraminidase prevents virus release and thus the spread of the virus (for review see Lleyssen *et al.*, 2008 and references cited in). Further rational design led to the development of the neuraminidase inhibitors zanamivir (Fig 1B) and oseltamivir (von Itzstein *et al.*, 1993). Both compounds are highly potent inhibitors of the influenza neuraminidase and influenza A and B virus replication *in vitro* and *in vivo* (mice, ferrets). They are both prophylactically and therapeutically effective against influenza in humans. The availability of an injectable neuraminidase inhibitor may be useful in the treatment of patients, hospitalized with severe and potentially life-threatening influenza as such a formulation may ensure appropriate dosing.

Ribavirin is a guanosine analogue and an inhibitor of IMP dehydrogenase (Fig 1C). It is a broad-spectrum inhibitor of RNA virus replication, which has been formally approved to treat HCV and RSV infections. The compound was found to inhibit the replication of a number of viruses *in vitro* and was shown to exert a protective effect in some experimental animal models (for review see Lleyssen *et al.*, 2008 and references cited in). Athough almost all RNA viruses are sensitive to the *in vitro* antiviral activity of ribavirin, some viruses are more susceptible than others. Several mechanisms were suggested for the antiviral activity of ribavirin; for influenza viruses an inhibition of RNA-dependent RNA polymerase activity was demonstrated (Bougie & Bisaillon, 2003).

Earlier we have established synergistic antiviral effects of the combinations of PC with Rim in different virus-cell model systems (Serkedjieva *et al.*, 1986; Uzunov *et al.*, 1991). We have also found that the mortality of white mice in EIVI was reduced synergistically by the simultaneous use of PC and Rim with respect to the individual substances (Gegova *et al.*, 1993).

All these considerations persuaded us to carry out additional investigations on the combined anti-influenza virus effects of PC and the three licensed selective antiviral drugs in cell cultures and in the EIVI in albino mice. Here we present the results from these experiments.

From dose-response dependence curves the EC_{50}–s of these preparations were found and the SI-s were calculated. The TC_{50}-s, EC_{50}-s, EC_{90}-s and SI-s of PC and Rim are presented in Table 1.

Table 1. Drug-susceptibility of A/Rostock and A/Aichi viruses to PC and Rim

Sample	MDCK	A/Rostock			A/Aichi		
	TC_{50} (μg/mL)	EC_{90} (μg/mL)	EC_{50} (μg/mL)	SI (μg/mL)	EC_{90} (μg/mL)	EC_{50}	SI
PC	75.0	3.1	1.1	68.2	5.2	2.2	34.1
Rim	250.0	4.8	10.0	100.0	> TC50	4.1	61.0
Zan	>200.0	0.7	0.2	>1000.0	5.0	2.9	>70.0
Rib	130.0	5.2	2.5	52.0	7.5	4.4	29.6

PC–polyphenolic complex, Rim - rimantadine hydrochloride, Zan - zanamavir, Rib - ribavirin. TC_{50} - 50% cell toxic concentration, EC_{50} - 50% effective concentration, EC_{90} - 50% effective concentration, SI - selectivity index

Further we tested the combined antiviral action of PC with each one of the three synthetic viral inhibitors on the replication of a range of influenza viruses in MDCK cells and CAM. The results are shown in Table 2.

Table 2. Inhibitory effect of individual and combined samples on influenza virus replication

Sample	Dose	IT	YA, YB	Sample	IT	YAB	YC	Combined effect
VC (PR8)			7.5					
PC1	0.2	4.5	0.6	PC1Rim	2.0	0.27	0.42	Synergistic
PC2	0.1	3.75	0.5	PC2Rim	0.2	0.027	0.35	Synergistic
Rim	0.025	5.25	0.7					
VC (Hong Kong)			6.0					
PC2	0.1	4.2	0.7	PC2Rim	2.7	0.49	0.5	Synergistic
PC3	0.05	4.4	0.73	PC3Rim	4.3	0.7	0.5	Antagonistic
Rim	0.025	4.2	0.7					
VC (A/Rostock)			7.0					
PC1	1.25	6.2	0.89	PC1Rim1	7.0	1.0	0.8	Antagonistic
PC2	0.62	7.0	1.0	PC1Rim2	7.0	1.0	0.89	Antagonistic
PC3	0.31	7.0	1.0	PC2Rim1	4.0	0.57	0.93	Synergistic
				PC2Rim2	4.5	0.64	1.0	Synergistic
Rim1	2.5	6.5	0.93	PC3Rim1	6.5	0.93	0.93	Indifferent
Rim2	1.25	7.0	1.0	PC3Rim2	7.0	1.0	1.0	Indifferent
VC (A/Aichi)		6.6						
PC1	2.5	6.0	0.9	PC1Rim1	5.2	0.79	0.75	Synergistic
PC2	1.25	6.6	1.0	PC1Rim2	5.2	0.79	0.81	Synergistic
PC3	0.62	6.6	1.0	PC2Rim1	5.3	0.8	0.83	Synergistic
				PC2Rim2	5.0	0.76	0.9	Synergistic
Rim1	5.0	5.5	0.83	PC3Rim1	5.2	0.79	0.83	Synergistic
Rim2	2.5	6.0	0.9	PC3Rim2	5.0	0.76	0.9	Synergistic
VC (A/Rostock)			6.0					
PC1	2.5	4.5	0.75	PC1Zan2	2.8	0.47	0.73	Synergistic
PC2	1.25	5.2	0.87	PC1Zan3	3.8	0.63	0.73	Synergistic
PC3	0.62	5.5	0.92	PC2Zan2	4.0	0.67	0.84	Synergistic
				PC2Zan3	4.8	0.8	0.84	Synergistic
Zan1	2.5	5.5	0.92	PC3Zan2	3.5	0.58	0.89	Synergistic
Zan2	1.25	5.8	0.97	PC3Zan3	4.1	0.68	0.89	Synergistic
Zan3	0.62	5.8	0.97					

Table 2. *Contd.*

Table 2. *Contd.*

Sample	Dose	IT	YA, YB	Sample	IT	YAB	YC	Combined effect
VC (A/Aichi)		6.3						
PC1	2.5	5.2	0.82	PC1Zan2	4.8	0.76	0.78	Synergistic
PC2	1.25	5.6	0.89	PC1Zan3	5.8	0.92	0.8	Antagonistic
PC3	0.62	5.9	0.94	PC2Zan2	6.3	1.0	0.89	Antagonistic
				PC2Zan3	5.8	0.92	0.86	Synergistic
Zan1	2.5	5.8	0.92	PC3Zan2	3.8	0.6	0.89	Synergistic
Zan2	1.25	6.0	0.95	PC3Zan3	4.5	0.71	0.91	Synergistic
Zan3	0.62	6.1	0.97					
VC (A/Rostock)			6.0					
PC1	2.5	4.5	0.75	PC1Rib2	3.4	0.57	0.69	Synergistic
PC2	1.25	5.2	0.87	PC1Rib3	3.5	0.58	0.69	Synergistic
PC3	0.62	5.5	0.92	PC2Rib2	3.5	0.58	0.8	Synergistic
				PC2Rib3	3.3	0.55	0.8	Synergistic
Rib1	5.0	5.3	0.83	PC3Rib2	3.5	0.55	0.85	Synergistic
Rib2	2.5	5.5	0.92	PC3Rib3	3.5	0.55	0.85	Synergistic
Rib3	1.25	5.5	0.92					
VC (A/Aichi)		6.4						
PC1	2.5	5.3	0.83	PC1Rib2	4.3	0.67	0.79	Synergistic
PC2	1.25	5.8	0.9	PC1Rib3	4.8	0.75	0.83	Synergistic
PC3	0.62	6.1	0.95	PC2Rib2	5.1	0.8	0.86	Synergistic
				PC2Rib3	4.4	0.7	0.9	Synergistic
Rib1	5.0	5.6	0.88	PC3Rib2	4.2	0.66	0.9	Synergistic
Rib2	2.5	6.1	0.95	PC3Rib3	4.2	0.66	0.95	Synergistic
Rib3	1.25	6.4	1.0					

VC – virus control, PC, Rim, Zan, Rib – as in Table 1. Y_A, Y_B - fractional yields of individual compounds,

Y_A, Y_{AB} fractional yields of the combinations, $Y_C = Y_A \times Y_B$; combined effect – calculated according to the formulae $Y_C=Y_A x Y_B$, if $Y_C>Y_{AB}$ - the effect was synergistic; if $Y_C=Y_{AB}$ - the effect was indifferent; if $Y_C<Y_{AB}$ - the effect was antagonistic.

The combined use of PC and Rim in doses, which by themselves do not suppress significantly viral replication, in most of the cases resulted in synergistic enhancement of the inhibition of A/Rostock and A/Aichi in MDCK cells. There were found also indifferent (PC3Rim1 and PC3Rim2) as well

antagonistic combinations (PC1Rim1 and PC1Rim2) with respect to A/Rostock replication. A synergistic enhancement of the inhibition of A/PR8 in CAM was registered. The effects on the replication of A/Hong Kong in CAM varied from antagonistic to synergistic.

We have established also synergistic and indifferent combined antiviral effects of PC with two more amantadine derivatives (adamantanamine glucuronide, Gl and its derivative, dGl; Uzunov *et al.*, 1991). In addition Rim, Gl, and dGl were combined with two more viral inhibitors of plant origin-the preparation SHS-174, obtained from three higher plants (Serkedjieva & Zgorniak-Nowosielska, 1993) and the infusion prepared from the flowers of *Verbascum thapsiforme* Schrad. (Serkedjieva, 2000). The combinations did not exhibit any virucidal effect and the cellular toxicity was not enhanced. As a rule the combinations showed increased inhibitory effects on the replication of several influenza A viruses in tissue cultures with respect to the individual components. Most of the combinations were synergistic.

The *in vitro* combined application of PC and Zan resulted in synergistic enhancement of the inhibition of A/Rostock and A/Aichi virus. Most of the combinations were synergistic, the exceptions were the antagonistic combinations of PC1Zan3 and PC2Zan2. As EC_{50}-s of the individual components in the effective combinations were reduced 4–8-fold, the SI-s were respectively raised.

The *in vitro* combined application of PC and Rib resulted in synergistic enhancement of the inhibitory effect on the replication of A/Rostock and A/Aichi in MDCK cells. As a rule the combinations showed a markedly increased virus-inhibitory effect with respect to the individual compounds. All combinations proved to be synergistic. The combined use of PC with Rib proved to be the most effective.

It is obvious that the *in vitro* assays could not be completely reliable to predict the effectiveness of combination therapy. For the evaluation of potential influenza virus inhibitors mouse models have been extensively used. The intranasal inoculation of A/Aichi-a virus to mice produces a damaging infection of the lungs which, depending on the dose of the viral inoculum, is highly lethal to the animals. The results from animal experiments could help to provide consistent evidence about the appropriate usage of combined treatment. Therefore the combined treatment with combinations of PC and the three selective anti-influenza drugs was tested in the EIVI of albino mice. Following viral challenge, numerous parameters of the infection were followed - % of mortality, respectively % of survival rates, protective indices, mean survival times, body weights of infected animals. A number of lung parameters were determined as well, because the lung represents the primary target organ for virus replication, namely – lung weights, indices, consolidation scores and infectious viral titres. The results are presented in Table 3 and Fig 2.

Table 3. Protective effect of individual and combined samples in the murine EIVI

Treatment group	Dosage (mg/kg)	Mortality (%)	Protective index# (%)	$E_{1,2}$	Combined effect[5]	MST (days)	Lung parameters (6th day p.i.) Weight (g)	Lung index[@] (%)	Score[+]	Titre[§] ($TCID_{50}$/mL)
BK		73.6				7.7	0.26	1.17	4.0	6.7
PC1	5.0	37.3	49.4			11.2	0.3	1.0	2.5	4.3
PC2	2.5	47.1	36.1			10.5	0.22	1.1	4.0	6.3
PC3	1.25	60.0*	18.4			9.1	0.21	1.2	4.0	6.7
Rim	10.0	55.7*	24.3			10.2	0.23	1.12	4.0	6.2
PC1 + Rim	5 + 10	10.0	86.4	0.61	Synergistic	12.3	0.22	0.92	1.0	3.0
PC2 + Rim	2.5 + 10	15.0	79.7	0.51	Synergistic	9.2	0.19	1.12	2.0	5.0
PC3 + Rim	1.25 + 10	45.6	38.0	0.38	Indifferent	8.1	0.21	1.0	3.0	5.6
BK		76.4			7.4	0.26	1.19	3.5	5.3	
PC1	5.0	41.5	45.7			11.1	0.3	1.0	2.5	4.3
PC2	2.5	47.0	38.4			9.4	0.23	1.17	4.0	4.0
PC3	1.25	56.4*	26.1			8.6	0.22	1.2	3.5	5.0
Zan	2.5	62.0*	18.9			10.5	0.19	1.1	2.0	2.5
PC1 + Zan	5.0 + 2.5	16.5	78.9	0.55	Synergistic	13.1	0.19	1.12	0.5	0.5
PC2 + Zan	2.5 + 2.5	27.5	63.5	0.56	Synergistic	12.2	0.21	1.14	1.0	1.3
PC3 + Zan	1.25 + 2.5	43.7	42.8	0.4	Synergistic	10.8	0.21	0.9	3.0	3.3
BK		73.2			8.0	0.28	1.2	4.0	5.1	
PC1	5.0	37.5	48.8			11.8	0.26	1.12	2.0	3.5
PC2	2.5	50.0	31.8			10.9	0.26	1.2	4.0	4.3
PC3	1.25	56.4*	22.9			9.1				
Rib	10.0	37.5	48.8			11.12	0.27	0.96	4.0	4.0
PC1 +Rib	5 + 10	6.25	91.6	0.74	Synergistic	13.6	0.3	1.0	3.0	3.5
PC2 +Rib	2.5 + 10	12.5	82.9	0.65	Synergistic	10.5	0.29	0.92	3.0	4.3
PC3 +Rib	1.25 + 10	46.7	36.1	0.67	Antagonistic	8.2	0.28	0.9	3.3	4.6

*The difference with virus control is not significant ($p<0.05$)

VC, PC, Rim, Zan, Rib – as in Table 2. PC was administered i.n. 3 h before virus challenge, Rim and Rib – orally, -24, +2, +24,+48,+72 h relative to virus infection, Zan – i.n.,-24,+2, +24,+48,+72 h with respect to infection. Experimental groups were of 12 animals each. The results are the mean of 3 experiments.

#Protective index = (PR-1)/PR x 100, where PR (protective ratio) is $M_{control}/M_{experiment}$ and M is mortality.

§Combined effect - calculated from $E_{1,2}=E_1+E_2-E_1 x\ E_2$, where $E_{1,2}= PI_{1,2}/100$, $E_1=PI_1/100$ and $E_2=PI_2/100$; the combined effect is synergistic if $E_{1,2}$ is >, indifferent if $E_{1,2}$ is = and antagonistic if $E_{1,2}$ is $<E_1+E_2-E_1 x\ E_2$.

@Lung index –lung weight/body weight x 100

+Consolidation scores 0-4, assigned to % visible consolidation. Samples taken on day +6.

§Infectious titres $\log_{10}$ 50% tissue culture infectious doses/0.5 mL. Samples taken on day +6.

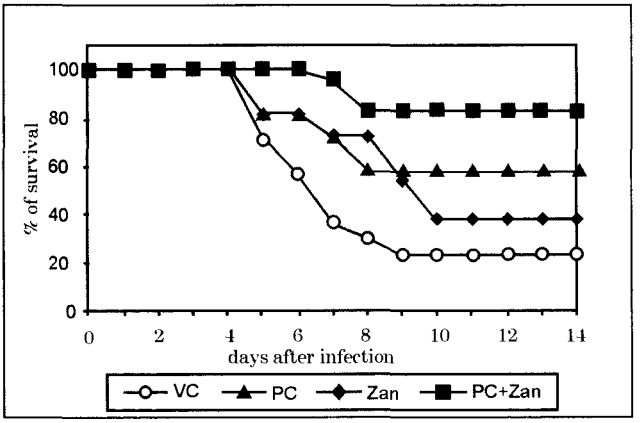


Fig 2. Effects of PC, Zan and their combination on the survival rate in the murine EIVI[a]. VC – virus control (*A/A* ichi-a), PC – polyphenolic complex, 5 mg/kg, i.n., -3 h, Zan - zanamavir, 2.5 mg/kg, i.n., -24, -2, +24, +48, +72 h, [a]the results are from one representative experiment

Administration of PC in combination with Rim in the course of the murine EIVI produced indifferent to synergistic rise of protection. The combination PC3+Rim, where both components were not significantly protective, caused a significant reduction of lung infections titre and lung score and a decrease of mortality rates of indifferent type with regard to virus control ($p<0.05$). The combined protective effect of PC1 and Rim1 induced a synergistic decrease in mortality rates (IP=86.4%), a distinct prolongation of mean survival time (+4.6 days) and a reduction of body weight loss (Table 3). A marked decrease of lung infectious virus titre in comparison to control virus-infected mice was achieved by the use of this combination ($\log_{10}TCID_{50}/mL=3.7$). Histological examination demonstrated that the mice, receiving combined treatment showed minimal pathological lesions in the lungs whereas control untreated animals had total hemorrhagic pneumonia. Thus the combined use of PC and Rim had a synergistic therapeutic effect in the animals, inoculated with a high dose of influenza virus. The obtained results are in accordance with previous observations about the combined application of PC and Rim in EIVI (Gegova *et al.*, 1993).

Clear evidence of protection was observed for animals receiving PC in combination with Zan in the EIVI. A synergistic decrease of mortality rates was achieved (PI=42.8–78.9%), MST was significantly prolonged (+3.4-5.7 days). The combination PC3+Zan, where neither individual component caused significantly protection, induced a significant reduction of lung infectious titre and lung score and a synergistic decrease of mortality rates with respect to virus control ($p<0.05$). A pronounced reduction of lung lesions and lung virus titres was achieved ($\log_{10}TCID_{50}/mL=2.0–4.8$). The marked increase of survival rates is illustrated in Fig 2.

Administration of PC in combination with Rib in the course of the EIVI in the mouse model produced antagonistic to synergistic rise of protection. Although the combination PC3+Rib caused a significant reduction of lung infectious titre and lung score (Table 3), the difference of mortality rates with virus control was not noteworthy and protection was increased in antagonistic manner. The combined protective effect of PC1 and Rib produced a synergistic protective effect: mortality rate was significantly decreased (PI=91.6%), MST was markedly prolonged (+5.5 days). A pronounced reduction of the lung lesions due to infection an of lung infectious virus titres in comparison to control virus infected mice was achieved ($\Delta\log_{10}$ $TCID_{50}$/mL=1.6).

In conclusion, the combined use of the plant preparation PC and any of the three synthetic selective anti-influenza drugs resulted in synergistic enhancement of the inhibition of A/Aichi influenza virus in MDCK cells. There were found also synergistic, indifferent as well antagonistic combined effects on A/Rostock replication (Table 2). The combined use of PC+Zan and PC+Rib resulted in synergistic increase of inhibition. The most pronounced enhancement in MDCK cells was achieved by the combinations of PC and Rim.

Our results demonstrated too that combination treatment in the murine EIVI exerted a greater antiviral effect than monotherapy with either agent (Table 3, Fig 2). In the EIVI the combinations of PC with the three synthetic anti-influenza drugs varied from antagonistic to synergistic and the most effective were the combinations of PC with Zan.

Taken together, our results support the findings that the appropriate combined use of antiviral agents with alternative modes of action is a promising approach for the treatment of influenza virus infection. An important conclusion from these results is that in order to achieve a synergistic virus-inhibitory effect of the combinations it is essential to precisely select the doses of the individual components. For a meaningful evaluation and interpretation of the effects of drug combinations it is necessary to consider the biological significance of the combinations, that is, the therapeutic effect of the combinations, the lack of cytotoxicity, the mechanism(s) of action of the individual constituents.

ACKNOWLEDGEMENTS

This study was supported by research grants K-1007 and K-1518 from the National Science Fund, Bulgaria. The author is thankful to Ms. A. Teodosieva and Ms. K. Todorova for the excellent technical assistance.

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Antihypertensive and Hypolipidemic Effects of Tuber of *Apios americana* Medikus in SHR

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ABSTRACT

The tuber of Apios americana Medikus, native to North America, become edible tubers. This tuber having high nutritional values attracts consumers' attention, and has recently become widely cultivated in northern Japan. In order to verify its antihypertensive and hypolipidemic effects, A. americana was investigated. In male spontaneously hypertensive rats (SHR) fed a diet containing 5% A americana powder for 3 weeks, blood pressure (BP) was lowered by 10%. Moreover, in SHR fed a diet containing 1% cholesterol, ingestion of 5% A. americana also decreased BP and plasma triacylglycerol (TG) and hepatic total-cholesterol (T-CHO), and showed a tendency to decrease plasma T-CHO. At 0.5 and 1 h after a single oral administration of water extract of A. americana at 200 mg/kg, 10% BP lowered in male SHR. This extract had weaker angiotensin-converting enzyme (ACE) inhibitory activity than that of lisinopril, however it was rich in proline. Several peptides that were digested by pepsin had strong ACE inhibition. Therefore, it was expected that proline-rich peptide, which was produced from protein of A. americana during digestion, contributed to antihypertensive action. Although identification of the active components of A. americana is progressing, it is suggested that A. americana, having no negative influence on growth, is a healthy food material for prevention of hypertension and hyperlipidemia.

Key words : *Apios americana*, antihypertension, spontaneously hypertensive rat (SHR), total cholesterol, triglyceride, angiotensine-converting enzyme (ACE), proline (Pro)

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INTRODUCTION

Apios americana Medikus is a perennial plant in the family of legumina. Its underground stem forms knots in intervals between 5 and 10 cm during growth, and they become to edible tubers (Hoshikawa *et al.*, 1995; Juliarni *et al.*, 1996, 1997). *A. americana*, native to North America, is distributed widely in Florida and Texas now. In Japan, it is a common belief that *A. americana* had been imported from the United States with the soil of apple young trees. *A. americana* is now cultivated as a crop in northern Japan (Fig 1A).

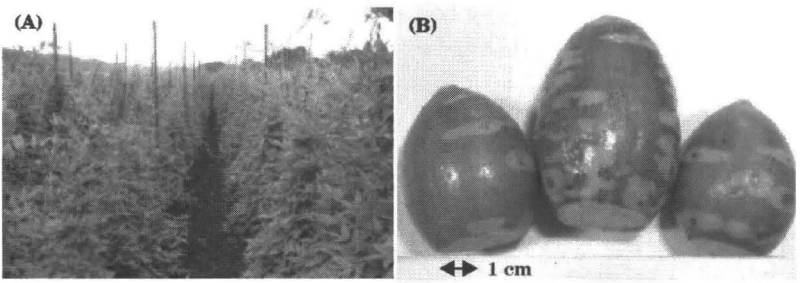

Fig 1. Cultivating field of *A. americana* (A) and its tubers (B)

Tuber of *A. americana* (Fig 1B) attracted much consumer's attention based on the research of Hoshikawa *et al.* (1995) and Juliarni *et al.* (1996, 1997), which indicated high nutritional value and traditional healthy benefits. Actually there are descriptions of some *A. americana* lovers getting rid of chronic constipation, positive effects on disorders before and after childbirth, on obesity and diabetes, improving energy, etc. On the other hand, nutritional composition (Walter *et al.*, 1986; Kinugasa *et al.*, 1992), lipid composition (Wilson *et al.*, 1986) and amino acid composition (Wilson *et al.*, 1987) of *A. americana* tubers were reported. Furthermore, DDMP-saponine (Okubo *et al.*, 1994) and genistein (Krishnan, 1998) were found in its tubers. However, there is no scientific reports and evidence about the various healthy effects described above.

In recent years, increase of life style related diseases has become a problem. Hypertension is one of the typical life style related diseases, and is well known that hypertension is an important risk factor for cardiovascular disease (Kannel, 1996). It is suggested that hypertension is closely related to food components, especially sodium chloride and protein intake. Therefore, it is very important to develop natural food-related compounds for the treatment and prevention of hypertension (Yoshikawa *et al.*, 2000). In recent years food has been considered not only a source of energy and basic

components for the maintenance and growth of the body, but also a source of bioactive compounds that may exert beneficial effects in humans. For example, peptides derived from various foods have been extensively studied for antihypertensive effects (Yamamoto, 1997; Takano, 1998). The hypotensive activity of peptides has also been demonstrated *in vivo* in spontaneously hypertensive rats (SHR) (Yamamoto *et al.*, 1994) and human volunteers (Sekiya *et al.*, 1992). This may be partially attributed to the recognition of the relationship between food and health, which is the focus of functional foods.

Therefore, we have an interest in the *A. americana* tuber that is familiar to us, and we have focused on its physiological effects in order to discover its healthy benefits. In this paper, physiological effects of *A. americana* on hypertension and lipid metabolism in SHR and its active components were investigated.

NUTRITION OF *A. AMERICANA*

Raw tubers of *A. americana*, which were harvested in the Kuraishi area of Gonohe Town in Japan, were used for this experiment, contained 55.3% moisture, 6.6% crude proteins, 0.6% crude fats, 1.7% ash, 9.8% fiber and 26.0% carbohydrate (Table 1).

Table 1. Nutirional composition of raw *A. americana* tuber

Nutrient	Content	Method
Moisture	55.3 g/100 g	Oven drying method
Crude protein	6.6 g/100 g	Kjeldahl method
Crude fat	0.6 g/100 g	Method of A.O.A.C.
Crude ash	1.7 g/100 g	Direct ashing method
Crude fiber	9.8 g/100 g	Method of A.O.A.C.
Carbohydrate	26.0 g/100 g	Moisture, protein, fat, ash and fiber were taken from 100 g.
Sodium	4 mg/100 g	Flame atomic absorption spectrometry
Potassium	720 mg/100 g	Flame atomic absorption spectrometry
Calcium	260 mg/100 g	Flame atomic absorption spectrometry
Iron	0.9 mg/100 g	Flame atomic absorption spectrometry
Vitamin C	17 mg/100 g	HPLC method
α-Tocopherol	1.1 mg/100 g	HPLC method

There are many reports on the characterization and cultivation of *A. americana* native to North America (Hoshikawa *et al.*, 1995; Juliarni *et al.*, 1996, 1997). Walter *et al.* (1986) and Kinugasa *et al.* (1992) had reported the

general nutrients of its tuber. We also had similar results on the nutrients of *A. americana* tuber, which was harvested in the Kuraishi area. These results showed that its tuber had more protein than potatoes and less fat than soybeans. Moreover, it was rich in calcium (720 mg/100 g) and potassium (260 mg/100 g), we have confirmed again that *A. americana* is healthy food material from the view point of nutrients.

EFFECT OF *A. AMERICANA* ON BLOOD PRESSURE

In order to estimate the antihypertensive effect of *A. americana* tuber, animal experiment was designed using diets and extracts of *A. americana*. Basal diet composition, which was based on the formulation of AIN-93G diet (Reeves *et al.*, 1993), was the normal diet (N) as a control. Cholesterol diet (C) and cholesterol-Apios diet (CA) contained 1% cholesterol, and normal-Apios diet (NA) and CA diets contained 5% *A. americana* powder respectively. Casein, cellulose and starch contents in the NA and CA diets were adjusted according to the protein, fiber and carbohydrate contents in the *A. americana* powder (Table 2). Male SHR (Japan Charles River Inc., Tokyo, Japan), 9 weeks old, were divided into 4 groups, and given free access to the experimental diets for 3 weeks. Blood pressure (BP) was measured prior to experiment and at 1-week intervals for a total of 3 weeks. The tail-cuff method was performed to measure systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) of unanesthetized SHR using a blood pressure analyzer (model BP-98A, Softron Co., Tokyo, Japan). The rats were warmed at 37°C for 10 min and allowed to rest quietly in a chamber before measurement of BP. The average of three trials was used as data.

Table 2. Compositions of the experimental diets (%)

Composition	Normal diet		Cholesterol diet	
	N	NA	C	CA
Caseina	20.0	19.2	20.0	19.2
α-Corn starch[a]	13.3	12.3	13.0	12.0
β-Corn starch[a]	39.95	36.95	39.0	36.0
Sucrose	10.0	10.0	10.0	10.0
Cellulose	5.0	4.8	5.0	4.8
Corn oil	7.0	7.0	7.0	7.0
Vitamin mixture[b]	1.0	1.0	1.0	1.0
Mineral mixture[b]	3.5	3.5	3.5	3.5
Choline bitartrate	0.25	0.25	0.25	0.25
Sodium cholate	0	0	0.25	0.25
Cholesterol	0	0	1.0	1.0
Apios powder	0	5.0	0	5.0

N, normal diet; NA, normal diet containing 5% *A. americana*; C, 1% cholesterol diet; CA, 1% cholesterol diet containing 5% *A. americana*. [a]Casein and starch were adjusted by the contents of proteins and carbohydrates in *A. americana* powder. [b]AIN-93-VX as a vitamin mixture and AIN-93G-MX as a mineral mixture were obtained from Oriental Yeast Co.

Fig 2 shows the SBP and DBP changes of SHR during the dietary experiment for 3 weeks. The both BP of N and C groups increased with aging. Although SBP of NA group elevated within 2 weeks, it was significantly lower than that of N group at 3 weeks (Fig 2A). NA group showed lower DBP than N group (Fig 2B). SBP of CA group was also significantly lower than that of C group (Fig 2C), and CA group also moderate the elevation of DBP (Fig 2D). During 3 weeks, SBP increased 46 to 65 mmHg in the N and C groups without *A. americana*, however the elevation of SBP in the NA and CA groups was less than 40 mmHg. There was no difference in the heart rate in all groups.

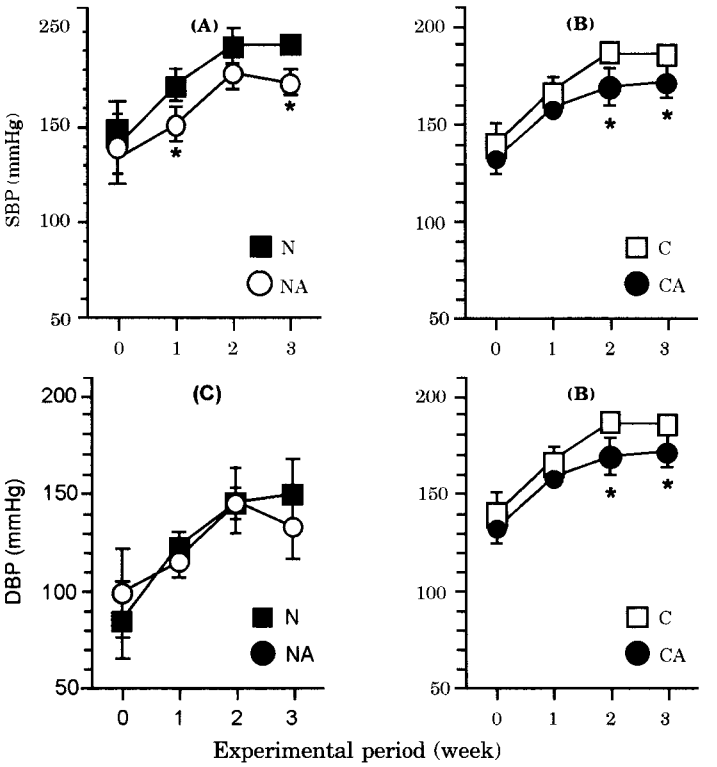

Fig 2. Changes of SBP and DBP in male SHR fed diets with or without *A. americana* for 3 weeks

(A) SBP in SHR fed basal diets; (B) SBP in SHR fed cholesterol diets; (C) DBP in SHR fed basal diets; (D) DBP in SHR fed cholesterol diets. N, normal diet; NA, normal diet containing 5% *A. americana*; C, 1% cholesterol diet; CA, 1% cholesterol diet containing 5% *A. americana*. Data are expressed as mean ± SD of five rats. *Significant difference is indicated between diets with and without *A. americana* at $p<0.05$.

Water extract (AWE) and methanolic extract (AME) of *A. americana* were prepared from the *A. americana* powder by extraction with 3-fold volume of distilled water and methanol, and their yields were 31.3% and 10.4%,

respectively. Male SHR and Wistar Kyoto rats (WKY), each 14 weeks old, were administered orally with extracts (200 mg/kg), and then BP was measured at 1 h after administration. Lisinopril (50 mg/kg) as a positive control drug, AWE and AME lowered significantly SBP and DBP from the control (administration of distilled water) and before administration in SHR. Lisinopril also lowered significantly BP in WKY, but the BP of WKY administered with AWE and AME were similar to those of the control. The heart rate was not altered in both rats by each administration (Table 3).

Table 3. Changes of SBP and DBP in SHR and WKY at 1 h after single oral administration of lisinopril, AWE and AME (mmHg)

	SHR		WKY	
	SBP	**DBP**	**SBP**	**DBP**
Control	198.6 ± 15.3	177.8 ± 8.5	134.4 ± 15.1	111.4 ± 9.7
Lisinopril	143.6 ± 19.6*	110.3 ± 14.7*	112.2 ± 7.4*	80.8 ± 10.5*
AWE	159.2 ± 20.1*	132.1 ± 17.6*	122.7 ± 9.3	102.7 ± 11.6
AME	162.8 ± 9.4*	133.9 ± 8.3*	115.8 ± 18.9	92.4 ± 20.8

AWE, *Apios americana* water extract; *AME*, *Apios americana* methanol extract. Dose: control, distilled water; lisinopril, 50 mg/kg; AWE and AME, 200 mg/kg. Rats were fasted for 20 h before administration. Data are expressed as mean ± SD of five rats. *Significant difference is indicated from control group at $p<0.05$.

Moreover, SBP and DBP of SHR, which was administered orally with AWE, were measured at 0.5 h intervals for 2 h after administration. At 0.5 and 1 h after administration of AWE, SBP and DBP lowered significantly from those before administration, and they then returned to the initial levels at 2 h (Fig 3). At each point, there was no change in the heart rate.

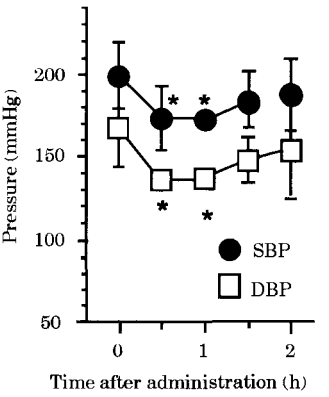

Fig 3. Time-coursed changes of SBP and DBP in male SHR after a single oral administration of AWE

Rats were fasted for 20 h before the administration. Data are expressed as mean ± SD of five rats. *Significant difference is indicated from initial level at $p<0.05$.

With findings concerning the empirical efficacy of *A. americana*, we focused on its antihypertensive effects. SHR has elevation of BP with aging

as the primmary characterization (Iriuchijima *et al.*, 1975; Iriuchijima, 1974), and in this study the elevation of BP was also observed in the experimental period. In SHR fed *A. americana* diets for 3 weeks, BP lowered about 10% as compared to SHR fed diet without *A. americana*. About a 10% decrease of BP was observed between 0.5 and 1 h after a single oral administration of crude water extract of *A. americana* (AWE), however it returned to the initial level at 2 h. At 1 h after administration, the lowering effect of AWE on BP was weaker than that of lisinopril. Therefore, it was considered that the antihypertensive effect of *A. americana* was mild. A dose of AWE at 200 mg/kg was equal to 639 mg/kg of *A. americana* powder, and the dose was calculated at 38.3 g of *A. americana* in a male adult of 60 kg body weight. On the other hand, in the experiment of *A. americana* diets, SHR fed 2.75 g/kg of *A. americana*, and the content of AWE was calculated to 861 mg/kg. Moreover, it took 2 weeks for antihypertensive action to appear in SHR fed *A. americana* diets. Therefore, it was thought that content or absorption of the antihypertensive component in *A. americana* was slight. Furthermore, the decrease of BP was not found in WKY after a single oral administration of *A. americana* crude extracts, and so it was suggested that the effect of *A. americana* was weak on normal BP.

EFFECT OF *A. AMERICANA* ON LIPID LEVELS

In the dietary experiment, effect of *A. americana* on lipid metabolism in rat was also studied. Blood was collected with heparinization from tail artery of SHR fastmed for 20 h on the 1st, 10th day and 21st day of the experiment of *A. americana* diets, and it was separated to plasma and red blood cells. Concentrations of total cholesterol (T-CHO) and triacylglycerol (TG) in the plasma of SHR were enzymatically measured by the commercial kits (Kano, 1987). The changes of plasma T-CHO and TG levels in SHR are shown in Fig 4. The plasma T-CHO in the NA group was similar to that in the N group during the experiment (Fig 4A). Cholesterol diet increased plasma T-CHO level in SHR during the experimental period and significantly higher concentration than normal diet groups at 3 weeks. On the other hand, CA group showed to suppress significantly plasma T-CHO level from C group, and its concentration was similar to that of normal diet groups (Fig 4B).

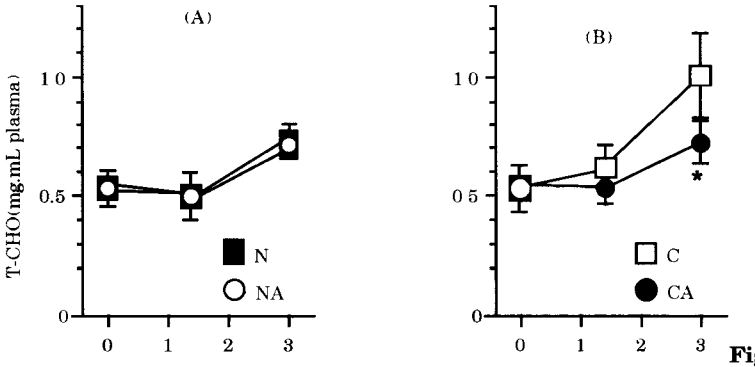

Fig 4. *Contd.*

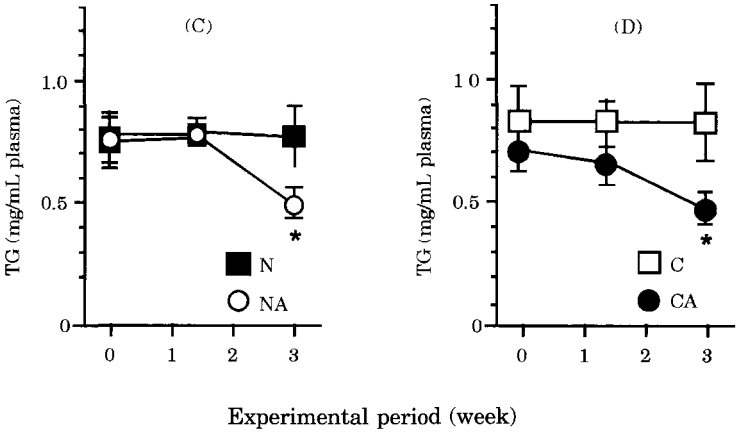

Fig 4. Changes of plasma T-CHO and TG levels in male SHR fed diets with or without *A. americana* for 3 weeks
(A) Plasma T-CHO in SHR fed basal diets; (B) plasma T-CHO in SHR fed cholesterol diets; (C) plasma TG in SHR fed basal diets; (D) plasma TG in SHR fed cholesterol diets. N, normal diet; NA, normal diet containing 5% *A. americana*; C, 1% cholesterol diet; CA, 1% cholesterol diet containing 5% *A. americana*. Data are expressed as mean ± SD of five rats. *Significant difference is indicated between diets with and without *A. americana* at $p<0.05$.

Normal diet groups showed a similar level of plasma TG within 10 days; however NA group showed significantly lower TG level than N group at the end of experiment (Fig 4C). Although plasma TG level in C group was not altered during experiment, CA group showed the tendency to decrease plasma TG level. At 3 weeks, the plasma TG level in CA group was significantly lower than that in C group (Fig 4D).

At the end of the experiment, all SHR were sacrificed by ether anesthesia after fasting for 20 h, and then liver, kidney and aorta were removed after blood collection. Crude lipid was extracted from the homogenates of these tissues with chloroform-methanol according to the method described by Bligh and Dyer (1959), and T-CHO in the crude lipid was enzymatically measured using a commercial kit (Richmond, 1973). Table 4 shows the concentration of T-CHO in the liver, kidney and aorta of male SHR fed experimental diets after 3 weeks. The NA group showed lower level of T-CHO in the liver than N group, and other tissue T-CHO levels in NA group were similar to those in N group. Although C group showed the tendency to increase concentration of T-CHO in all tissues, T-CHO levels in CA group were similar to those of N group, except for the liver.

A. americana showed the tendency to suppress the increase of T-CHO in plasma and tissues, which was induced by the addition of cholesterol into

Table 4. Concentration of T-CHO in plasma and organs of male SHR fed *A. americana* diets for 3 weeks[#]

Organ	N	NA	C	CA
Plasma (mg/mL)	0.738 ± 0.063	0.706 ± 0.048[b]	1.002 ± 0.241[b]	0.790 ± 0.048
RBC (mg/mL)	1.414 ± 0.119	1.539 ± 0.080	1.432 ± 0.134	1.574 ± 0.047
Liver (mg/g organ)	9.758 ± 1.203	5.634 ± 1.059[ab]	15.344 ± 6.259	10.266 ± 2.079[c]
Kidney (mg/g organ)	3.757 ± 0.293	3.754 ± 0.343	4.110 ± 0.464	3.593 ± 0.143
Aorta (mg/g organ)	5.079 ± 0.702	4.031 ± 0.494	6.964 ± 3.045	3.934 ± 0.459

N, normal diet; NA, normal diet containing 5% *A. americana*; C, 1% cholesterol diet; CA, 1% cholesterol diet containing 5% *A. americana*. [#]*Rats* were fasted for 20 h after experimental period for 3 weeks. Data are expressed as mean ± SD of five rats. Significant difference is indicated from [a]N, [b]C and [c]NA groups at $p<0.05$.

diet. In the case of a diet without cholesterol, *A. americana* also lowered the hepatic T-CHO and plasma TG. These results suggested that *A. americana* had some effects on lipid absorption or metabolism. It is well known that soybean isoflavones such as daidzein and genistein have lowering effects on plasma T-CHO and TG, estrogen-like actions, antioxidant effects and antihypertensive actions (Lichtenstein, 1998; Demonty *et al.*, 2002; Martin *et al.*, 2001). On reflection that genistein and saponin were already found in *A. americana* tuber (Okubo *et al.*, 1994; Krishnan, 1998), its effects on lipid metabolism was expected to be caused by isoflavones, however the antioxidant actions were not observed markedly in this experiment. Consequently, it is needed to investigate the effect of *A. americana* isoflavones on lipid metabolism and the influence of its fibers on intestinal microflora for elucidation of lipid lowering effects.

EFFECT OF *A. AMERICANA* ON LIPID PEROXIDATION

For the purpose of estimate the antioxidant activity of *A. americana*, concentration of lipid peroxide was measured as a thiobarbituric acid reactive substance (TBARS) in SHR fed experimental diets. Fig 5 shows the changes of TBARS in plasma and RBC of SHR during 3 weeks. There is no significant difference in both TBARS values among the four groups. There was also no difference in tissue TBARS levels between N and NA groups (Table 5). The C group showed significantly higher hepatic TBARS than that of other groups, and showed the tendency to increase TBARS in other tissues. However, the CA group showed significantly lower levels of hepatic TBARS than C group.

Table 5. Concentration of lipid peroxidation (TBARS) in plasma and organs of male SHR fed *A. americana* diets for 3 weeks#

Organ	N	C	NA	CA
Plasma (nmol/mg protein)	0.130 ± 0.009	0.156 ± 0.031	0.137 ± 0.022	0.117 ± 0.014
RBC (nmol/mg protein)	0.021 ± 0.002	0.026 ± 0.004	0.019 ± 0.005[b]	0.020 ± 0.002
Liver (nmol/mg protein)	0.069 ± 0.012	0.151 ± 0.016[a]	0.077 ± 0.017[b]	0.142 ± 0.010[a c]
Kidney (nmol/mg protein)	0.179 ± 0.079	0.320 ± 0.157	0.162 ± 0.034	0.240 ± 0.050
Aorta (nmol/mg protein)	0.123 ± 0.012	0.262 ± 0.105	0.180 ± 0.062	0.249 ± 0.187

N, normal diet; NA, normal diet containing 5% *A. americana*; C, 1% cholesterol diet; CA, 1% cholesterol diet containing 5% *A. americana*. #Rats were fasted for 20 h after experimental period for 3 weeks. Data represent the mean ± SD of five rats. Significant difference is indicated from [a]N, [b]C and [c]NA group at $p<0.05$.

It has been reported that genistein and saponins, natural antioxidants, were found in tuber of *A. americana* (Okubo *et al.*, 1994; Krishnan, 1998), however, antioxidant effects was not found in SHR fed *A. americana* diets in this study. Therefore, it is suggested that antioxidant activity of *A. americana* tuber including isoflavones and saponins is slight.

INFLUENCE OF *A. AMERICANA* ON GROWTH

In the dietary experiment that male SHR were given free access to the experimental diets, there was no significant difference among the body weights of the four groups, and all groups showed linear and normal changes of body weight (Table 6). Body weight gain, dietary intake and food efficiency of NA group were similar to those of N group (control). C group showed a tendency to gain a large amount of body weight with little dietary intake. Consequently, the food efficiency of C group was significantly higher than that of N group. However, the food efficiency of CA group was similar to that of N group. After 3 weeks, although hepatic hypertrophy was found in C and CA groups, there was no difference in kidney weight in the four groups.

Table 6. Body weight gain, dietary food intake and organ weight of male SHR fed normal and *A. americana* diets for 3 weeks#

	N	NA	C	CA
Body weight gain (g/d)	4.89 ± 0.54	4.61 ± 0.24	5.39 ± 0.72	4.81 ± 0.45
Dietary food intake (g/d)	27.58 ± 0.69	27.97 ± 1.65	25.99 ± 1.13	29.00 ± 1.32[c]
Food efficiency (%)	17.19 ± 1.46	16.49 ± 0.70[c]	20.81 ± 3.01	16.58 ± 0.96[c]
Liver weight (g)	8.588 ± 0.261	7.764 ± 0.432[c]	14.018 ± 0.840[b]	12.745 ± 0.735[bd]
Kidney weight (g)	1.916 ± 0.052	1.839 ± 0.048	1.929 ± 0.054	1.926 ± 0.068

N, normal diet; NA, normal diet containing 5% *A. americana*; C, 1% cholesterol diet; CA, 1% cholesterol diet containing 5% *A. americana*. #Rats were fasted for 20 h after experimental period for 3 weeks. Data are expressed as mean ± SD of five rats. Significant difference is indicated from [a]N, [b]C and [c]NA groups at $p<0.05$.

The national average of potatoes intake is 4.7% and that of legumes intake is 5.1% by the National Nutrition Survey in Japan 2000 (Office for Life-Style Related Diseases Control, 2002). On the basis of this data, the content of *A. americana* powder was determined. The report of Ameny *et al.* (1994), which the 10% protein diet containing *A. americana* was given to weanling rats, showed lower body weight gain, protein efficiency ratio and food efficiency of an *A. americana* diet than those of a casein diet, and furthermore they pointed out the insufficiency of essential amino acids in the *A. americana* diet. However, it was expected that the deterioration of growth was caused by a decrease of food intake in their study. In our study, the experimental diet contained 5% *A. americana* powder, and the composition of nutrients were same as the control diet adjusting amounts of protein and starch. As a result of feeding for 3 weeks, there were no differences in body weight change, food intake, and liver and kidney weights of SHR. Although hepatic hypertrophy was observed in SHR fed cholesterol diets, it was expected to be induced by the addition of 1% cholesterol not 5% *A. americana*. The differences in the plasma biochemical parameters (aspartate aminotransferase and alanine aminotransferase) as indexes of hepatic function were not found between SHR fed diets with and without *A. americana* (the data are not shown). Moreover, the deterioration of other plasma biochemistry was not observed in SHR fed *A. americana* diets. These results suggest that *A. americana* has no negative influence such as undernourishment and organ dysfunction.

BIOACTIVE COMPONENTS OF *A. AMERICANA*

So, we investigated the bioactive component of *A. americana*. The composition of amino acids in *A. americana* was analyzed by the JLC-500 Auto Amino Acid Analyzer (JEOL Ltd., Akishima, Japan). Table 7 shows the compositions of amino acids in the hydrolysate (total) and water solute (free) of *A. americana* powder, AWE and AME. In the hydrolysate of powder, aspartic acid (Asp) was the major amino acid, and glutamic acid, glycine, leucine and proline (Pro) were also present in high content. In the AWE, Asp was also the major amino acid, and the next was Pro, and AME also had the largest amounts of Pro. The powder and both extracts were rich in Pro as a free amino acid. Wilson *et al.* (1987) had reported that Asp and glutamic acid were the predominant amino acids in the raw tuber of *A. americana*, and that other amino acids presented in significant quantities were leucine and Pro. This study had also shown a similar amino acid composition result for *A. americana* powder. However, the most abundant amino acid in hydrolysate of AWE was Asp (34%), and the next was Pro (17%). Furthermore, the most abundant free amino acid in the powder and both extracts was Pro (33-49%).

Table 7. Amino acid compositions of powder and extracts of *A. americana* tuber

Amino acid	Hydrolysate (nmol/mg)			Free (nmol/mg)		
	Powder	AWE	AME	Powder	AWE	AME
Glycine	63.00	16.12	4.53	3.22	0.13	0
Alanine	43.25	14.14	13.92	1.68	7.30	9.65
Valine	11.61	6.83	2.73	1.56	0.77	1.71
Leucine	56.85	5.57	1.22	0	0.46	0.76
Isoleucine	33.76	4.19	1.60	0	0.53	1.14
Serine	52.34	15.61	9.14	2.69	0	7.71
Threonine	42.25	6.46	3.19	0	8.56	3.86
Aspartic acid	100.83	115.78	55.67	14.12	9.99	0
Asparagine	0	0	0	9.37	39.13	23.62
Glutamic acid	75.11	43.77	23.45	1.82	25.96	14.07
Cysteine	2.10	2.64	0	1.31	0	0.25
Lysine	26.00	6.43	0.41	0	1.09	0
Arginine	18.25	5.51	0	0	2.93	0
Histidine	18.27	32.87	18.11	3.01	5.09	2.39
Phenylalanine	30.93	3.57	0	0	0.36	0.79
Tyrosine	13.18	3.64	2.54	0	0.06	1.21
Proline	55.34	59.32	79.13	19.08	53.24	65.14

AWE, *Apios americana* water extract; AME, *Apios americana* methanol extract. Glutamine, methionine and tryptophane were not detected.

Angiotensin-converting enzyme (ACE) inhibition, which was one of the hypotensive actions (Matsui, 2002), of AWE and AME was measured according to the spectrophotometric method (Hernández-Ledesma *et al.*, 2003) using hippuryl-histidyl-leucine as a substrate. ACE inhibitory activity was observed in AWE, but its activity (IC_{50}=127 mg/mL) was far weaker than that of lisinopril (IC_{50}=4.47 X 10^{-6} mg/mL) (Li *et al.*, 1997; Inoue *et al.*, 1992), similar to the results of a single oral administration. It was considered that the extracted ingredients had no ACE inhibitory activity, but the AWE was found to be rich in Pro by amino acid analysis. Several peptides containing Pro were already reported to have ACE inhibitory effect (Nakamura *et al.*, 1995; Sipola *et al.*, 2001; Chen *et al.*, 2003). In many peptides having ACE inhibitory activity, one of the characteristics of strong inhibitory peptides is the presence of Pro at C-terminal (Cheung *et al.*, 1980). So, it was suggested that the peptide containing Pro, which was derived from proteins of *A. americana* during the process of digestion and absorption, participated the antihypertensive effect, because the appearance of antihypertensive action of *A. americana* was moderate, and its crude extract had a weak ACE inhibitory activity and was rich in Pro.

Furthermore, we examined pepsin digestion of *A. americana*, and ACE inhibitory fractions were isolated and purified by Sep-Pak cartridge and high performance liquid chromatography. Several tetra- and hexa-peptides having Pro and strong ACE inhibition were found in the active fractions. The identification of these peptides is progressing. A future subject is detail assignment of these peptides, synthesis of these peptides and elucidation of characterization.

CONCLUSIONS

It was distinct that *A. americana* tuber had a moderate antihypertensive effect. Its action was expected to be induced by ACE inhibition of Pro-rich peptides, which were derived during digestion of *A. americana*, however that is a future subject. Moreover, it was found that *A. americana* tuber lowered lipid concentrations, and the participation of isoflavones in the tuber was expected. The analyses of these active components are progressing now. It was scientifically clarified that *A. americana* was a healthful material for functional food having preventing actions on hypertension and hyperlipidemia.

ACKNOWLEDGEMENTS

We are grateful to H. Uchisawa (Aomori Industrial Research Center) for measurements of amino acid compositions.

ABBREVIATIONS

BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; AWE, water extract of *A. americana*; AME, methanolic extract of *A. americana*; ACE, angiotensin-converting enzyme; T-CHO, total-cholesterol; TG, triacylglycerol; Asp, aspartic acid; Pro, proline.

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8

Anti-Ulcer Effects of Aqueous Extract of *Persea americana* Mill (Avocado) Leaves in Rats

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ABSTRACT

The aqueous leaves extract of Presea americana was investigated for anti-ulcerogenic effects using ethanol / HCI and indomethacin as the ulcerogens. The effect of the extract on gastric acid secretion was also investigated. The extract was administered orally at the doses of 100 and 200 mg / kg for the experimental groups while the control and reference groups received saline (10 mL / kg) and cimetidine (32 mg / kg) respectively. The results show that the extract significantly ($p < 0.001$) reduced the ulcer index from 4.52 ± 0.22 to 0.45 ± 0.09 and from 3.95 ± 0.19 to 0.14 ± 0.09 in the ethanol / HCI and indomethacin induced ulceration respectively. The extract also significantly ($p < 0.05$) reduced the gastric acid secretion. In conclusion this study has shown that the aqueous extract of P. americana has anti-ulcer effects which might be due to its ability to reduce gastric secretion. The findings from this study also justify the folkloric uses of P. americana for the treatment of ulcer.

Key words : Avocado, aqueous extract, anti-ulcer, gastric acid, leaves, plants, rats

INTRODUCTION

Persea americana mill (Lauraceae) commonly called Avocado is a medium-sized, erect and deciduous tree ranging from 15–20 m in height (Ojewole *et al.*, 2007). Different parts of the plant are used in folk medicine for the treatment of several ailments such as hypertension, diabetes, inflammation, etc. (Gill, 1992; Adeyemi *et al.*, 2002; Lans, 2006; Bartholomew *et al*, 2007).

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Specifically the fruit is used as vermifuge, for treatment of dysentery and as aphrodisiac (Watt & Breyer-Brandwijk, 1962; Bartholomew *et al.*, 2007). The leaves are used extensively in the treatment of hypertension (Gill, 1992; Lans, 2006), sore throat, haemorrhage and inflammatory conditions (Bartholomew *et al.*, 2007).

Some of the scientifically validated activities of the plant leaves include its antihypertensive activity (Owolabi *et al.*, 2005; Ojewole *et al.*, 2007), anti-convulsant effect (Ojewole & Amabeoku, 2006), analgesic and anti-inflammatory activities (Adeyemi *et al.*, 2002).

The aim of the present study is to evaluate the anti-ulcer effect of the aqueous extract of the leaves of this plant. This is based on the widespread practice of prescribing the aqueous extract of the plant for the treatment of gastric ulcer by traditional medical practitioners.

MATERIALS AND METHODS

Plant Material and Preparation of Extract

The leaves of *Persea americana* used for this study were collected from the Ministry of Agriculture, Ilorin, Nigeria. Identification of the plant was carried out by T.K. Odewo at the Forestry Research institute of Nigeria (FRIN, Ibadan). The voucher specimen (FHI 107767) was subsequently deposited in the herbarium of the institute. The harvested leaves were air dried and milled to fine powder. 1000 g of the powdered leaves was extracted by cold maceration using 4 L of distilled water. The mixture was evaporated in a carefully regulated water bath (maintained at 65°C) to yield 50 g of a dark solid extract. The extract was stored in a refrigerator at 4°C and reconstituted in normal saline for pharmacological studies.

Animals

Male Wistar rats weighing 200 ± 15 g were used for the study. The animals were bred and housed in the animal house of the Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin. Animals were kept in clean and standard cages with good ventilation and 12-h light/dark cycle. They were provided with standard mouse cubes (Bendel feeds) and water *ad libitum* prior to the commencement of anti-ulcer studies.

Anti-ulcer Studies

Ethanol Induced Ulcer

The method used by Mizui and Douteuchi, (1983) was also used in this study. Ulceration was induced in 24 h fasted rats by the oral administration of 1 mL of ethanol/hydrochloric acid (0.3 m HCl in 60% ethanol) thirty min after the extract of *P. americana* (100 and 200 mg/kg), cimetidine (34 mg/kg) and

saline were administered. The animals were sacrificed 1 h after the ethanol-acid administration. The abdominal cavities and subsequently the stomachs of the animals were dissected out. Gastric lesions were observed using a hand held lens (x 10) and an ulcer score was calculated for each animal according to the arbitrary scale used by Singh *et al.* (1997), where 0 = no lesion, 0.5 = hyperaemia, 1 = one or two slight lesions, 3 = very severe lesions, 4 = mucosa full of lesions. Ulcer index was calculated as mean ulcer scores (Tan *et al.*, 1996).

Indomethacin Induced Ulcers

Indomethacin induced ulcer was carried according to the method described by Parmar & Parmar, (1993). Indomethacin (10 mg/kg) was administered (orally) 30 min after the extract of *P. americana* (100 and 200 mg/kg), cimetidine (34 mg/kg) and saline (10 mL/kg) administration. Administration of indomethacin was repeated after 15 h. All the rats were sacrificed 1 h after the last dose of indomethacin and the stomachs were dissected in order to evaluate the level of mucosal irritation. The ulcer index was determined as described earlier in ethanol-acid induced ulcers.

Evaluation of Gastric Acidity

The gastric juice collected from the dissected stomachs of the animals in indomethacin induced ulcer group was washed using saline and centrifuged at 3000 g for 5 min. The clear supernatant was titrated against, 0.01 mol/L solution of sodium hydroxide (NaOH) to pH 7.0. The acid content of the stomach was calculated according to the method of Shay *et al.* (1954) and expressed as MEq/L.

Statistical Analysis

In this study values are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using the Student's t-test. Values with $p<0.05$ compared with the control group were considered as being significantly different.

RESULTS

Anti-ulcer Studies

The results of the anti ulcer studies are shown in Table 1 and Fig 1. Table 1 shows that the extract of *P. americana* significantly ($p<0.001$) reduced the ulcer index from 3.95 ± 0.19 (control) to 0.14 ± 0.09 (200 mg/kg) in the indomethacin induced ulceration group. Likewise in Fig 1, the result show that the extract significantly ($p<0.001$) reduced the ulcer index from 4.52 ± 0.22 (control) to 0.45 ± 0.09 (200 mg/kg) in the ethanol/HCl induced ulceration group.

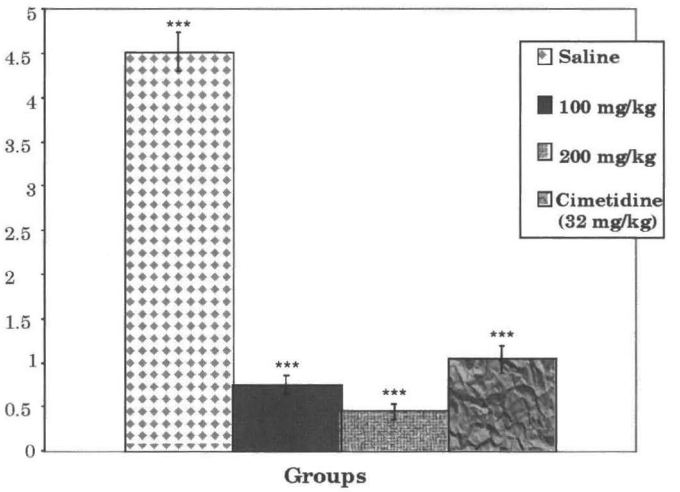

Fig 1. Effect of aqueous extract of *Persea americana* leaves on ethanol-acid induced gastric ulceration. Each value is the mean ± S.E.M. of 5 rats. ***p<0.001 compared with control; Student's t-test

Gastric Acidity

The extract of *P. americana* significantly (p<0.001) reduced the level of gastric acid from 141.0 ± 3.14 (control) to 42.3 ± 2.3 MEq/L (200 mg/kg). Likewise the result show that the volume of gastric juice was significantly reduced (Table 1).

Groups	Dose (mg/kg)	Ulcer index (%)	Protection	Gastric juice (mL)	Gastric acidity (MEq/L)
Control (saline)	-	3.95 ± 0.19	0	3.6 ± 0.1	141.0 ± 3.1
P. americana	100	0.25 ± 0.11***	93.7	1.8 ± 0.2***	71.4 ± 1.7***
P. americana	200	0.14 ± 0.09***	96.5	1.2 ± 0.2***	56.1 ± 2.5***
Cimetidine	32	0.34 ± 0.12***	91.4	3.04 ± 0.1*	71.4 ± 1.7***

DISCUSSION AND CONCLUSIONS

In the present study, the cytoprotective effects of the aqueous extract of *P. americana* was investigated using two important models (ethanol-acid and indomethacin induced ulcerogens). The ethanol-acid ulcerogens caused severe gastric mucosal ulceration either by acting directly on the gastric mucosal or indirectly by increasing the release of vasoactive products such as histamine from mast cells (Szabo, 1987; Oates & Hakkinen, 1987; Goulart *et al.*, 2005). Histamine is a potent stimulator of acid secretion via the H_2 receptor and this forms the basis of using antacids and H_2 receptor antagonists (Mitra *et al.*, 1996). The results obtained from ethanol-acid induced ulcer

model show that the extract at the doses of 100 and 200 mg/kg significantly ($p<0.05$) reduced the necrotizing effects of the ulcerogen and thus exhibits cytoprotective effects. The highest dose (200 mg/kg) produced better cytoprotection compared with the standard reference drug (cimetidine).

The indomethacin induced ulcer model represents a form of gastric irritation resulting from the inhibition of prostaglandins synthase. Indomethacin is an example of non steroidal anti-inflammatory drugs which produces their effects by inhibiting prostaglandins synthesis (Vane, 1971; Steinmeyer, 2000). Acute toxic doses of indomethacin (*e.g.* 20 mg/kg) or lower chronic doses can produce gastric irritation (Goulart *et al.*, 2005; Steinmeyer, 2000). Increase in prostaglandins especially PGE_2 and PGI_2 has been associated with cytoprotection (Neal, 1991; Deshpande *et al.*, 2003). Therefore, agents that inhibit the effects of non-steroidal anti-inflammatory drugs (NSAIDs) *e.g.* indomethacin will exhibit cytoprotection. The results obtained from using the indomethacin ulcer model showed that the extract can significantly inhibit the gastric effect of indomethacin (10 mg/kg) and therefore further exhibiting cytoprotection.

Generally, the activities of the extract may not be unconnected with the anti secretory effects of the extract. Since, the volume of gastric juice and the acidity were significantly reduced by the extract. Most of the studies on *Persea americana* leaves were carried out using aqueous extract (Adeyemi *et al.*, 2002; Ojewole & Amabeoku, 2006, Bartholomew *et al*, 2007; Ojewole *et al.*, 2007). Notable among these studies is the report by Adeyemi *et al.* (2002) showing, that the aqueous extract of *P. americana* possesses analgesic and anti-inflammatory activities. Usually some substances like the NSAIDS produce gastric mucosal irritation in addition to various degrees of analgesic, anti-inflammatory and antipyretic effects (Neal, 1991; Steinmeyer, 2000). On the other hand some plant products such as flavonoids produce anti-inflammatory activities without gastric mucosal irritation (Graziani *et al.*, 2005; Zayachkivska *et al.*, 2005). Therefore, the results obtained in the present study are not only complimentary to those of previous worker on the plant, but it further reveals an important biologic effect (anti-ulcer) of the plant. This indicates that *P. americana* can be subjected to further studies in order to isolate the active ingredients in the leaves as well as determine the mechanism of action of such active ingredients. These may ultimately yield better analgesic agents than we have in the market today.

In conclusion, the present study have established the anti-ulcer effects of aqueous extract of *P. americana* leaves and further justifies the folkloric uses of the decoction of the leaves for the treatment of ulcer.

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9

Anti-Asthmatic Medicinal Plants

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ABSTRACT

Despite the availability of a good number of oral and inhaled medications, the incidence, prevalence of asthma and its associated morbidity and mortality continues to rise. This has turned attention to the use of herbal medicine in the management of asthma. Conventional antiasthmatics such as cromoglycate and theophylline are of plant origin. In the quest to meet the challenges of developing new, safe and effective anti-asthma therapies there is the need to tap the potentials of naturally occurring substances through logical drug development. This article provides a comprehensive literature survey of plants used ethnomedicinally in the management of asthma. These plants have been evaluated for pharmacological activities beneficial in the management of asthma. Their possible mechanisms of action have been investigated, while bioactive compounds have been isolated from some of them.

Key words: Anti-allergic, anti-asthmatic, anti-inflammatory, bronchospasmolytic, medicinal plants

INTRODUCTION

Asthma is a chronic inflammatory disease affecting millions of people worldwide. It is characterized by recurrent and reversible obstruction, inflammation and hyper-responsiveness of the airways. Asthma is also associated with structural changes of the airways, including infiltration of inflammatory cells, epithelial desquamation, sub-epithelial fibrosis, and increased thickness of the smooth muscle layer (Joubert & Hamid, 2005) and the airflow limitation may become permanent (Cookson, 1999). The

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prevalence of asthma is rapidly increasing around the world, especially in young children, and it has become a significant cause of morbidity and mortality in developed countries (Braman, 2006).

The pathogenesis of this disease is believed to be atopic in more than 50%, and at least 80% of affected adults and children (WHO, 2002), and it is a global health problem that results from a complex interplay between genetic and environmental factors (Phillip, 2003). Common triggers of an asthmatic attack include common allergens, irritants, drugs *e.g.* non-steroidal anti-inflammatory drugs (NSAIDs) and upper respiratory tract infections; oftentimes there is no identifiable trigger.

In sensitized individuals, the already inflamed airways respond to an asthma trigger through bronchoconstriction causing recurrent episodes of wheezing, dyspnea, and cough particularly at night and/or early morning. Narrowing of the airway occurs because of inflammation and mucus hypersecretion, and is exacerbated as the smooth musculature in the bronchiolar walls becomes hyperresponsive, leading to intermittent airway constriction. Intermittent airway constriction gives rise to the asthmatic symptoms of wheeze, cough, chest tightness and shortness of breath. Therefore, the pathophysiological components that define asthma include inflammation, bronchoconstriction, airflow limitation and airway hyperresponsiveness.

The airway inflammation in asthma is due to an immune-mediated process in which inflammatory cells and inflammatory mediators enter airway tissues to cause disordered lung function. Both inflammatory mediators (histamine, leukotrienes, platelet-activating factor, cytokines, etc.) and inflammatory cells (mast cells, T lymphocytes, eosinophils, basophils, etc.) are involved in the pathogenetic mechanisms of asthma (Barnes *et al.*, 1988, 1998; Busse & Rosenwasser, 2003). The activation of these inflammatory cells and structural cells (such as airway epithelial cells, smooth muscle cells, endothelial cells, and fibroblasts) leads to the release of inflammatory mediators that effect the typical pathophysiological changes of asthma (Barnes, 1996; Barnes *et al.*, 1998). More than fifty different mediators have been identified in asthma (Barnes *et al.*, 1998).

An imbalance between T-helper 1 (Th1) (*e.g.* IFN-γ) and T-helper 2 (Th2) leads to the clinical expression of allergic disease. Th2 cytokines, including interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13), typically increase in allergic diseases and have important effects on airway infiltration, eosinophil activation, induction of immunoglobulin E (IgE) production, mucus secretion and the release of a variety of inflammatory mediators (Woodfolk, 2006). Eosinophils are commonly associated with allergic inflammation, and act as effector cells in the pathogenesis of this disease by releasing cytotoxic granule proteins (Kay, 2001).

In recent years, the treatment of asthma has been improved by the greater understanding of its pathogenetic mechanisms and implementation of management guidelines. Inhaled corticosteroids and β_2-agonists are used as the first line drugs in the therapy of asthma, reducing airway inflammation and bronchial constriction effectively. Many other medicines such as theophylline, anticholinergics, leukotriene modifiers and the cromones are also used to control asthma. However, the effects of those drugs are limited in clinical practice, because of local or systemic side effects. Also, the prevalence of asthma is rapidly increasing around the world, especially in young children, and it has become a significant cause of morbidity and mortality in developed countries (Braman, 2006). There is need for new or alternative approaches to the control of asthma and the development of better and safer drugs. Many useful anti-asthmatic drugs such as cromoglycate and theophylline, are of plant origin, thus herbal medicine should be a promising area for the search of novel anti-asthma agents. To date, there has been a growing interest in, and usage of herbal medicines both in developed and in third world countries for the management of various diseases including asthma (Akah *et al.*, 1997; Ernst, 1998; Ikawati *et al.*, 2001).

The plants presented in this review have been identified as being used in ethnomedicine for the management of asthma, and have been assessed for different pharmacological properties beneficial in the pharmacotherapy of asthma. Their pharmacological mechanisms of action range from anti-allergic effect, bronchodilatation, modulation of airway hyperresponsiveness, to various modes of anti-inflammatory actions. Some act through one mechanism, while others, exhibit an overlap of two or more mechanisms.

ANTI-ASTHMATIC MEDICINAL PLANTS

Plants that Relieve Bronchoconstriction

Adhatoda vasica

Adhatoda vasica Nees (Acanthaceae) has been recommended by Ayurvedic physicians for the management of various types of respiratory disorders for thousands of years (Chemexil, 1992). The leaves of the plant were found to contain an essential oil and the quinazoline alkaloids vasicine, vasicinone and deoxyvasicine. The roots contain vasicinolone, vasicol, peganine and 2'-hydroxy-4-glucosyl-oxychalcone. The flowers contain β-sitosterol-D-glucoside, kaempferol and its glucosides, as well as the bioflavonoid, quercetin (Chemexil, 1992). The leaves are used in the treatment of respiratory disorders in Ayurveda. Alkaloids, vasicine and vasicinone present in the leaves, possess respiratory stimulant activity (Amin & Mehta, 1959; Chopra, 1925). Vasicine, at low concentrations, induced bronchodilation and relaxation of the tracheal

muscle (Chopra, 1925). At high concentrations, vasicine offered significant protection against histamine induced bronchospasm in guinea pigs. Vasicinone, the auto-oxidation product of vasicine has been reported to cause bronchodilatory effects both *in vitro* and *in vivo* (Cambridge *et al.*, 1962). In another study, vasicine showed appreciable bronchodilatory effect and marked respiratory stimulant activities whereas vasicinone showed relaxation of the tracheal muscle *in vitro* and bronchoconstriction *in vivo* (Atal, 1980). Of the two alkaloids, vasicinone was found to be more potent than vasicine, with potential anti-asthmatic activity comparable to that of disodium cromoglycate.

Aegle marmelos

Aegle marmelos Corr. (Rutaceae) is used commonly for the treatment of asthma and heart diseases in the Indian system of medicine. The ethanol extract of *A. marmelos* leaves produced a dose-dependent inhibition of acetylcholine- and histamine-induced contractions of the isolated guinea pig trachea, suggesting that the antagonism of histamine and acetylcholine receptors may partly account for the beneficial use of the plant in the management of asthma (Arul *et al.*, 2004).

Albizzia lebbeck

Albizzia lebbeck Benth. (Mimosaceae) is a large, erect, deciduous spreading tree, well known in the Indian subcontinent for its range of medicinal uses. In the indigenious system of medicine it is purportedly effective in asthma, bronchitis, other respiratory disorders, snakebite and other ailments (Kumar *et al.*, 2007). The decoction of the bark and flower of *A. lebbeck* protected guinea pigs against histamine and acetylcholine induced bronchospasm, also chronic treatment with the bark decoction protected the sensitized guinea pigs against antigen challenge (Tripathi & Das, 1977). The plant extracts have also been demonstrated to possess anti-inflammatory (Thenmozi *et al.*, 1989; Kumar *et al.*, 2007) and immunomodulatory (Baruah *et al.*, 2000) activities, inhibit passive cutaneous anaphylaxis and mast cell degranulation in rat dose-dependently, and protect the sensitized guinea pig from antigen induced anoxic convulsion (Baruah *et al.*, 1997).

Amburana cearensis

Amburana cearensis A.C. Smith (Fabaceae) is a medicinal plant popularly used in Northeastern Brazil to treat respiratory tract diseases including asthma (Braga, 1976). Several compounds such as 3,4-dihydroxybenzoic acid (protocatechuic acid), a mixture of glucosylated β-sitosterol and stigmasterol, coumarin, four flavonoids (isokaempferide-IKPF, kaempferol, afrormosin, and 4′-methoxyfisetin) and the phenol glucosides, amburosides A and B,

were isolated from the trunk bark of this plant (Bravo *et al.*, 1999; Canuto & Silveira, 2000). The hydroalcoholic extract, coumarin and the flavonoid fraction (with IKPF as the main constituent) from *A. cearensis* have anti-inflammatory activity and were able to relax the isolated guinea-pig tracheal smooth muscle pre-contracted by carbachol, histamine or KCl (Leal *et al.*, 2000, 2003). The flavonoid, isokaempferide (IKPF; 5,7,4'-trihydroxy-3-methoxyflavone), from *A. cearensis* caused a direct and epithelium-independent relaxation of the isolated guinea pig trachea, possibly due to the opening of Ca^{2+} and ATP-sensitive K^+ channels. These results further indicate that IKPF may be partly responsible for the bronchodilatory activity of *A. cearensis* (Leal *et al.*, 2006).

Artemisia caerulescens

In a study of the bronchodilatory effect of the butanol extract of *Artemisia caerulescens subsp.* Gallica on anaesthetized guinea pigs *in vivo* and *in vitro* preparations of the guinea pig trachea, the extract antagonized histamine- and acetylcholine- induced bronchoconstriction. Also the dose-response curves obtained in the *in vitro* preparations were shifted to the right in the presence of β-adrenergic blockers (propranolol or ICI 118.551), indicating that agonist action on β_2 receptors may contribute to the bronchodilatory effect of *A. caerulescens* (Moran *et al.*, 1989).

Asystasia gangetica

The leaves of *Asystasia gangetica* (L.) T. Anderson *subsp.* Micrantha (Nees) Ensermu (Acanthaceae) is a traditional asthma remedy in many parts of Nigeria. In a study to investigate the anti-asthmatic potential of the leaves; results indicated that the extracts elicited a dose-related inhibition of acetylcholine-, serotonin- and histamine-induced contractions of the isolated guinea pig trachea, and also ameliorated the inflammatory response (Akah *et al.*, 2003; Ezike *et al.*, 2008).

Bacopa monniera

Bacopa monniera (L) syn. *Herpestis monniera* Linn (Scrophulariaceae), a herbaceous plant widely distributed throughout the Indo-Pakistan subcontinent, is used in traditional medicine for the cure of various ailments including bronchitis, inflammation and asthma (Nadkarni, 1976). Ethanol extracts of the leaves and stalks of *B. monniera* had no effect on the basal tone of the guinea pig trachea, but elicited a dose-related relaxation in the presence of histamine (Dar & Channa, 1997). The results further suggested that the relaxation may also involve prostacyclin compounds and β_2 receptors (Dar & Channa, 1997).

Bunium persicum

The seed of *Bunium persicum* (Boiss.) B. Fedtsch. (or *Carum persicum*) has bronchodilatory activity acting through inhibition of histamine (H_1) receptors (Boskabady & Moghadas, 2004).

Carum copticum

Carum copticum is a grassy, annual plant with white flowers and small brownish seeds, which grows in the east of India, Iran and Egypt. The seeds of this plant have an odour similar to thymol; and its essential oil contains terpinene, p. cymene, α-pinene, β-pinene and other substances such as thymol and carvacrol (Ballba *et al.*, 1973; Nagalakshmi *et al.*, 2000; Gersbach & Reddy, 2002). The seeds of *C. copticum* have several therapeutic effects including anti-asthma, anti-dyspnea, diuretic, anti-vomiting, analgesic, and others (Avesina, 1985). Studies have demonstrated that the plant exhibits antibacterial (Srivastava *et al.*, 1999; Singh *et al.*, 2002), bronchodilatory and anticholinergic (Boskabady *et al.*, 1998), histamine (H_1) receptor antagonist (Boskabady & Shikhi, 2000), β-adrenergic agonist (Boskabady & Moemeni, 2000) and probably xanthine-like (Boskabady & Karachian, 2000) effects. A recent study suggests that the bronchodilatory effect of essential oil from *C. copticum* is mainly due to carvacrol (Boskabady *et al.*, 2003); carvacrol has bronchodilatory effect (Boskabady & Jandaghi, 2003). Interestingly an earlier study showed the muscarinic effect of roasted *Carum copticum* (Devansankaraih *et al.*, 1972).

Cecropia glaziovii

The leaf extracts of *Cecropia* specie are used in Latin America for the treatment of cough, asthma and bronchitis (Delarcina *et al.*, 2007). The aqueous extract of the leaves of *Cecropia glaziovii* Sneth (Cecropiaceae) exhibits bronchodilatory activity probably related to β-adrenergic activity (Delarcina *et al.*, 2007).

Clerodendrum petasites

In the traditional system of medicine of Thailand, the aerial part of *Clerodendrum petasites* S. Moore (Lamiaceae), locally known as 'Thao yaai mom', is prepared as a tea or an alcoholic extract and used to treat asthma. The ethanol (96%) extract of the aerial parts (2.25–9.0 mg/mL) elicited a dose-dependent relaxation of the guinea pig trachea pre-contracted with histamine. A bronchodilatory flavonoid, hispidulin (4′,5,7-trihydroxy-6-methoxy-flavone) was isolated on bioassay-guided fractionation of the crude alcoholic extract (Hazekamp *et al.*, 2001).

Coleus forskohlii

Coleus forskohlii is a perennial member of the mint (Lamiaceae) family and grows in the subtropical temperate climates of India, Nepal, Sri Lanka, and Thailand. The plant has long been used in the Hindu and Ayurvedic traditional medicine to treat various disorders including asthma, and various preclinical and clinical studies support the ethnomedicinal use of the plant. *C. forskohlii* is the only known natural source of the unique adenyl cyclase activating drug, forskolin, which is of benefit in the therapy of asthma (de Souza, 1991).

Cynanchum komarovii

The aqueous and ethanol extracts of *Cynanchum komarovii* ('Lao Gua Tou' in Chinese) were shown to promote expectoration and alleviate acetylcholine- and histamine-induced asthma in guinea pigs (Lu *et al.*, 1997).

Ding-Chuan-Tang

Ding-Chuan-Tang (DCT), a traditional Chinese medicine has been used in the treatment of bronchial asthma for several centuries. In a study to elucidate the mechanism of its antiasthmatic effect, it was observed that DCT caused dose-dependent relaxation of carbachol-induced contractions of the guinea pig trachea, and significantly inhibited ovalbumin-induced immediate- and late- asthmatic responses. The results suggest that the antiasthmatic effect of DCT is mainly due to bronchodilation, inhibition of eosinophil migration into the airway, and prophylactic effect against allergen-induced airway inflammation (Kao *et al.*, 2004).

Drosera madagascariensis

The dried aerial parts of *Drosera madagascariensis* used in the therapy of infections and other disorders of the respiratory tract, was shown to elicit spasmolytic effect via antagonism of acetylcholine M_3 and histamine H_1 receptors. *D. madagascariensis* also inhibited human neutrophil elastase activity (Melzig *et al.*, 2001), this may prevent subsequent progression of lung injury (Kawabata *et al.*, 2000). It had no effect on $PGF2_{\alpha}$- induced contraction of the guinea pig trachea (Melzig *et al.*, 2001).

Foeniculum vulgare

The ethanol extract and an essential oil from *F. vulgare* have bronchodilatory effect as shown by their significant relaxant effect on methacholine-induced contraction of the isolated guinea pig trachea (Boskabady *et al.*, 2004).

Gnaphalium liebmannii

Several species of the genus *Gnaphalium* are used in Mexico for the management of respiratory disorders such as asthma, bronchitis, cough, cold and other bronchial ailments (Aguilar *et al.*, 1994). The extracts of the inflorescences of *G. liebmannii* have bronchodilatory effects as shown by a dose-related inhibition of carbachol-induced contractions of the guinea pig trachea; this may contribute to its use as an anti-asthmatic agent (S´anchez-Mendoza *et al.*, 2007).

Hedera helix L. (Ivy)

Preclinical studies suggest that ivy leaf extracts have spasmolytic, bronchodilating and antibacterial effect which are mainly attributable to the triterpene saponins contained in them (Cioaca *et al.*, 1978; Haen, 1996; Trute *et al.*, 1997; Bedir *et al.*, 2000). Various clinical trials have shown that three formulations of dried ivy leaf extracts (drops, syrup, suppositories) can be safe and effective in improving respiratory function in children suffering from mildly to moderately severe, reversible chronic respiratory tract diseases (obstructive or non-obstructive) (Gulyas *et al.*, 1997; Mansfeld *et al.*, 1997, 1998). These findings are consistent with the results of another trial in adults with simple or obstructive chronic bronchitis (Meyer-Wegener *et al.*, 1993).

Nevertheless, more far-reaching conclusion(s) on the efficacy and safety of ivy leaf extract in respiratory disorders is hampered by the limitations of the conducted trials; therefore there is need for additional placebo-controlled trials with appropriate methodology and larger sample size (Hofmann *et al.*, 2003).

Ilex latifolia

Ilex latifolia Thunb. ('Kudingcha' in Chinese) significantly inhibited the contraction of the isolated guinea pig trachea induced by acetylcholine, histamine and calcium chloride (Jiang *et al.*, 2001).

Mangifera indica

Mangifera indica L. (Anacardiaceae) is widely distributed in Africa, and many traditional healers in Togo use the stem bark to treat asthma. Aqueous extract of the stem bark of *M. indica* (1–4 mg/kg) elicited a dose-dependent relaxation of the isolated rat trachea pre-contracted with acetylcholine (Agbonon *et al.*, 2002), this may partly account for its ethnomedicinal use in the management of asthma.

Moringa oleifera

Moringa oleifera Lam. (Moringaceae) is used by many Ayuverdic practitioners for the treatment of asthma and chronic rheumatism (Fahey, 2005). *M.*

oleifera, a medium-sized tree found wild in the sub-Himalayan tract is reported to elicit good clinical response in children suffering from upper respiratory tract infection and skin infection. Studies have shown the seeds to possess antispasmodic and anti-inflammatory activities (Udupa *et al.*, 1994; Guevara *et al.*, 1999). An alkaloid from the plant, moringine, is useful in the therapy of asthma; it relaxes the bronchioles and it has a similar pharmacological activity to ephedrine (Kirtikar & Basu, 1975; Agrawal & Mehta, 2006).

Clinical studies on *M. oleifera* showed that the plant elicited appreciable decrease in the severity of symptoms and improvement of lung function parameters (Agrawal & Mehta, 2006, 2008).

Nigella sativa

The seeds of *N. sativa* Linn. (Ranunculaceae) have been used traditionally in the Middle East in ancient Iran for its therapeutic effects in respiratory disorders such as asthma, dyspnea and other ailments (Nadkarni, 1976; Sayed, 1980; Lautenbacher, 1997). Extracts of *N. sativa* seeds have bronchodilatory activity as shown by their anticholinergic (Boskabady & Shahabi, 1997) and histamine (H_1) receptor antagonist (Boskabady & Shiravi, 2000) effects on the guinea pig tracheal chains. *Nigella sativa* seed oil seems not to have an immunomodulatory effect on Th1 and Th2 cell responsiveness to allergen stimulation (Bu¨yu¨ko¨ztu¨rk *et al.*, 2005), but may modify leukotriene synthesis and inhibit histamine release (Chakravarty, 1993).

Onion

Onion has been demonstrated to possess antiasthmatic and anti-inflammatory effects, which may partly depend on the thiosulfinate moiety (Dorsch *et al.*, 1988, 1989).

Passiflora incarnata

In many countries, *Passiflora incarnata* Linn. (Passifloraceae) (Passion flower) has been used traditionally to treat severe asthma, bronchitis and other respiratory disorders (Lutomski *et al.*, 1981; Dhawan *et al.*, 2003). The leaf extract was shown to exhibit significant antitussive activity at a dose of 100 mg/kg, against SO_2-induced cough in mice (Dhawan & Sharma, 2002). Using a 7-day treatment regimen, Dhawan *et al.* (2003) demonstrated a significant prevention of dyspnoea-related convulsions in the animals treated with a 100 mg/kg dose of the methanol extract of the plant; no preventive effect was exhibited by the 50 mg/kg dose; and at a higher dose (200 mg/kg), the preventive effect was reduced.

Pimpinella anisum

Essential oil, aqueous and ethanol extracts of *P. anisum* were shown to have bronchodilatory effects due to inhibitory effects on muscarinic

receptors (Boskabady & Ramazani-Assari, 2001), also the antispasmodic activity may be dependent on the activation of the NO-cGMP pathway (Tirapelli *et al.*, 2007).

Polygala cyparissias

Incubation of the hydroalcoholic extract (0.125–1 mg/mL), and the purified xanthone (1,7-dihydroxy-2,3-dimethoxy xanthone) (2.5 to 80 microg/mL) isolated from *Polygala cyparissias* with the guinea pig trachea for 20 min, had no effect on the basal tone, but elicited a dose-related, reversible and non-competitive inhibition of contractions induced by acetylcholine, histamine, compound 48/80, bradykinin, substance P, and prostaglandin E_2. The extractives also caused a dose-related inhibition of ovalbumin-induced contractions of trachea isolated from sensitized guinea pigs. At higher concentrations, the xanthone also antagonised contraction induced by potassium chloride in guinea-pig trachea. The results lend credence to the ethnomedicinal use of *P. cyparissias* in the management of asthma, allergy and inflammation, and suggest that 1,7-dihydroxy-2,3-dimethoxy xanthone is an active antiasthmatic principle of the plant (El Sayah *et al.*, 1999).

Portulaca oleracea

P. oleracea L. (Portulacaceae) an annual plant which grows in many areas of the world is indicated in ancient Iranian medical books for the management of respiratory diseases (Malek *et al.*, 2004). Aqueous extract of *P. oleracea* has a relatively potent but transient bronchodilatory effect on asthmatic airways, when compared with theophylline syrup and salbutamol inhaler (Malek *et al.*, 2004). In addition, *P. oleracea* exhibited relaxant effect on skeletal (Okwuasaba *et al.*, 1986, 1987) and intestinal smooth muscles (Parry *et al.*, 1987), anti-inflammatory (Chan *et al.*, 2000), antioxidant (He *et al.*, 1997; Simoponlos *et al.*, 1992) and potassium channel opening effects (Habtemariam *et al.*, 1993).

Propolis

Propolis, a naturally occurring substance, is a red or yellow-brown to dark-brown lipophilic mixture that is produced by mixing the exudates collected from various plants by honeybees (Miyataka *et al.*, 1997). Propolis, also known as bee glue, is a traditional remedy that is widely used in many countries for the management of numerous diseases, including airway disorders (Paulino *et al.*, 2002). Numerous studies indicate that propolis and its constituents exert a wide range of pharmacological actions including immunomodulatory and anti-inflammatory effects (Ivanovska *et al.*, 1995), and antibacterial activity (Bankova *et al.*, 1995) which may be beneficial in

asthma therapy. Furthermore, propolis induces a relaxant effect on the guinea-pig isolated trachea by means of several mechanisms that may involve the release of nitric oxide probably from sensory neurons, the activation of soluble guanylate cyclase, activation of Ca^{2+} and ATP-sensitive K^+ channels, and stimulation of β_2-adrenergic and vasoactive intestinal peptide (VIP) receptors (Paulino *et al.*, 2002).

Pluchea ovalis

Pluchea ovalis (Pers.) DC. (Asteraceae) is distributed in the swamp of Senegal, Mauritania, Ghana, Mali and Togo. The root of *P. ovalis* is also used by Togolese traditional healers in the management of asthma. In an evaluation of the pharmacological basis for its use in asthma, the ethanol extract of the roots of *P. ovalis* (0.25–1 mg/mL) produced a dose-dependent relaxation of the rat tracheal smooth muscle strip pre-contracted by acetylcholine (0.055 mmol/L) (Agbonon *et al.*, 2002).

Terminalia bellerica

Terminalia bellerica Roxb. (Combretaceae) commonly known as "belleric myrobalan" and locally as "bahera" is a large deciduous tree, found throughout Central Asia and some other parts of the world (Kapoor, 1990). Its fruit is used in folk medicine to treat asthma, inflammation, rheumatism, cancer, colic, cough, diarrhea, dyspepsia, dysuria, headache, hypertension amongst other ailments (Gilani *et al.*, 2008). In a clinical study, *T. bellerica* was found to possess antispasmodic, antiasthmatic and antitussive effects (Trivedi *et al.*, 1979). A 75% aqueous-methanol extract of *T. bellerica* fruit elicited a dose-dependent inhibition of carbachol-induced increase in respiratory pressure in anaesthesized rats. On the isolated guinea pig trachea, the extract caused inhibition of carbachol (1 μM)-induced contractions with IC_{50} of 3.3 mg/mL compared to that against K^+ (80 mm)-induced contractions with IC_{50} of 8.5 mg/mL. The inhibitory effect of the plant extract is mediated possibly through anticholinergic and calcium antagonist action. Bioactivity-guided fractionation revealed that the Ca^{2+} antagonist component was concentrated in the aqueous fraction, while anticholinergic agent was distributed both in organic and aqueous fractions, with the ethyl acetate fraction exhibiting the most potent antispasmodic effect (Gilani *et al.*, 2008).

Vitex trifolia

The n-hexane extract of *V. trifolia* has tracheospasmolytic activity, and on fractionation yielded two compounds; viteosin-A and vitexicarpin. These compounds blocked histamine-induced contraction of the guinea pig trachea, however only vitexicarpin blocked the effects of histamine released from mast cells in murine model of ovalbumin-induced asthma (Alam *et al.*, 2002).

The leaves of *V. trifolia* were demonstrated to inhibit mast cell degranulation (Ikawati *et al.*, 2001), and hence histamine release.

Plants that Reduce Airway Inflammation

Airway inflammation plays a central role in the pathogenesis of a number of lung diseases, including asthma, chronic bronchitis, bronchiectasis, and chronic obstructive pulmonary disease (COPD). Allergic diseases are known to occur with an imbalance in T-helper (Th) cells with a shift to Th2 subtype predominance and a decrease in Th1 subpopulation. One of the therapeutic objectives in asthma is to reduce the airway inflammatory response through the reduction of antigen-induced inflammatory cells, inhibition of synthesis and function of inflammatory cytokines and other mediators, immunomodulation, *i.e.* reducing Th2 proliferation and providing sufficient Th1 response (Gore & Custovic, 2004) amongst others. Plants reported to possess beneficial anti-inflammatory property in the pharmacotherapy of asthma include:

Ailanthus altissima

Ailanthus altissima (Simaroubaceae), commonly known as the 'tree of heaven', is used in traditional medicine in many parts of Asia to treat respiratory and gastric diseases. Anti-inflammatory quassinoids have been isolated from *Ailanthus altissima* (Okano *et al.*, 1990). The ethanol extract of the leaf and branch of *A. altissima* reduced the eosinophil infiltration into the airway and the eotaxin, interleukin-4 (IL-4), and interleukin-13 (IL-13) mRNA expression levels in ovalbumin (OVA)-sensitized mice. In an *in vitro* anti-inflammatory assay using bone marrow-derived mast cells from male BALB/c mice, the extract inhibited generation of the cyclooxygenase-2 (COX-2) dependent phases of prostaglandin D_2 (PGD_2) in a concentration-dependent manner with an IC_{50} value of 214.6 μg/mL, but did not inhibit COX-2 protein expression up to a concentration of 400 μg/mL; indicating a direct inhibition of COX-2 activity. In addition, *A. altissima* inhibited leukotriene C_4 (LTC_4) production with an IC_{50} value of 25.7 μg/mL and degranulation reaction in a dose dependent manner, with an IC_{50} value of 27.3 μg/mL. The anti-inflammatory activity of *A. altissima* in OVA-induced lung inflammation may occur in part via the down regulation of Th2 cytokines and eotaxin transcripts as well as the inhibition of inflammatory mediators (Jin *et al.*, 2006).

Astragalus membranaceus

Astragalus membranaceus (AM) is a traditional Chinese herb which has been used for more than 2000 years in China. Physicians in TCM (Traditional Chinese Medicine) system used AM formulated as injection (regarded as a potent tonic for increasing energy levels and stimulating the immune system)

to boost the body's general vitality and strengthen resistance to exogenous pathogens (Block, 2003). A murine model of ovalbmin-induced chronic asthma was used to investigate the effects of AM injection on allergen-induced airway inflammation, mucus secretion and hyperreactivity. The results showed that the administration of AM significantly inhibited airway hyperreactivity, mucus hypersecretion, and eosinophil infiltration in the airway. Elevated IL-5, IL-13 in bronchoalveolar lavage fluid (BALF) in asthmatic mice were markedly decreased while increased interferon-γ (IFN-γ) was observed (Shen *et al.*, 2008). The results suggest a beneficial role for AM in alleviating airway inflammation.

Chrysanthemum sibiricum

The ethanol extract of the aerial parts of *Chrysanthemum sibiricum* var. *latilobum* Kitamura (Compositae), (*Chrysanthemi sibirici herba*) commonly called 'Gu-Jul-Cho' in Korea, has been used as a traditional folk remedy for the treatment of pneumonia, bronchitis, cough, common cold, pharyngitis amongst other ailments (Lee *et al.*, 2004). The extract was demonstrated to effectively suppress an *in vivo* anaphylactic reaction induced by compound 48/80; in addition to inhibition of the degranulation in RBL-2H3 mast cells and the expression of tumor necrosis factor-α (TNF-α) in a dose-related manner (Lee *et al.*, 2004).

Crinum giganteum

The hot water extract of *Crinum giganteum* (Amaryllidaceae) is widely used in Nigeria for the treatment of asthma and related respiratory tract disorders. The aqueous extract of the bulb of *C. giganteum* has been shown to possess significant anti-inflammatory and anti-lymphocytic activities which may be beneficial in the therapy of asthma (Kapu *et al.*, 2001).

Drymis winteri

Drymis winteri J.R. et Forster (Winteraceae) is a native plant found in southern Brazil and in some other South American countries. Infusion of its barks has been commonly used in Brazil as a remedy for the treatment of respiratory diseases, such as asthma, allergy and bronchitis (El Sayah *et al.*, 1997). It is also used as an anti-inflammatory, antispamodic, antipyretic and cicatrizant remedy (Morton, 1981). The hydroalcoholic extract of *D. winteri* elicited a dose-related antagonism of contractions of the guinea pig trachea induced by compound 48/80, and inflammatory mediators of asthma such as bradykinin, prostaglandin E_2 (PGE_2) and substance P. However, the extract had little or no effect on acetylcholine- and histamine-induced contractions of the isolated guinea pig trachea. In addition, the extract caused a dose-related inhibition of ovalbumin-mediated contractions of the trachea isolated

from sensitized guinea pigs (EI Sayah *et al.*, 1997). These effects are partly due to the sesquiterpene, polygodial, isolated from the bark of *D. winteri* (EI Sayah *et al.*, 1998).

Duchesnea chrysantha

Duchesnea chrysantha (Rosaceae) a herb with anti-oxidative, anti-inflammatory and immune enhancing properties was screened for inhibitory effect on lung inflammation using the murine model of ovalbumin-induced asthma. The results showed that ethanol (80%) extract of *D. chrysantha* significantly reduced the elevated infiltration of inflammatory cells ($p<0.05$), and inhibited leukocytosis, the production of eotaxin and eosinophilia in BALF ($p<0.01$). Eotaxin a potent chemoattractant for eosinophils produced by epithelial cells in response to IL-4 and IL-13, is generally elevated after asthma induction (Blanchard *et al.*, 2005; Yang *et al.*, 2007). The extract also elicited a non-significant reduction in the increased mucus secretion, a slight reduction of total IgE level and a marked reduction of ovalbumin-specific IgE level of serum and BALF. These indicate that the extract may inhibit the asthmatic response and offer a useful therapeutic approach in the management of allergic airway diseases (Yang *et al.*, 2007).

Euphorbia stenoclada

Euphorbia stenoclada Baill. (Euphorbiaceae) is used in Madagascar as herbal remedy for asthma and bronchitis. The ethanol extract of aerial parts of *E. stenoclada* totally abolished the IL-1β-induced proliferation of human airway smooth muscle cells (IC_{50} = 0.73 ± 0.08 g/mL), and quercetin has been identified as the major anti-proliferative compound of *E. stenoclada* (Chaabi *et al.*, 2007). This effect may be beneficial in prevention and alleviation of asthma-associated airway structural changes. However, this result is in accordance with numerous studies showing the anti-proliferative activity of quercetin in other cell types, such as in cancer cell lines (Kanadaswami *et al.*, 2005; Lambert *et al.*, 2005).

Galphimia glauca

The methanol extract of *Galphimia glauca* Cav. (Malpighiaceae) (p.o) inhibited acute bronchial reactions to inhalation challenge by allergen (ovalbumin, 10 mg/mL) and platelet-activating factor (PAF) (1 μg/mL), but not to histamine or acetylcholine in spontaneously breathing guinea pigs. In addition, PAF-induced bronchial hyperreactivity was markedly reduced (Dorsch *et al.*, 1992). Also, Dorsch *et al.* (1992) reported the isolation of gallic acid, methyl gallate, quercetin and tetragalloylquinic acid from *G. glauca*; these compounds were identified as responsible for the antiasthmatic effect. Gallic acid, methyl gallate and quercetin showed significant effects after a single oral dose of 45

mg/kg, compared to tetragalloylquinic acid after 5 mg/kg. In another study, the ethylacetate fraction of the methanol extract of the aerial parts of *G. glauca* significantly inhibited both the contraction induced by ovalbumin and by LTD_4 in the guinea pig trachea. The fraction had no effect on the contractions induced by LTC_4, histamine, carbachol, and potassium chloride (Campos *et al.*, 2001). The selective inhibition of LTD_4 provides a lead for drug development in the light of the finding that though LTC_4, LTD_4 and LTE_4 contract human airways, only one receptor, that for LTD_4 has been demonstrated as the most pharmacologically relevant for contraction (Buckner *et al.*, 1986).

Gleditsia sinensis

Gleditsia sinensis LAM. (Leguminosae) is a perennial shrub widely distributed throughout China. The anomalous fruits of *G. sinensis* have long been known in traditional Chinese medicine as a saponin-rich herbal medicine for treating various diseases, including asthma, productive cough and headache (Hou *et al.*, 2006). Studies have demonstrated that extracts of the anomalous fruits of *G. sinensis* attenuated IgE-mediated immediate allergic reactions such as passive cutaneous anaphylaxis in rats (Dai *et al.*, 2002), inhibited histamine release from mast cells (Fu *et al.*, 2003), inhibited nasal vascular permeability (Fu *et al.*, 2003), and attenuated picryl chloride-induced delayed type hypersensitivity and IL-2 production in mice (Hou *et al.*, 2006). These inhibitory effects on immuno-inflammation may partly account for the use of *G. sinensis* in asthma.

Ledum groenlandicum

Ledum groenlandicum Retzius (Ericaceae) (Labrador tea) is widely distributed in North America. It is a 30–120 cm high shrub with densely tomentose twigs, which grows from Alaska to Groenland and spreads south to Pennsylvania. The leaves and twigs were used in Native American traditional medicine to treat several ailments such as inflammatory diseases (Rousseau, 1947; Moerman, 2000), asthma (Chandler *et al.*, 1979), rheumatism (Gunther, 1973), burns (Leighton, 1985) and diseases of the liver (Burgesse, 1944) and kidney (Chandler *et al.*, 1979; Turner *et al.*, 1980). The crude methanol extracts of the leaves and twigs of *L. groenlandicum* possess significant antioxidant and anti-inflammatory activities (Dufour *et al.*, 2007), which may partly be responsible for its ethnomedicinal use in asthma.

Liriope platyphylla

Liriope platyphylla is a popular herb used in oriental medicine for treatment of asthma and bronchial and lung inflammation (Lee *et al.*, 2005). Tai *et al.* (2002) reported that it improves airway mucociliary clearance, acting partly through the amelioration of airway mucus secretion. Evaluation of the effects

of the aqueous extract of the fruit (tuber) of *L. platyphylla* on pulmonary eosinophilic inflammation using OVA-induced airway inflammation in murine model of asthma, showed that the extract reduced total lung leukocytes, eosinophils, IL-4, IL-5, IL-13 and IgE levels in the BALF and serum. The extract modulated Th1/Th2 cytokine imbalance, and also decreased eosinophil CCR3 (chemokine receptor) expression and CD11b expression in lung cells. These results suggest that *L. platyphylla* is immunomodulatory producing marked inhibitory effects on airway inflammation and hyperresponsiveness (Lee *et al.*, 2005).

Lomatia hirsuta

The aqueous extract of the stem and leaves of *Lomatia hirsuta* (Lam.) Diels ex macbr. (Proteaceae) (Radal) is used in the traditional medicine of Chile for treatment of cough, bronchial ailments and asthma (San Martin, 1983). The leaf extract was shown to possess mild anti-inflammatory activity against carrageenan-induced paw edema in guinea pigs (Erazo *et al.*, 1997).

Oryza sativa

The seed of *Oryza sativa* L. var. *japonica* (Poaceae) (syn. Glamineae), which is commonly known as 'Heuk-Mi' (black rice) in South Korea, is a dark purple-colored major rice crop in South Asia and China. It is enriched rice and has been used as a traditional medicine for various allergic disorders such as chronic dermatitis and bronchitis (Kim *et al.*, 1999). The therapeutic potential of an ethanol extract of black rice (DA-9201) as an anti-asthmatic, was evaluated on an OVA-induced mouse model of asthma. Balb/c mice immunized with OVA were administered with DA-9201 (30, 100 or 300 mg/kg, *p.o.*) or dexamethasone (3 mg/kg, *p.o.*), and challenged with 1% aerosolized OVA for 30 min. The effects on airway inflammation, airway hyperresponsiveness (AHR), antibody profiles and cytokines were evaluated. DA-9201 treatment significantly reduced the number of eosinophils in BALF and ameliorated the AHR. Lung histological features also showed that DA-9201 reduced airway inflammation. Furthermore, DA-9201 treatment decreased IFN-γ as well as IL-4, IL-5 and IL-13 levels in the supernatant of cultured splenocytes, and suppressed the level of OVA-specific IgG, IgG2a, IgG1 and total IgE in plasma. DA-9201 showed anti-asthmatic effects by suppressing unnecessary immune responses, airway inflammation, eosinophilia, AHR and IgE level. These results suggest DA-9201 might be beneficial in the treatment of asthma (Lee *et al.*, 2006a).

Perilla frutescens

Perilla frutescens BRITTON (Labiatae) (Perilla) is one of the components of Saiboku-to a Kampo medicine used in the treatment of bronchial asthma.

Intraperitoneal injection of a decoction of dried leaves of *P. frutescens* suppressed antigen-specific IgE production in mice (Imaoka *et al.*, 1993), histamine-release from peritoneal mast cells in rats (Shin *et al.*, 2000) and induced Th1-type cytokines in mice (Ishihara *et al.*, 1999) suggesting that *Perilla* has anti-inflammatory and anti-allergic activities. Likewise, oral administration of *Perilla* decoction suppressed allergic reaction in mice, and the active constituents are rosmarinic acid and apigenin-diglucuronide (Makino *et al.*, 2001, 2003).

Picrorhiza kurroa

The underground parts of *Picrorhiza kurroa* have been used in the traditional Indian system of medicine since ancient times to treat liver troubles and bronchial problems. *P. kurroa* extracts have been demonstrated to elicit anti-allergic activity, probably mediated through mast cell stabilizing activity (Mahajani & Kulkarni, 1977) and inhibition of PAF (Dorsch *et al.*, 1991). Androsin, a phenolic glycoside isolated from *Picrorhiza kurroa,* has been attributed with anti-asthmatic properties, as it inhibits PAF, thereby relieving bronchial hyperreactivity and subsequently bronchial obstruction (Dorsch *et al.*, 1991).

Prunus armeniaca

In traditional oriental medicine, *Armeniacae semen* has been used for the treatment of asthma, bronchitis, emphysema, constipation, nausea, leprosy, and leucoderma. *Armeniacae semen* is the seed of *Prunus armeniaca* L. var. *ansu* MAXIM (Rosaceae). Chang *et al.* (2005) reported that *Armeniacae semen* exerts anti-inflammatory and analgesic effects probably by suppressing COX-2 and inducible nitric oxide synthetase (iNOS) expressions, and consequently inhibition of prostaglandin E_2 (PGE_2) and nitric oxide (NO) synthesis.

Rhamnus nakaharai

Rhamnus nakaharai Hayata (Rhamnaceae) is used as a folk medicine in Taiwan for treating asthma, inflammation, constipation, and other ailments. A main constituent of the plant, 3-*O*-Methylquercetin (3-MQ), has been reported to have potential for use in the treatment of asthma (Jiang *et al.*, 2006).

Saururus chinensis

The aerial part of *S. chinensis* is used in Korean folk medicine for the treatment of several inflammatory diseases and other ailments. The ethanol (70%) extract of *S. chinensis* inhibited generation of the COX-2 dependent phases of prostaglandin D_2 in bone marrow-derived mast cells in a

concentration-dependent manner with an IC_{50} value of 14.3 mg/mL, and also inhibited LTC_4 production with an IC_{50} value of 0.3 mg/mL. In an *in vivo* evaluation of anti-asthmatic activity using ovalbumin-induced asthma in mouse, the oral administration (50–200 mg/kg) of the ethanol extract of *S. chinensis* reduced the number of infiltrated eosinophil in BALF and inhibited the eotaxin and IL-4 mRNA expression levels at 100 mg/kg. These results suggest that the anti-asthmatic activity of *S. chinensis* might in part occur via the inhibition of eicosanoid generation, as well as the down regulation of IL-4 and eotaxin mRNA expression (Lee *et al.*, 2006b).

Saussurea costus

Saussurea costus (Falc.) Lipschitz, syn *Saussurea lappa* C.B. Clarke is a well known and important medicinal plant widely used in several indigenous systems of medicine for the treatment of various ailments, including asthma, inflammatory diseases such as chronic lung inflammation, chest congestion, ulcer and stomach problems (Tsarong, 1994; Pandey *et al.*, 2007). The extracts of *S. costus* have been demonstrated to possess antispasmodic and anti-inflammatory activities (Pandey *et al.*, 2007).

Semen armeniacae amarum (SAA)

Semen armeniacae amarum has long been used in Korean traditional medicine to manage asthma. Oral administration of the aqueous extract of SAA significantly reduced airway hyperreactivity and inflammation. SAA reduced the level of IL-4 in BALF, and elicited a selective inhibition of Th2 response to allergen (Do *et al.*, 2006).

Solanum xanthocarpum and *Solanum trilobatum*

Solanum xanthocarpum and *Solanum trilobatum* (Solanaceae) are widely used to treat respiratory diseases in Southern Indian traditional medicine. In a pilot study to investigate the clinical efficacy and safety of the herbs in mild to moderate bronchial asthma, it was observed that a single oral dose (300 mg) of powdered whole plant of either *Solanum xanthocarpum* or *Solanum trilobatum* significantly improved various parameters of pulmonary functions (Govindan *et al.*, 1999). The methanol extracts of the root and leaf of *S. xanthocarpum* also inhibited the synthesis of LTB_4 (Kumar *et al.*, 2000).

Tetrapleura tetraptera

Tetrapleura tetraptera Taub (Fabaceae) is used in the traditional medicine of tropical Africa to manage a number of diseases including inflammatory conditions like asthma and arthritis (Ojewole & Adewunmi, 2004). The aqueous extract of its fruit was shown to possess anti-inflammatory activity

against fresh egg albumin-induced pedal edema in rats (Ojewole & Adewunmi, 2004).

Tussilago farfara

The flower buds of *Tussilago farfara* L. (Compositae), also known as "Kwandong Hwa" in Korea and China, have long been used in traditional Oriental medicine for the treatment of bronchitis and asthma (Namba, 1980). *T. farfara* has been shown to exhibit anti-inflammatory properties, acting via inhibition of arachidonic acid metabolism (Murase *et al.*, 1996) and nitric acid synthesis (Ryu *et al.*, 1999).

Tylophora indica

The leaves of the plant *Tylophora indica* or *T. asthmatica* have been used in Ayurveda, the traditional Indian system of medicine, to treat various allergic and inflammatory disorders like bronchial asthma, rhinitis, whooping cough, catarrh et cetera (Chopra *et al.*, 1958). Various extracts of the leaves of *T. indica* were found to have antiasthmatic and antiallergic properties (Shivpuri *et al.* 1968, 1969, 1972). Tylophora extracts also inhibited Schultz-Dale reaction and the release of inflammatory mediators in experimental systems (Bhide *et al.*, 1974; Haranath *et al.*, 1975), and also evoked inhibition of systemic anaphylaxis, immunoadherence and adjuvant induced arthritis (Gopalakrishnan *et al.*, 1980), and suppression of delayed type hypersensitivity reaction in rats and mice (Ganguly & Sainis, 2001).

Anti-Allergic/Mast Cell Stabilizers of Plant Origin

Conventional anti-asthmatic compounds with anti-allergic and mast cell stabilizing properties have also been developed from plant isolates; for example, cromolyn was developed from the furanochromone – khellin (visammin) – found in the Asian plant *Amni visnaga*.

Crinum glaucum

Crinum glaucum (Amaryllidaceae), popularly known among the Yoruba of South West Nigeria as 'isumeri' (Gbile, 1984), is a bulbous plant with thick, stiff, erect, glaucous leaves. It has been reported as an effective remedy in the relief of cough, asthma and convulsions, by traditional medicine practitioners (Okpo & Adeyemi, 2002). The aqueous extract of *C. glaucum* bulbs evoked a non-specific relaxant effect on gastrointestinal smooth muscles (Okpo & Adeyemi, 1998), and has analgesic and anti-inflammatory effects (Okpo *et al.*, 2001).

In a bid to elucidate the pharmacological basis for the ethnomedicinal use of the plant in asthma, Okpo and Adeyemi (2002) investigated the effects of aqueous extract of *C. glaucum* bulb on rat passive cutaneous anaphylactic

reaction, rat peritoneal mast cell degranulation and allergic bronchoconstriction in the guinea pig. The extract significantly inhibited dextran- and antigen- induced degranulation of rat peritoneal mast cells and passive cutaneous anaphylaxis (PCA) in rats, comparable to the standard anti-allergic drug, sodium cromoglycate (Okpo & Adeyemi, 2002). The extract also significantly protected guinea pigs against egg albumin-induced allergic bronchoconstriction (Okpo & Adeyemi, 2002).

Kaempferia parviflora

In Thai traditional medicine, the alcohol decoction of *Kaempferia parviflora* powder is reported to cure allergy, asthma, diabetes. The anti-allergic effects of *K. parviflora* in RBL-2H3 cell line model has been reported (Tewtrakul & Subhadhirasakul, 2007; Tewtrakul *et al.*, 2008), and the methoxyflavone derivatives (5-hydroxy-3,7,3',4'-tetramethoxyflavone, 5-hydroxy-7-methoxyflavone and 5-hydroxy-7,4'-dimethoxyflavone) have been identified as the potent anti-allergic constituents of the plant (Tewtrakul & Subhadhirasakul, 2007; Tewtrakul *et al.*, 2008). The results indicated that the anti-allergic mechanism involves inhibition of cell degranulation mainly via the inhibition of Ca^{2+} influx to the cells via Ca^{2+} release-activated Ca^{2+} (CRAC) channels (Tewtrakul & Subhadhirasakul, 2007; Tewtrakul *et al.*, 2008).

Morus alba

Morus alba (Moraceae) has long been used for the control of diabetes, inflammation and asthma (Chai *et al.*, 2005). The aqueous root bark extract was shown to possess strong antihistamine and antiallergic activity as shown by inhibition of compound 48/80-induced systemic allergic reaction and anti-CGG (anti-chicken gamma globulin) IgE-induced local allergic reaction (Chai *et al.*, 2005).

HERBAL MIXTURES

Gakani

Gakani is a polyherbal drug commonly used in Western Nigeria to treat asthma and related respiratory disorders. It is an equal mixture of powders from the roots of six plants (*Cenchrus biflorus* Roxb., *Olax subscorpioidea* Oliv., *Piper guineense* Schum et Thonn, *Psorospermum guineense* Hochr., *Securidaca longipedunculata* Tresen, *Syziygium aromaticum* (L.) Merr. et Perry). The aqueous extract of Gakani unsurmountably blocked the effects of histamine and isoprenaline on the guinea pig tracheal chain; the blockade of isoprenaline effect is a paradox. The extract also exhibited good anti-inflammatory effect, causing a dose-related inhibition of rat paw edema. These activities may account for the use of Gakani in the management of asthma (Akah *et al.*, 1997).

Gyokuheifusan (GHS)

Gyokuheifusan (GHS; Yu-Ping-Feng-San in Chinese) a classical formula of traditional Chinese medicine (TCM) is the extract of a mixture of *Astragalus* root, *Atractylodes* rhizome, and *Saposhnikovia* root. It is widely prescribed in China, Japan and Korea to treat respiratory tract diseases such as respiratory infection, allergic rhinitis, bronchial asthma, and other similar conditions (Fang *et al.*, 2005). In TCM, GHS is believed to increase human vigor and to enhance the protective function of the lungs, defending the body against invasion by external pathogenic influences (Bensky & Barolet, 1990). Fang *et al.* (2005) studied the immunomodulatory effects of GHS on the Th1/Th2 balance in OVA-induced asthma model in mice; they demonstrated that GHS down-regulates the over-production of IgE and IL-4 via a significant and persistent increase of IFN-γ. Earlier studies show that *Astragalus* root reduced the secretion of IL-4 by regulating the shift of Th1 to Th2, and decreased the total IgE produced by B cells.

Saiboku-To

Saiboku-to, a herbal medicine prepared from ten herbs (*Bupleuri* radix, *Pinelliae* tuber, *Hoelen*, *Scutellariae* radix, *Magnoliae* cortex, *Zizyphi* fructus, *Ginseng* radix, *Glycyrrhizae* radix, *Perillae* herba and *Zingiberis* rhizoma), has long been used for the therapy of bronchial asthma and bronchitis in Japan (Nagano *et al.*, 1988). The clinical effectiveness of Saiboku-To (normally formulated as freeze-dried granules) in bronchial asthma, chronic bronchitis and bronchiectasis has been established by various trials (Nagano, 1990; Kimura, 1991; Egashira & Nagano, 1993; Nakajima *et al.*, 1993; Nishizawa *et al.*, 1997; Tohda *et al.*, 1999). The effects of Saiboku-To on patients with allergic disease, especially bronchial asthma may partly be due to inhibition of IgE-mediated allergic reactions (Koda *et al.*, 1982, 1993).

Shinpi-To

Shinpi-To (TJ-85) is a freeze-dried granular Chinese herbal medicine that is prepared from the extract of seven medicinal herbs; Ephedrae herba *(Ephedra sinica* Stapf), Armeniacae semen *(Prunus armeniaca* Linne), Magnoliae cortex *(Magnolia obovata* Thunberg), Aurantii nobilis pericarpium *(Citrus unshiu* Markovich), Glycyrrhizae radix (*Glycyrrhiza uralensis* Fischer), Bupleuri radix *(Bupleurum falcatum* L.), and Perillae herba *(Perilla frutescens* Britton var. *acuta* Kudo). Although it has been used in treating childhood asthma (Tubaki *et al.*, 1994), the pharmacological basis of its action is unclear. Results indicate that Shinpi-To inhibits leukotriene synthesis by inhibiting phospholipase A_2 (PLA_2) and 5-lipoxygenase (5-LO) activities (Hamasaki *et al.*, 1997).

So-Cheong-Ryong-Tang (SCRT) / Xiao-Qing-Long-Tang / Sho-Seiryu-To

So-Cheong-Ryong-Tang (SCRT) also called Xiao-Qing-Long-Tang or Sho-Seiryu-To, contains eight species of medicinal plants (Ephedra Herba, Paeoniae Radix Alba, Schizandrae Fructus, Pinelliae Rhizoma, Asari Herba Cum Radice, Zingiberis Rhizoma, Cinnamomi Ramulus, Glycyrrhizae Radix), and has been used as herbal medicine to treat allergic rhinitis and asthma for hundreds of years in Asian countries. Among several Chinese herbal medicines, SCRT is the most frequently prescribed for treating allergic asthma and rhinitis. The effects of SCRT have been investigated *in vivo*, *in vitro*, and at the clinical level. A broad range of pharmacologic evidence including decreasing antigen-induced eosinophil infiltration in guinea pigs (Sakaguchi *et al.*, 1997), suppressing allergen- induced bronchial inflammation in mite-sensitized mice (Kao *et al.*, 2000), and decreasing serum IgE levels in allergic rhinitis patients (Yang *et al.*, 2001; Tanaka *et al.*, 1998) have consistently demonstrated the beneficial effect of SCRT in allergic diseases (Ko *et al.*, 2004a).

In an effort to elucidate its pharmacologic mechanism, it was demonstrated that SCRT decreases the expression of the IL-4 mRNA that plays a pivotal role in Th2 cell development, while it increases IFN-γ expression (Ko *et al.*, 2004a).

The most difficult obstacle in developing antiallergic agents is probably the general immunosuppressive effects as seen with cortisol or cyclosporin A. On the contrary, SCRT does not suppress the T cell immune reaction, but rather enhances the TCR-triggered T cell response and IL-2 expression, suggesting that SCRT is not a general immune suppressor (Ko *et al.*, 2004b) Furthermore, the results also indicate that SCRT extract has selective immunomodulatory effects on Th2 cells but not on Th1 cells. Therefore SCRT extract may be useful for preventing the onset of allergies or improving allergic symptoms (Ko *et al.*, 2004a, 2004b).

Wu-Hu-Tang

Wu-Hu-Tang (WHT), is a Chinese formulation which consists of seven crude drugs, namely; *Ephedra sinica* Stapf., *Prunus armeniaca* L., Gypsum, *Glycyrrhiza uralensis* Fisch., *Morus alba* L., *Asarum sieboldii* Miq., and *Zingiber officinale* (Willd.) Rosc. It has been used for the treatment of asthma for hundreds of years. In a bid to elucidate the pharmacological basis for the ethnomedicinal use of the formulation, it was demonstrated that the aqueous extract of WHT produced a significant inhibition on the homologous passive cutaneous anaphylaxis (PCA) in rats and the heterologous PCA in mice, decreased the degranulation of mast cells of calvarial periosteum in rats, inhibited the release of anaphylactic mediators from sensitized lung tissues

of guinea pigs and the contraction of isolated guinea pig ileum induced by histamine. These results indicated that the therapeutic activity of WHT in asthma may be related to its inhibitory effects on immediate hypersensitivity *i.e.* anti-allergic activity (Dai *et al.*, 1997).

3.0 ISOLATED ANTIASTHMATIC PHYTOCONSTITUENTS

A number of bioactive compounds with pharmacological activities which may be beneficial in asthma have been isolated from various plants, and they include flavonoids (Harborne & Baxter, 1993; Hazekamp *et al.*, 2001), terpenes (Tsukawaki *et al.*, 1987) and coumarins (Zhao *et al.*, 1997) amongst others. Naturally occurring progenitors of standard drugs used in the management of asthma include khellin, vasicine, modification of which yielded cromones and bromhexine respectively. Studies on the structure-activity relationships of these phytoconstituents may yield compounds with higher efficacy and modified characteristics in the treatment of asthma. The isolated phytoconstituents include:

Androsin

Androsin, a phenolic glycoside isolated from *Picrorhiza kurroa,* is a potential anti-asthmatic agent, demonstrated to inhibit PAF, thereby relieving bronchial hyperreactivity and subsequently bronchial obstruction (Dorsch *et al.*, 1991).

Amurensin H

Amurensin H, a resveratrol dimer having a benzofuran moiety, is an isolate of *Vitis amurensis* Rupr. (Vitaceae), which grows in northeast and central parts of China. In a study to explore the anti-inflammatory effects of amurensin H on asthma-like reaction induced by allergen in sensitized mice, BALB/c mice were sensitized by ovalbumin (i.p) on days 0 and 14 and challenged with 1% OVA on days 18 to 22. Mice developed airway eosinophilia, mucus hypersecretion, and elevation in cytokine levels. Mice were daily administered amurensin H (49, 70, or 100 mg/kg) orally from day 15 to the last day, and BALF was collected at 24 h and 48 h after the last OVA challenge. Levels of tumor necrosis factor-α (TNF-α), interleukin 4 (IL-4), interleukin 5 (IL-5), and interleukin 13 (IL-13) in BALF were measured using ELISA method, and differential cell counts of macrophages, lymphocytes, neutrophils and eosinophils were performed. Lung tissue sections of 6-μm thickness were stained with Mayer's hematoxylin and eosin for assessment of cell infiltration, mucus production, and tissue damage. Results showed that oral administration of amurensin H significantly inhibited OVA-induced increases in total cell counts, eosinophil counts, and TNF-α, IL-4, IL-5 and IL-13 levels in BALF. In addition, amuresin H remarkably decreased OVA-induced lung tissue damage and mucus production (Li *et al.*, 2006).

Eucalyptol (1,8-cineol)

As the active agent and main component of eucalyptus oil, 1,8-cineol exhibits remarkable anti-inflammatory activity, including the modulation of cytokine production. The monoterpene 1,8-cineol exhibits a steroid-like suppression of arachidonic acid metabolism, tumor necrosis factor- α (TNF-α), and IL-1β production in human blood monocytes *in vitro* (Juergens *et al.*, 1998a). It also significantly inhibited production of TNF-α, IL-1β, IL-4, IL-5 in human unselected lymphocytes, and TNF-α, IL-1β, IL-6, and IL-8 in lipopolysaccharide-stimulated monocytes (Juergens *et al.*, 2004). Furthermore, a noncontrolled study showed significant inhibition of LTB_4 and IL-1β in stimulated monocytes *ex vivo* after additional therapy with 200 mg 1,8-cineol (*tid*) administered in enteric-coated capsules (Juergens *et al.*, 1998b). Also long-term systemic therapy with 1,8-cineol had a significant steroid-saving effect in steroid-dependent asthma (Juergens *et al.*, 2003).

The anti-inflammatory mechanism of eucalyptol probably involves a concentration-dependent inhibition of early growth response factor-1 (Egr-1) expression in lipopolysaccharide-stimulated human monocyte THP-1 cell line (Zhou *et al.*, 2007).

Forskolin

Forskolin, a diterpene derivative isolated from the roots of *Coleus forskohlii* was shown to relax guinea pig airway smooth muscle *in vitro* and *in vivo* independently of beta-adrenoceptors by raising tissue cyclic AMP levels (Tsukawaki *et al.*, 1987). In addition, forskolin caused dose-dependent relaxation of LTC_4-, LTD4 - and carbachol-induced contraction of isolated guinea pig trachea. Forskolin was shown to reverse the tachyphylaxis to the bronchodilator effects of salbutamol; this suggests a potential use of forskolin probably in combination with β_2 agonists as an alternative therapy to increased agonist dose due to tachyphylaxis. In addition, such a combination may offer the advantage of eliminating the serious side effects resulting from use of high dose β_2 agonists (Yousif & Thulesius, 1999).

The poor water solubility and the low oral activity of forskolin are drawbacks to its clinical usage both as an intravenous and an oral formulation (Bauman *et al.*, 1990). NKH477, 6-(3-dimethylaminopropionyl) forskolin hydrochloride, is a novel and potent water-soluble forskolin derivative (Hosono *et al.*, 1992; Takeuchi *et al.*, 1995; Satake *et al.*, 1998). This agent, like forskolin, enhanced adenyl cyclase activity in cardiovascular tissues, and reduced cardiac β-adrenoceptor density (Takeuchi *et al.*, 1995), attenuated the contraction induced by either high K^+ solution, noradrenaline, prostaglandin or acetylcholine in a concentration-dependent manner with a potency comparable to or greater than that of forskolin (Himeta *et al.*, 1991;

Shafiq *et al.*, 1992; Takeuchi *et al.*, 1995). NKH477 also has a potent bronchorelaxant action, which may result at least in part from activation of large-conductance Ca^{2+}- activated K^+ (BK_{Ca}) channels (Satake *et al.*, 1998), which causes hyperpolarization of smooth muscle cell membranes and a secondary decrease in Ca^{2+} influx through voltage-dependent calcium channels (Small *et al.*, 1993; Kaczorowski & Jones, 1995). Studies using patch clamp techniques have shown that BK_{Ca} channels are distributed abundantly in the surface of airway smooth muscle cells (McCann & Welsh, 1986; Kume *et al.*, 1989), and that these channels are stimulated via cyclic AMP-dependent phosphorylation as well as by a cyclic AMP-independent, membrane-delimited signal transduction process (Kume *et al.*, 1989, 1992, 1994).

Hispidulin

A bioassay-guided fractionation of the ethanol (96%) extract of the aerial parts of *Clerodendrum petasites* led to the isolation and identification of hispidulin as the antispasmolytic and anti-asthmatic principle of the plant. The EC_{50} values of the crude extract, aminophylline and hispidulin were found to be 4.8 mg/mL, 26 µg/mL and 9 µg/mL respectively (Abdalla *et al.*, 1988; Hazekamp *et al.*, 2001).

Hispidulin, first isolated from *Digitalis lanata* L. by Doherty *et al.* (1963) has been described in species of several plant families, *e.g.* Asteraceae, Papilionaceae, Lamiaceae and Verbenaceae (Hazekamp *et al.*, 2001).

Isokaempferide (IKPF; 5,7,4'-trihydroxy-3-methoxyflavone)

Isokaempferide (5,7,4'-Trihydroxy-3-methoxyflavone or IKPF) was isolated from the trunk bark extract of *Amburana cearensis* (Fabaceae) (Canuto &Silveira, 2000; Leal *et al.*, 2006), a Brazilian traditional remedy used in therapy of asthma and other respiratory disorders (Braga, 1976). It has been also isolated from other species such as *Genista ephedroides* (Pistelli *et al.*, 1998), *Combretum quadrangulare* (Banskota *et al.*, 2000a) and *Dracocephalum subcapitatum* (Saeidnia *et al.*, 2005). It has anti-inflammatory and bronchorelaxant effects (Leal *et al.*, 2000, 2003, 2006), antibacterial and antiviral activities (Wang *et al.*, 1989; De Meyer *et al.*, 1991), potently inhibited TNF-α-induced cell death (Banskota *et al.*, 2000b), and also inhibited the growth of tumor cell lines and sea urchin cell develpment (Costa-Lotufo *et al.*, 2003).

Knipholone

Knipholone, a binary compound composed of the anthraquinone chrysophanol and an acetylphloroglucinol, was originally isolated from the roots of *Kniphofia foliosa* Hochst (Asphodelaceae) together with chrysophanol (Dagne

& Steglich, 1984). Knipholone was demonstrated to inhibit leukotriene biosynthesis *in vitro* by inhibition of the 5-lipoxygenase activating protein (FLAP) or as a competitive (non-redox) inhibitor of the enzyme and could be a potential candidate for a new anti-asthma drug (Wube *et al.*, 2006).

Polygodial

Polygodial, the main sesquiterpene isolated from the bark of *D. winteri* is responsible for most, if not all, of the pharmacological action relevant in the therapy of asthma observed in the plant (El Sayah *et al.*, 1997; El Sayah *et al.*, 1998). Thus, polygodial could be of potential value in the development of a new drug for the treatment of asthma (El Sayah *et al.*, 1998).

Scoparone

Scoparone (6,7-dimethoxycoumarin) was isolated and purified from the flowers and buds of *Artemisia scoparia* Waidst. et Kit (Zhao *et al.*, 1997). It significantly relaxed tracheal smooth muscle (Liu *et al.*, 2001), and directly reduced intracellular calcium ion concentrations in isolated guinea-pig tracheal smooth muscle (Liu *et al.*, 2002). The later activity is also underscored by the observations that elevations in intracellular calcium ion concentration is the main reason for contraction of the bronchi and increased sensitivity to bronchi sensitizers (Tanaka *et al.*, 1996; Hall, 2000). In addition, scoparone significantly prolonged asthmogenic latent periods in asthmatic guinea-pigs by spray inhalation (Zhao *et al.*, 2000; Liu *et al.*, 2000).

Pharmacokinetic evaluation of scoparone showed it to exhibit a short elimination half-life (Kano *et al.*, 1994; Fang *et al.*, 2003).

Tetragalloylquinic Acid

Tetragalloylquinic acid which was isolated together with other compounds (gallic acid, methyl gallate, and flavonoid acylglycosides) from the alcohol extract of *G. glauca*, showed the highest activity against bronchial hyperreactivity and allergic reactions (Dorsch *et al.*, 1992; Neszmelyi *et al.*, 1993).

Viteosin-A and Vitexicarpin

Viteosin-A and vitexicarpin, isolated from the n-hexane extract of *Vitex trifolia,* blocked histamine-induced contraction of the guinea pig trachea, however only vitexicarpin blocked the effects of histamine released from mast cells in murine model of ovalbumin-induced asthma (Alam *et al.*, 2002).

Quercetin

The flavonoid, quercetin and its derivatives, is a constituent of various plants used in the management of asthma (Erazo *et al.*, 1997; Ko *et al.*, 2002, 2004c).

Various experimental studies have shown benefits of quercetin in the treatment of asthma. It has been shown to inhibit bronchial obstruction and airway hyperresponsiveness in the guinea pig (Dorsch *et al.*, 1992), to display *in vitro* relaxant effects on guinea pig trachea pre-contracted with histamine, carbachol or KCl (Ko *et al.*, 1999, 2002, 2003), to inhibit the release of histamine *in vitro* from rat peritoneal mast cells (Haggag *et al.*, 2003) and elicit anti-proliferative effects (Kanadaswami *et al.*, 2005; Lambert *et al.*, 2005; Chaabi *et al.*, 2007).

Quercetin derivatives having one or more methyl substitution were shown to be more active than quercetin itself in relaxing the isolated guinea pig trachea contracted with histamine, carbachol or potassium chloride (Ko *et al.*, 1999). These authors reported further that 3-*O*-methylquercetin isolated from *Rhamnus nakaharai* (Hayata) a species used as folk medicine in Taiwan for the treatment of inflammation and asthma, exhibited relaxant activity on the guinea pig trachea *in vitro* by inhibition of phosphodiesterases (Ko *et al.*, 2002). The same group reported that 3-*O*-methylquercetin inhibited inflammation and airway hyper-responsiveness in a murine model of asthma (Ko *et al.*, 2004c), and also inhibited iNOS DNA transcription (Jiang *et al.*, 2006).

CONCLUSIONS

Though a large number of medicinal plants are used in the therapy of asthma, relatively few have been evaluated for pharmacological activity, and even fewer bioactive constituents have been isolated. Of the isolated few, apart from the earlier ones like ephedrine and cromoglycate, none has been approved for therapeutic use as an orthodox anti-asthmatic drug.

Medicinal plants offer a largely untapped source of new leads for the development of new antiasthmathic agents. There is need for intensified research into the pharmacological and therapeutic potentials of these natural products, as quite a number of them have not been scientifically evaluated for activity.

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Hepatoprotective Effects of *Pimpinella anisum* Seed Extract in Rats

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ABSTRACT

We investigated the role of Pimpinella anisum seed extract (PFO) in the prevention of carbon tetrachloride (CCl_4) induced liver injury. Thirty-six Sprague-Dawley rats were allocated into five groups. Animals in group I, II, and III received isotonic saline solution, olive oil and CCl_4, respectively. Silibinin and CCl_4 were administered to the animals in the group IV while group V animals received PFO and CCl_4. The rats were sacrificed at the end of the eighth day and histopathological and biochemical examinations were performed. Body weights of the animals were measured daily. CCl_4 induced acute liver damage as observed by increases in serum ALT and AST levels and histopathological findings. In the PFO group serum AST and ALT levels were lower compared to those in the CCl_4 group, although this was not as low as those in the silibinin group. However there was no difference in the histopathologic findings between the CCl_4 and PFO groups. In conclusion, PFO could show hepatoprotective effects though less effective than silibinin.

Key words : *Pimpinella anisum* L., carbon tetrachloride, hepatoprotective effect, rats

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INTRODUCTION

Pimpinella anisum is a member of the Umbelliferae and is found in the North-Eastern Anatolia (Zeybek, 1960). In Turkish folk medicine, *Pimpinella* species have been used as analgesic, antiinflammatory, appetizing, hypnotic, expectorant, antibacterial and hepatoprotective agents and also to increase milk secretion (Oztürk *et al.*, 1991; Pamuk, 1998; Baytop, 1999). The seeds of *P. anisum* contain 1.5–6% essential oil, 10–20% fixed oil and 18% proteins. The main constituents of the essential oil are 90% anethole, 2–4% gamma-himachalene, <1% p-anisaldehyde, 0.9–1.5% methylchavicol, 3% cis-pseudoisoeugenyl 2-methylbutyrate and 1.3% trans-pseudoisoeugenyl 2-methylbutyrate (Rodrigues *et al.*, 2003). The essential oil of *P. anisum* was not found effective against carbon tetrachloride-induced acute hepatotoxicity in rats (Erdogan *et al.*, 2004). Hypoglycemic effect of the essential oil of *P. anisum* has been reported by Ceylan *et al.* (2005). It was found to have an acaricidal effect against *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* (Lee, 2004). Its anti-diuretic effect was reported by Kreydiyyeh *et al.* (2003). Essential oil of *P. anisum* was found effective against some bacteria (Singh *et al.*, 2002). Sahraei *et al.* (2002) reported that the essential oil of the *P. anisum* may reduce the morphine effects via a GABAergic mechanism (Sahraei *et al.*, 2002). Bronchodilatory effects of the essential oil, aqueous, and ethanol extracts of *P. anisum* through its inhibitory effects on muscarinic receptors have been shown (Boskabady & Ramazani-Assari, 2001). It has been reported that the essential oil of the *P. anisum* suppressed tonic convulsions induced by pentylenetetrazole (PTZ) or maximal electroshock (MES) (Pourgholami *et al.*, 1999) and it has been considered to be an active estrogenic agent (Albert-Puleo, 1980). Özbek *et al.* (2002) reported that median lethal dose of PFO was 3.152 mL/kg in mice.

No reports are available on the evaluation of *P. anisum* fixed oil for its hepatoprotective activity. In this study, the fixed oil of *P. anisum* was investigated for its hepatoprotective effect on carbon tetrachloride-induced liver injury in rats.

MATERIALS AND METHODS

Plant

The seeds of *P. anisum* were purchased from a local herbal store in Van, Turkey. The plant samples were kept at room temperature until grinding process. *P. anisum* was identified by the botanists in the herbarium of Yuzuncu Yil University, Van and the specimen number of the plant is B-16.

Isolation of Tested Material

The seeds of *P. anisum* were grounded in a mixer. Ground plant material was macerated in diethyl ether for 2 h. The solvent was evaporated (Büchi

RE 111 rotavapor and Büchi 461 water bath, Switzerland). The fixed oil content of the seeds was 14%.

Chemicals

Carbon tetrachloride (CCl_4) was obtained from Merck KgaA (64271 Darmstadt, Germany), diethyl ether was obtained from Kimetsan (Ankara-Turkey), silibinin was obtained from Sigma-Aldrich (Steinheim-Germany) and olive oil obtained from Fluka (Steinheim-Germany).

Animals

Sprague-Dawley rats of both sexes (150–200g) were used in this study. The animals were housed in standard cages with food and water *ad libitum*, at room temparature (20 ± 2°C) with 12 h light-dark cycle. The animals were kept under controlled environment following the standard operating procedures of the animal house facility of the Faculty of Medicine (University of Yuzuncu Yil), and provided with pelleted food (Van Animal Feed Factory, Van-TURKEY). The prior approval of Animal Ethics Committee was also obtained.

Carbon Tetrachloride Model for Evaluation of Acute Hepatotoxicity

The CCl_4 model of hepatotoxicity described by Handa, Sharma and Lershin was used for scheduling the dose regimen (Lershin, 1971; Handa & Sharma, 1990). Intraperitoneal (i.p.) injection of carbon tetrachloride (0.8 mL/kg) diluted in olive oil (1:1 dilution) was employed for inducing acute liver toxicity.

Experimental Procedure

Thirty-six Sprague-dawley rats were divided into six groups of six animals each. Animals in group I (ISS group) received i.p. injection of 0.2 mL isotonic saline solution (ISS); Animals in group II and group III received olive oil (0.8 mL/kg, *i.p.*) and CCl_4 (0.8 mL/kg, *i.p.*), respectively. Group IV (Silibinin group) animals were injected with silibinin (50 mg/kg, *i.p.*) and CCl_4 (0.8 mL/kg, *i.p.*) (Horváth *et al.*, 2001), while Group V (PFO group) animals received PFO (0.5 mL/kg, *i.p.*) and CCl_4 (0.8 mL/kg *i.p.*). The first two groups served as control groups. Injections were given once a day for seven days. All animals were observed daily and dead animals were subjected to post-mortem examination to find the cause of death. At the end of the treatment, blood samples were collected by direct cardiac puncture and the serum was used for the assay of marker enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

Body weights of the rats were measured everyday for eight days and daily percentage changes in body weights were recorded.

Assessment of Liver Function

The serum AST and ALT concentrations were determined with a commercial slide (Vitros) by Vitros DT60II Autoanalyzer.

Histopathological Examination of the Liver

The liver of the experimental animals were fixed in 10% neutral buffered-formalin prior to routine processing in paraffin-embedded blocks. Four µm thick sections were cut and stained using Haematoxylin-eosin (HE) stain.

Statistical Analysis

Results of the biochemical analyses were reported as mean ± Standard Error of Mean (SEM). The total variation was analysed by performing one-way analysis of variance (ANOVA). Tukey's Honestly Significant Difference test (Tukey's HSD test) was used for determining significance. Probability levels of less than 0.05 were considered significant.

RESULTS

Effects of PFO on AST and ALT Levels

Results of the biochemical analyses in all groups are presented in Table 1. There were no significant differences in AST and ALT levels between the ISS and olive oil groups. Serum AST and ALT levels were significantly higher in the CCl_4 group compared to the control groups. In the silibinin group serum AST and ALT levels were significantly lower compared to those in the CCl_4 group while serum AST level was higher compared to the control groups. Serum AST and ALT levels in the PFO group were significantly lower as compared to those in the CCl_4 group while these were significantly higher when compared to those in the control groups.

Table 1. The Effects of *P. anisum* extract (PFO) on serum levels of AST and ALT in rats

Treatment	AST	ALT
	Serum (U/L)	Serum (U/L)
ISS*	177.0 ± 015.6	43.5 ± 2.1
Olive oil	127.8 ± 16.9	46.8 ± 3.3
CCl_4	1727.0 ± 225.8^{ab}	969.0 ± 166.4^{ab}
Silibinin	767.8 ± 179.4^{abc}	248.2 ± 93.1^{c}
PFO	1032.8 ± 155.6^{abc}	417.4 ± 69.8^{abc}
F-value	22.246	19.059
p-value	0.000	0.000

*ISS: Isotonic saline solution

The values represent the mean ± S.E.M. (Standard Error of Mean).

Post-hoc Tukey's HSD test: [a]: $p<0.05$ with respect to control (ISS) group; [b]: $p<0.05$ with respect to olive oil group; [c]: $p<0.05$ with respect to CCl4 group

Percentage changes in weight were 8.87% in the ISS group, -1.72% in the olive oil group, -12.40% in the CCl_4 group, -4.60% in the silibinin group and -12.11% in PFO group. The animals in the CCl_4 and PFO groups showed a larger weight loss compared to those in the control group.

Histopathological Changes

There were no pathological changes in the liver of the rats in the ISS and olive oil treatment groups. In the carbon tetrachloride group and the PFO group, drastic alterations were observed in the liver. Histopathological examinations showed diffused and ballooning degeneration (Fig 1). Ballooned hepatocytes were of different sizes and much larger than normal hepatocytes and occasionally appeared as confluent areas. Histopathological changes in the liver of the PFO group were similar to those of the CCl_4 group (Fig 2).

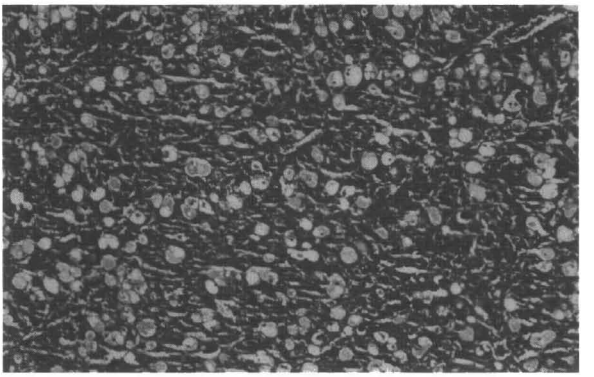

Fig 1. Ballooning degeneration in liver in CCl4 group (HE, X10)

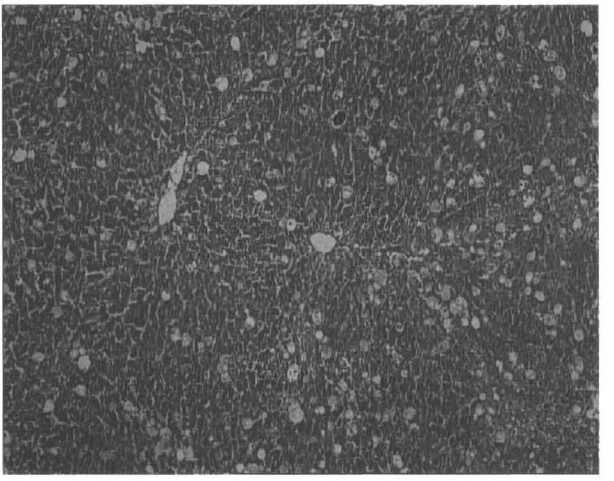

Fig 2. Ballooning degeneration in liver in PFO group (HE, X5)

DISCUSSION

CCl_4 induce centro lobular necrosis in rat liver and it is transformed to tricholoromethyle free radical (CCl_3) by sitocrom-P450. CCl_3 reacts with oxygen to form peroksil radical (CCl_3O_2), which powerfully induce lipid peroxidation. Thus, CCl_4 causes oxidative destruction of liver cell membranes and serious tissue damage in rats (Akkus, 1995).

Biochemical findings showed that serum ALT and AST levels were significantly lower in the PFO group compared to CCl_4 group. Decreased serum AST and ALT levels suggest that PFO could prevent liver cell damage (Goldfrank, 1998).

Silibinin, which is known to have hepatoprotective effects on liver significantly decreased serum AST and ALT levels compared to CCl_4 (Horváth, 2001). Although serum ALT level was lower in the silibinin group than those in the PFO group the difference however, was not statistically significant. Considering the body weight changes and histopathological findings in the PFO group, it is suggested that silibinin has a better hepatoprotective effect than PFO. Histopathological findings were similar in all groups although balloon degeneration in the groups other than the CCl_4 group was less widespread.

In the light of these findings it is concluded that PFO can have hepatoprotective effect in CCl_4 induced hepatotoxicity and it may be less effective than silibinin. To find out which chemical in the PFO is responsible for its effects a chromatographic analysis of PFO needs to be performed and it ingredients should be evaluated in the CCl_4 induced hepatotoxicity model.

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Safety and Efficacy Study of Polyherbal Formulations "Mersina", "Limit" and "Eumil" in Experimental Animals

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ABSTRACT

Study was undertaken to investigate the safety and efficacy of antidiabetic polyherbal formulations Mersina, Limit and Emuil in normal and diabetic experimental animals. The formulations were evaluated for acute and subacute toxicity (OECD, 2001) study. The poly herbal formulations were administered orally in different groups of normal and diabetic rats. Modulation of β-cell destruction action was studied by pretreatment of formulations to animals followed by alloxan treatment. Haematological, hepatic, metabolic, kidney function tests, urinalysis, gross pathological and histopathological tests were performed in test and control group of animals. Glibenclamide is used as a reference standard. In acute and subacute toxicity studies, no mortality was observed. There were no significant changes occurred in any of the tests performed. Treatment with Mersina, Limit and glibenclamide in diabetic animals resulted in a significant reduction of blood glucose and improvement in haematological, liver and kidney function parameters compared with control animals. Eumil has shown slight antihyperglycemic activity. Pretreatment with formulations (Mersina and Limit) produced protective effect against alloxan action. The effects were comparable with that of glibenclamide. The formulations shown antioxidant activity, were found to be safe in acute and subacute toxicity study and thus beneficial in improving the conditions of diabetes and its complications.

Key words : Antidiabetic, Eumil, hypoglycemia, Limit, Mersina, polyherbal

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INTRODUCTION

Herbal medicines are popular remedies for diseases used by a vast majority of the World's population. In the traditional system of Indian medicine, plant formulation and combined extracts of plants are used as drug of choice rather than individual. Presently there is growing interest in antidiabetic, nootropic, hepatoprotective and lipid lowering agents. Plant remedies have been and are being used by diabetic patient's throught the world. Studies suggest that using an antidiabetic plant in whole form or as complex extracts may offer many benefits due to the presence of multiple active components (Kuruvilla, 2002). Various herbal formulations such as Coagent db, Hyponidd and Diasulin (Pari, 2002; Poongothai, 2002; Saravanan, 2005) are well known for their antidiabetic effects. Diabetes mellitus is a disease that results in a reduction of endogenous antioxidants and an increase in oxidative stress in the human body. Antioxidants have been shown to reduce the risk of diabetes onset, improve glucose disposal and improve some of the associated complications (Laight, 2000).

The pharmacological effects of many plants have been studied in various laboratories, where as there are many limitations regarding the safety and efficacy of these formulations (Guigliano, 1997). Mersina, Limit and Eumil are polyherbal antidiabetic formulations used in traditional medicine to treat type II diabetes, contains both antidiabetic and antioxidant principles.

In the present study, the safety and efficacy of polyherbal formulations Mersina, Limit and Eumil were investigated in experimental animals.

MATERIALS AND METHODS

Animals

Wistar rats (180–210 g) and mice (25–35 g) of either sex were used in the study. They were maintained at a room temperature of 23 ± 2°C with 12 h light/dark cycle and 45–55% relative humidity. The animals had free access to Amruth brand standard pellet diet (Nav Maharashtra Chakan Oil Mills Ltd. India) and water *ad libitum*. The animals were fasted overnight but were allowed free access to water. The experimental protocols were approved by Institutional Animal Ethics Committee (CPCSEA/SPTM/P-18/2008 and CPCSEA/SPTM/P-19/2008).

Chemicals and Drugs

Antidiabetic Ayurvedic poly herbal formulations: Mersina, Limit and Eumil (Table 1).

Table 1.

Name of the formulation	Ingredients	Manufacturer
Mersina	*Gymnema sylvestre, Momordica charantia, Cassia auriculata, Syzigium cumini, Phyllanthus emblica, Trigonella foenum graceum, Coccinia indica, Tinospora cordifolia, Melia azadarichta.*	J & J Dechane Laboratories Pvt Ltd., Hyderabad.
Limit	*Momordica charantia, Enicostemma littorale, Gymnema sylvestre, Eugenia jambolana, Tribang Bhasma, Myristica fragrans, Trikatu, Withania somnifera, Trigonella foenum graceum, Suddha Shilajit.*	Ayulabs Pvt. Ltd. Rajkot.
Eumil	*Ocimum sanctum, Withania somnifera, Emblica officinalis, Asparagus racemosus.*	Envin Bioceuticals Pvt. Ltd., Shaharanpur.

Glibenclamide, DPPH (1, 1'– diphenyl–2– picrylhydrazyl), Ascorbic acid, Methanol

Standardisation of the Formulations

The ethanolic extracts obtained from the authenticated specimens were mixed in the right proportion and maintained as reference standard. The batches were compared with the reference standard by TLC analysis. One gram of the plant material was refluxed with 20 mL of alcohol in a water bath for 1 h. It was then filtered and the filtrate was concentrated to 5 mL. The concentrate was spotted and developed in a mobile phase consisting of toluene, ethyl acetate and glacial acetic acid (6:4:0.4). The dried plate was then scanned at 254 nm. The R_f value of the batches was compared with the reference standard.

Drug Administration

Formulations were prepared as a suspension in 1% (w/v) carboxyl methyl cellulose (CMC) using mortar and pestle, administered orally through feeding needle.

Experimental Induction of Diabetes in Rats

The rats were injected intraperitoneally with alloxan monohydrate dissolved in ice cold normal saline at a dose of 150 mg/kg body weight. Animals with blood glucose levels in the range of 150–250 mg/dl and above 350 mg/dl were used for the experiment.

Experimental Design

Acute Toxicity

The acute toxicity of the formulation was evaluated in mice using OECD procedure (OECD, 2001). Mice of either sex (three females and three males, weight: 25–35 g, age: 6–8 weeks) received formulation starting at 2 g/kg orally by gavage. The animals were observed for toxic symptoms continuously for the first 4 h after dosing. Finally, the number of survivors was noted after 24 h and these animals were then maintained for further 13 days with observations made daily.

Repeated Dose Toxicity

Experimental Animals

The repeated dose toxicity of the formulation was evaluated in rat using OECD procedure (OECD, 2001). Thirty rats (weight: 150–250 g; age: 6–8 weeks old) were randomly assigned into three groups (n=10), five females and five males were housed in each group. Treatments were administered orally by oral gavage once a day for 4 weeks. The first group of rats, serving as control, received 1% CMC (5 mL/kg); the second and third group received the formulation at doses of 1 and 2 g/kg, respectively. All rats were observed daily for physiological and behavioral changes. Any rat that died during the test period was tested pathologically, and all animals were examined at the end of the test period.

Dose Determination Study

Albino rats were used in the experiment. The rats were divided into control and treatment groups and given suspension of the formulations (50–650 mg/kg, Mersina and Limit; 50–800 mg/kg, Emuil).

Determination of Antihyperglycemic Activity and Modulation of β-Cell Destruction Action

The rats were divided into different groups of six rats each. Group 1: Normal treated rats. Group 2 and 3: Diabetic rats (blood glucose 150–250 mg/dl) given suspension of the formulation Mersina (300 and 600 mg/dl). Group 3 and 4: diabetic rats (blood glucose above 300 mg/dl). Group 5 and 6: normal rats pretreated with the formulation mersina (300 and 600 mg/kg) followed by alloxanisation. Similar method is employed for the formulation Limit.

Determination of OGTT in Normal and Diabetic Animals

Different groups of rats, six in each were used in the experiment. Group 1: normal control Group 2: diabetic control Group 3 and 4: diabetic rats (blood

glucose 150–250 mg/dl) given suspension of the formulation Mersina (300 and 600 mg/dl). Group 5 and 6: diabetic rats (blood glucose above 300 mg/dl) given suspension of the formulation Mersina (300 and 600 mg/dl). Similarly Limit (300 and 600 mg/kg) and Eumil (400 and 750 mg/kg).

Observation and Examination Methods

Body weight and food intake were recorded weekly. Biochemical estimations prothrombin time, red blood cell count, hemoglobin concentration (Sahli's method), white blood cell count and white blood cell differential count, glucose (GOD POD method), creatinine (Jaffe's method), blood urea nitrogen (GLDH urease method), Serum glutamate pyruvate transaminase (IFCC method), Serum glutamate oxaloacetate transaminase (IFCC method), Alkaline phosphatase (Tris carbonate buffer method), bilirubin (Diazo method), triglyceride (GPO Trinder method) and total protein (Biuret method) were estimated by using Autoanalyser. After completion of the experiment all the animals were necropsied and subjected for the following analysis.

Urine Samples

On 27th day, 3–5 mL of urine was collected for a period of 4–6 h for immediate urinalysis.

Blood Analysis

On day 29th all surviving animals were fasted overnight, and anesthetized afterwards for blood collection from the right ventricle. Blood samples were collected into three tubes: (1) 3.2% buffered sodium citrate tubes; (2) heparinized centrifuge tubes; (3) dry non-heparinized centrifuge tubes. A blood analysis (hematology, coagulation and chemistry) was carried out. The blood in the sodium citrate tubes was used for prothrombin time (PT) estimation. The heparinized blood was used for a hematological study which included red blood cell count (RBC), hemoglobin concentration (Hb), white blood cell count (WBC) and white blood cell differential count. The nonheparinized blood was allowed to coagulate before being centrifuged and the serum separated. The serum was assayed for glucose, creatinine, blood urea nitrogen (BUN), serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), bilirubin, triglyceride and total protein.

Tissue Analysis

Vascular perfusion was performed for tissue fixation using isotonic saline (250 mL) followed by 10% buffered formalin solution (250 mL) without delay after collecting the blood samples. The organs of the body such as liver, brain, heart,

spleen, pancreas and kidneys, were detached and weighted immediately on an electronic balance for subsequent analysis. Tissues from the control group and the group treated with the high dose of Mersina (2 g/kg) were embedded in paraffin and subjected to hematoxillin-eosin staining. The pathological observations of all tissues were performed on gross and microscopic bases. When lesions were observed in the high dose group, the affected organs were also examined in the low dose group of Mersina (1 g/kg).

Determination of Free Radical Scavenging Activity

The free radical scavenging activity of the formulation was measured by DPPH assay.

$$\% \text{ Free radical scavenging (Inhibition)} = \frac{(\text{Absorbance of DPPH} - \text{Absorbance of Test}}{\text{Absorbance of DPPH}} \times 100$$

The concentration of formulation at which it shows 50% inhibition of free radicals is known as IC_{50} value of that formulation.

Statistical Methods

All the data were presented as mean ± S.E.M. and analysed using one way analysis of varience (ANOVA) and compared using Dunnett. A value of $p<0.05$, $p<0.001$ was considered statistically significant.

RESULTS

Acute Toxicity

Over the study duration of 14 days, there were no deaths recorded in the male and female animals given 2 g/kg of the formulation orally. During the observation period animals did not produce any variations in the general appearance.

Subacute Toxicity

During this study, no deaths were observed; no significant clinically relevant changes were observed in general behavior, gross necroscopy and histopathological studies.

Dose Determination Study

The formulations Mersina and Limit at 150 and 300 mg/kg showed a slight significant effect in lowering blood glucose levels. A dose of 600 mg/kg shown significant effect in lowering blood glucose compared to control animals. Even a dose of 650 mg/kg bw had shown more or less the same effect as that with 600

mg/kg bw. Thus 600 mg/kg is the effective dose of the formulations Mersina and Limit on FBG of normal rats. Eumil showed slight glucose lowering effect at 300 and 600 mg/kg. Further increase in dose showed similar effect.

Effect of Mersina and Limit on OGTT in Normal and Diabetic Rats

The blood glucose levels of the normal rats reached a peak at 30 min after the oral administration of glucose (1 g/kg) and gradually decreased to the pre-glucose load level. The pretreatment with formulations Mersina and Limit (300 and 600 mg/kg), Eumil (400 and 750 mg/kg and glibenclamide (4 mg/kg) produced blood glucose levels significantly lower than that of the normal control group (Figs 1, 2).

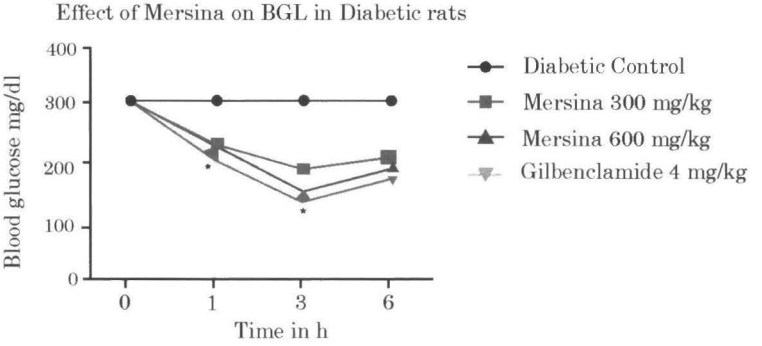

Fig 1. Effect of Mersina on BGL in diabetic rats N = 6 in each group, values are mean ± S.E.M, $p<0.05$ compared to normal control group

In diabetic animals the formulation has shown significant increase in glucose tolerance. The formulation (300 and 600 mg/kg) and glibenclamide (4 mg/kg) significantly depressed the peak of blood glucose level at 30 min after glucose loading (1 g/kg). The pattern of glucose tolerance curve was altered by herbal formulations and glibenclamide.

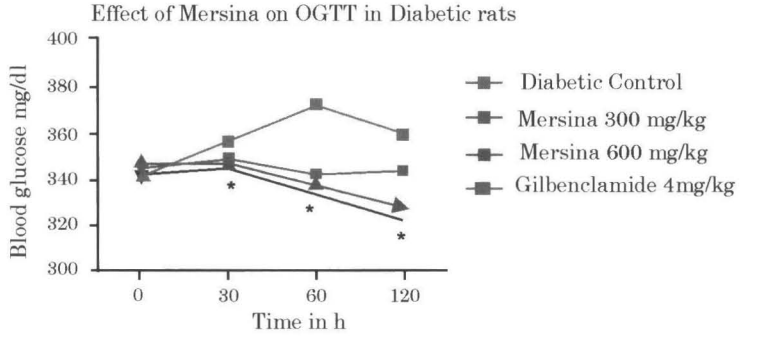

Fig 2. Effect of Mersina on BGL in diabetic rats N = 6 in each group, values are mean ± S.E.M, $p<0.05$ compared to normal control group

Antihyperglycemic Activity in Diabetic Animals

Single Dose Short Term Study

A single dose administration of the formulations Mersina and Limit (300 and 600 mg/kg) in diabetic (blood glucose level 150–250 mg/dl) animals, showed significant reduction in blood glucose level after 1 and 3 h interval. At 6 h the blood glucose level slightly increased as compared to 3 h blood glucose levels. The formulation Eumil has not shown significant reduction in blood glucose levels at 400 and 750 mg/kg in diabetic animals compared to control animals. (Figs 3, 4).

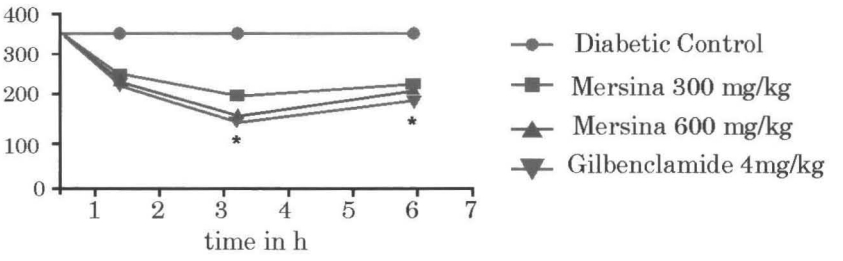

Fig 3. Effect of Limit on BGL in diabetic rats

N = 6 in each group, values are mean ± S.E.M, $p<0.05$ compared to normal control group

Effect of Limit on OGTT in normal Albino rats

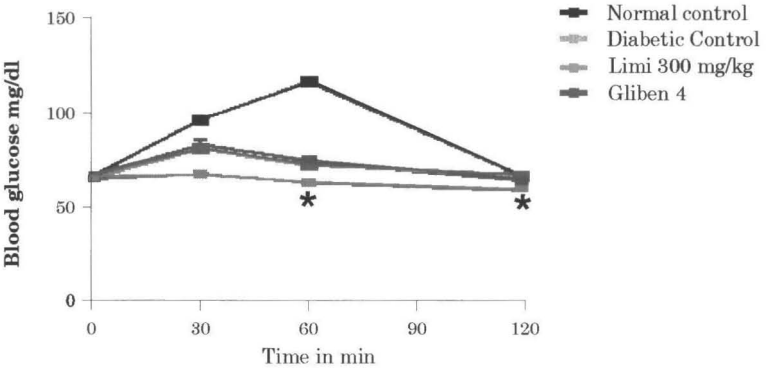

Fig 4. Effect of Mersina on BGL in diabetic rats

N = 6 in each group, values are mean ± S.E.M, $p<0.05$ compared to normal control group

Multi-Dose Long Term Study

The herbal formulation/glibenclamide administered orally for 30 days in alloxan treated rats. There was significant elevation in blood glucose level

with significant improvement in haematological, liver and kidney function parameters in alloxan diabetic rats compared with control rats. Administration of Mersina and Limit and glibenclamide tended to bring blood glucose and other parameters towards near normal levels. The effect of Mersina and Limit at 600 mg/kg were significantly better than 300 mg/kg; therefore the higher dose was used for further biochemical studies.

Determination of Free Radical Scavenging Activity

In the present investigation, the formulation Limit showed highest activity with the lowest IC_{50} value (217.35) followed by Mersina (318.51) and Eumil (437.06). Results shown the role of phytoconstituents- as potent antioxidants (polyphenolic flavonoids, xanthones, tannins and organic acids). Our investigations have revealed a strong correlation between the contents of formulations (*Limit, Mersina and Eumil)* their respective antioxidant activities which may have a protective role in diabetic complications (Figs 7, 8 and 9)

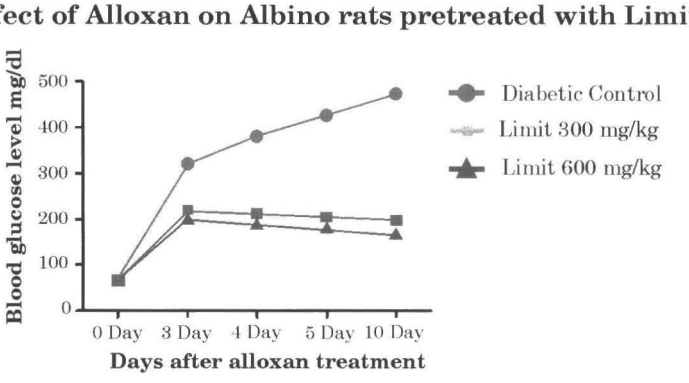

Fig 5. Effect of alloxan on Albino rats pretreated with Mersina
N = 6 in each group, values are mean ± S.E.M, $p < 0.05$ compared to normal control group

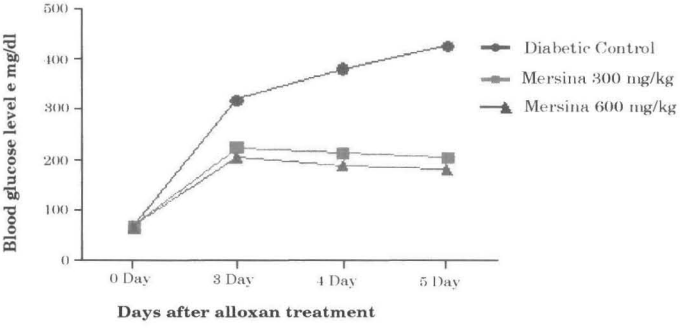

Fig 6. Effect of alloxan on Albino rats pretreated with Mersina
N = 6 in each group, values are mean ± S.E.M, $p<0.05$ compared to normal control group

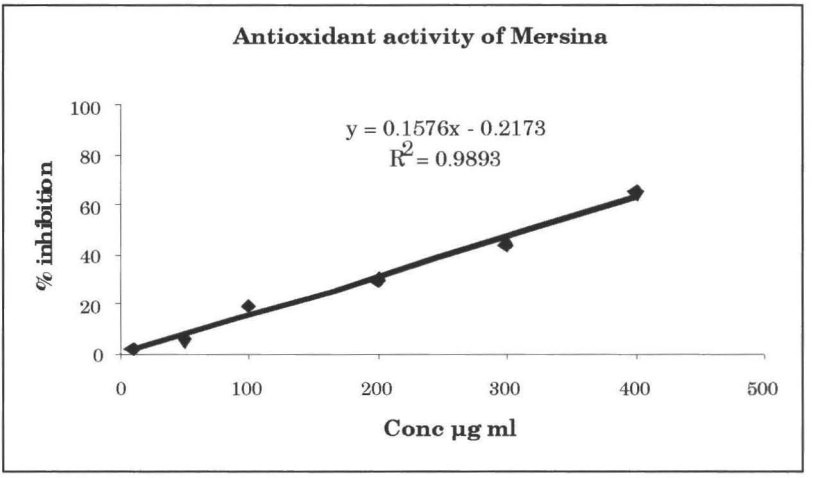

Fig 7. Antioxidant activity of Mersina

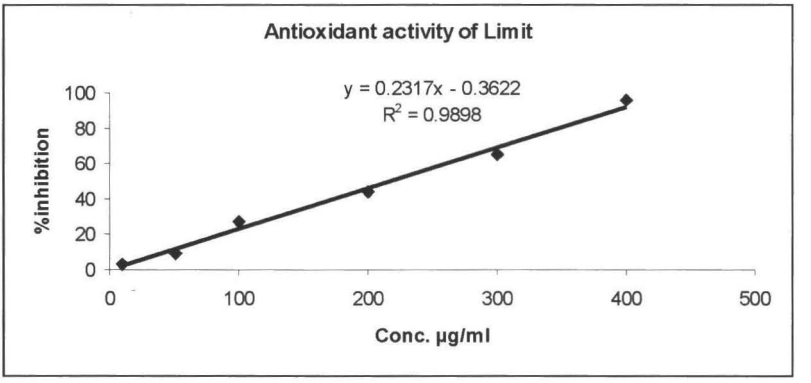

Fig 8. Antioxidant activity of Limit

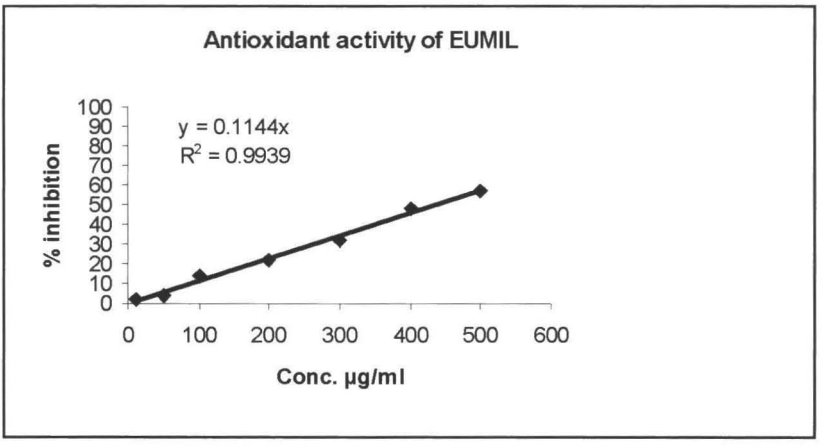

Fig 9. Antioxidant activit of Eumil

DISCUSSION

Diabetes mellitus is a long-term disorder associated with a number of clinical problems causing ill health and death. Disease affecting the small blood vessels in the retina, kidney and peripheral nerves appears to be most directly related to the duration and severity of the raised blood glucose. Large blood vessels of the heart, brain and peripheral circulation are also affected. Thus leading to blindness, chronic renal failure, foot deformity (microvascular complication) and coronary heart disease, stroke, peripheral vascular disease (macrovascular complication). Alloxan, a beta cytotoxin, induces "chemical diabetes" in a wide variety of animal species by damaging the insulin secreting pancreatic β-cell, resulting in a decrease in endogenous insulin release, which paves the way for the decreased utilization of glucose by the tissues.

In our study, we have observed that Mersina and Limit significantly reduced blood glucose levels, improvement in haematological, biochemical and metabolic actions in alloxan induced diabetic rats. Further pretreatment of the formulations shown protective action. The possible mechanism of action could be correlated with the reminiscent effect of the hypoglycemic suphonylureas that promote insulin secretion by closure of K+- ATP channels, membrane depolarization and stimulation of Ca^{2+} influx, an initial key step in insulin secretion. In this context, number of other plants has also been reported to have antihyperglycemic and insulin stimulatory effects. Glibenclamide also produced significant reduction in blood glucose levels of alloxan diabetic rats. Since alloxan is known to destroy pancreatic β-cells, the present findings appear to be in consonance with the earlier suggestion that sulphonylureas have extra-pancreatic antihyperglycemic mechanism of action secondary to their insulin secreting effect and the attendant glucose uptake into, and utilization by the tissues.

Apart from the regulation of carbohydrate metabolism, insulin also plays an important role in the metabolism of lipids. Insulin is potent inhibitor of lipolysis. Since it inhibits the activity of hormone sensitive lipases in adipose tissues and suppresses the release of free fatty acids. During diabetes, enhanced activity of this enzyme increases lipolysis and increases more free fatty acids into the circulation. Increased fatty acids concentration also increases the β-oxidation of fatty acids, producing more acetyl CoA and cholesterol during diabetes.

The increase in oxygen free radicals in diabetes could be primarily due to increase in blood glucose levels, which upon autoxidation generate free radicals and secondarily due to the effects of diabetogenic agent alloxan. In diabetes, hypoinsulinaemia increases the activity of the enzyme, fatty acyl coenzyme, coenzyme A oxidase, which initiates β-oxidation of fatty acids resulting in lipid peroxidation. Increased lipid peroxidation impairs membrane functions by decreasing membrane fluidity and changing the activity of

membrane-bound enzymes. Its products *viz.*, lipid radicals and lipid peroxide, are harmful to the cells in the body. Administration of Mersina, Limit and glibenclamide reduced the lipid peroxidative markers in liver and kidney tissues of diabetic rats. This indicates that formulations have inhibited oxidative damage due to the presence of antiperoxidative effect of ingredients. This could be correlated with previous study that reported that *Phyllanthus emblica*, *Trigonella foenum graceum*, *Coccinia indica*, *Tinospora cordifolia*, *Melia azadarichta* and Javakhar (ingredients of formulations) have antiperoxidative and antihyperlipidemic effect on diabetic animals.

Antidiabeic and antihyperlipidemic effect of formulations may be due to the effect of active constituents of different plants *viz.*, alkaloid and pectins from *Coccinia indica*, alkaloids from *Tinospora cordifolia*, emlicanin A and B from *Phyllanthus emblica*, trigonelline and scopoltin from *Trigonella foenum graceum*, *Melia azadarichta* and Javakhar which may be responsible for scavenging free radicals liberated by alloxan in diabetic rats (Platel, 1997; Subash, 2004; Prince, 1998; Grover, 2002).

Antioxidants in medicinal plants may help control diabetic complications arising in prediabetics and possibly reduce the onset of diabetes. This along with a tolerable safety profile makes herbals promising candidates for further studies.

In summary the polyherbal formulations, Mersina and Limit exert a significant antidiabetic effects. The formulations Mersina, Limit and Eumil have shown antioxidative effect. This could be due to different types of active principles, each with a single or a diverse range of biological activities, which serves as a good adjuvant in the present armamentarium of antidiabetic drugs. The formulation Eumil has not shown significant antihyperglycemic effects at the dose tested.

ACKNOWLEDGEMENTS

Thanks to Dr. Meena C, Assoc Prof., School of Pharmacy & Technology Management, NMIMS University, for her untiring help and co-operation during the course of the study and Prof. R.S. Gaud, Dean, School of Pharmacy & Technology Management, NMIMS University, Mumbai, for providing necessary facilities. J & J Dechane Laboratories Pvt Ltd., Hyderabad, Ayulabs Pvt Ltd., Rajkot and Envin Bioceuticals Pvt Ltd., Shaharanpur for providing the herbal samples.

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A Review of Biological Effects of Berberine and Croatian Barberry (*Berberis croatica* Horvat) as a New Source of Berberine

IVAN KOSALEC*, MARIJANA ZOVKO[1], DARIO KREMER[1], NADA ORŠOLIC[2] AND KSENIJA KARLOVIC[3]

ABSTRACT

Berberine is an alkaloid from the isoquinoline group of alkaloids, and it can be found in various Berberis spp., Coptis spp. and Hydrastis canadensis. As such, it is an important ingredient of many formulations of traditional Chinese medicine, and other traditional medicines. This review will focus on plant source of berberine, and on documented biological effects of berberine. Mechanism of bactericidal activity of berberine and inhibition of multiple-resistance pumps in bacteria in barberry species will be explained. In addition to antimicrobial activity, the review will concentrate on other biological activities of berberine such as hepatoprotective, glucose and cholesterol lowering, antiplatelet, antioxidant, anti-inflammatory, aetylcholinesterase inhibitory as well as cytostatic / cytotoxic and pro-apoptotic activity. Possible mechanisms for some of the above mentioned activities will be discussed. Furthermore, in search for phytochemical profile of Croatian barberry (Berberis croatica Horvat), which is an endemic illyric-balcanic species; berberine was found as bioactive alkaloid in roots, twigs and leaves of wild-collected specimens. This is the first report of Croatian barberry as a new and a rich source of biologically active alkaloid berberine.

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Key words : Berberine, *Berberis* spp., *B. croatica*, bioactivity, alkaloid, isoquinoline

INTRODUCTION

Barberry species have been used in ethnopharmacology for more than 3.000 years. Barberry species and other plants which contain alkaloid berberine are used in Ayurvedic and Traditional Chinese Medicine (Birdsall, 1997). Nowadays, some barberry species such as common or European barberry (*Berberis vulgaris* L.) are used for ornamental purposes. However, ethnopharmacological data collected from the phytomedical use of barberry species showed that these species are popular folk remedy for various conditions including diarrhoea, urinary tract diseases, ulcers and heartburn, liver dysfunction, gallbladder problems and as medicine against many microbial diseases. Extract from barberry was used as effective fever-reducing natural remedy (Saeed Arayne *et al.*, 2007). The main biological active alkaloid isolated from barberry species is berberine. Berberine is a yellow crystalline bitter alkaloid, and it is also found in goldenthread (*Coptis* sp.), and goldenseal (*Hydrastis canadensis).* The stem, root bark, and fruit of barberry species contain isoquinoline alkaloids (*e.g.* berberine), which are the main active ingredients of barberry (Gorval & Grishkovets, 1999). However, with the exception of berberine, various compounds have been isolated from *Berberis* species, such as alkaloids (aromoline, berbamine, berbamunine, berlambine, bervuleine, columbamine, hydroxycanthine, isocorydine, jatrorrhzine, lambertine, magniflorine, oxyberberine, oxycanthine, palmatine), phenolic compounds (chlorogenic acid, quercetine, rutin), etc. (Saeed Arayne *et al.*, 2007).

In this review, some interesting biological effects of barberry and berberine will be discussed. Our recent research found Croatian barberry (*Berberis croatica* Horvat) as a new source of berberine. New localities of this endemic barberry will be displayed.

BOTANIC SOURCE OF BERBERINE: A GENUS *BERBERIS*

Genus barberry (*Berberis* L.) encloses about 450 species distributed in Europe, North Africa, America, East and Central Asia (Erhardt *et al.*, 2002; Wielgorskaya, 1995). Two of them are present in Croatia: common barberry (*B. vulgaris* L.) and Croatian barberry (*B. croatica* Horvat). Common barberry is widely distributed in Croatia, such as in the most continental part of Europe, but rare in the Mediterranean region (Akeroyd & Webb, 1993). Croatian barberry is an endemic illyric-balcanic species, which grows on rocky slopes, at the upper limit of the wooded zone in Croatia, Bosnia and Herzegovina, Montenegro, and Macedonia (Kušan, 1969; Trinajstić, 1973; Šilić, 2005). In literature, Croatian barberry is also known under the following names: *B. aetnensis* var. *brachyacantha* (Borbás, 1886), *B. vulgaris emarginata*

(Anić, 1946), *B. vulgaris* var. *aetnensis* (Kušan, 1955), *B. vulgaris* var. *alpestris* (Trinajstić, 1973), *B. aetnensis* (Domac, 1984), *B. illyrica* (Borzan *et al.*, 1992). According to Martinis (1994) it is distributed on Dinaric mountains (from Mt. Učka and Gorski kotar, across Mt. Velebit, Lička Plješivica Mt., Dinara Mt., Troglav and Mt. Kamešnica to Biokovo). Present distribution of Croatian barberry according to Kremer *et al.* (2008) is presented on Fig 1.

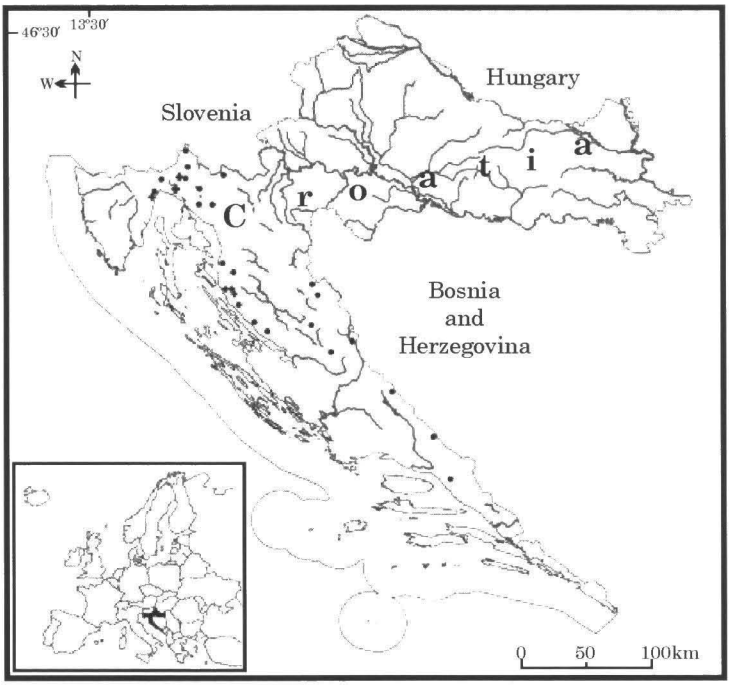

Fig 1. Distribution of *Berberis croatica* Horvat in Croatia: points –Martinis (1994); crosslets – new finding localities (Kremer *et al.*, 2008)

Croatian barberry is a deciduous, up to 60 cm high, spiny shrub with short shoots. Spines are 3–5-fid. Leaves are oblong-obovate or oblong-elliptic shaped, 1–3 cm long, subacute or blunt at apex, with spinulose-serrate margins, and short petiole. Yellow flowers are up to 1 cm in diameter, with perianth composed of 6 sepals and 6 petals; 3 inner petals have 2 nectaries beside the base. Flowers are grouped (5 to 12 flowers, rarely more) in erect or suberect racemes. Fruit is an oblong berry, red in color, up to 0.6 cm long and with 1–2, obovate seeds (Trinajstić, 1973).

ANTIMICROBIAL ACTIVITY

Berberine has a wide spectrum of antibacterial and antifungal activities (Amin *et al.*, 1969; Hahn & Ciak, 1976; Lesnau *et al.*, 1990; Subbaiah *et al.*, 1967). The ethnopharmacological use of berberine-rich plants against bacterial species which cause diarrhoea has been confirmed with several studies.

Several mechanisms of bactericidal action of berberine were involved in decreasing of symptoms of watery diarrhoea. The mechanisms of microbicidal activity of berberine are summarised in Table 1.

Table 1. Possible mechanisms of bactericidal activity of berberine

Mechanism of action	Reference(s)
Metabolic inhibition of microbes	Birdsall *et al.*, 1997
Inhibition of bacterial endotoxin formation (on *Vibrio cholerae* and *Escherichia coli*, both enterotoxin-producing strains)	Bradley & Froehlich, 1982.
Inhibition of adhesion of bacteria	Sun *et al.*, 1988
DNA-inhibitory activity	Yi *et al.*, 2007
Inhibition of arylamine N-acetytransferase activity/gene expression (on *Salmonella* Typhi)	Wu *et al.*, 2005

According to Yi *et al.* (2007), berberine acts as DNA-damaging alkaloid, and mechanism was detected using principal component analysis by means of LS/ESI-MS and comparison with synthetic antibiotics. In the last few years, lot of research has been focused on synergistica action of berberine and other compounds in barberry extracts, or on detection of inhibitory effects of low-molecular-weight metabolites in barberry against multi-drug pump (MDR) inhibitor in bacterial cell walls.

Berberine-rich endemic species *Berberis aethnensis* C. Presl. showed antibacterial activity against *Staphylococcus aureus*, but lower MIC values (indicating more potent antibacterial activity) was found in fractions rich with 5'-methoxyhydnocarpin-D (a flavolignan). It is interesting that these substances do not express bactericidal activity. But in the mixture with berberine, the antibacterial activity increased, and even potentiated bactericidal activity of ciprofloxacin (Musumeci *et al.*, 2003). In *S. aureus* strains with NorA MDR pump, when berberine was incubated with 5'-methoxyhydnocarpin-D, strong inhibition of MDR pumps were recorded. Consequently the level of accumulation of berberine increased strongly, which support hypothesis that 5'-methoxyhydnocarpin-D act as MDR-pump inhibitor (Stermitz *et al.*, 2001). With the exception of flavolignan 5'-methoxyhydnocarpin-D which are found in *B. aquifolium, B. fremontii, B. trifoliolata* and *B. repens* (Stermitz *et al.*, 2001), the porphyrin pheophorbide *a*, also MDR-pump inhibitor was isolated from *B. trifoliolata* (Stermitz *et al.*, 2001). Some other MDR-pump inhibitory substances from natural origin were also isolated (Stavri *et al.*, 2007).

Medical plants containing berberine were traditionally used as remedy against food-borne pathogens. When the influence of berberine was compared among patients with clinically manifested cholera (watery diarrhoea), analysis by factorial design equations, showed a reduction in diarrhoeal

stools by one litre and a reduction in cyclic adenosine monophosphate concentrations in stools by 77% in the groups given berberine (Khin-Maung *et al.,* 1985).

Berberine showed also inhibitory activity against uro-genital falagellate *Trichomonas vaginalis* which could be compared with activity of metronidazole (Soffar *et al.,* 2001). Berberine acts also antiparasitic against *Giardia lamblia, Entamoeba hystolitica, Leishmania donovani* (Kaneda *et al.,* 1990; Kaneda *et al.,* 1991). As very potent amoebicidal compound, berberine causes encystation, degeneration and lyses of amoebae (Subbaiah & Amin, 1967).

ANTIDIABETIC ACTIVITY

Antidiabetic activity of berberine was investigated in many studies. For example, the *in vitro* glucose-lowering action of berberine was investigated on HepG2 cell line (Yin *et al.,* 2002). Berberine significantly increased glucose consumption. The glucose-lowering effect of berberine decreased as the glucose concentration increased. That effect was not dependent on insulin concentration. Another study demonstrated that administration of berberine to normal and experimental diabetic rats for 12 days caused reduction in fasting blood glucose levels. Berberine caused significant increase of glycolytic enzymes in treated diabetic rats while a significant decrease was observed in the levels of the gluconeogenic enzymes. Oral administration of berberine in streptozotocin-nicotinamide induced type 2 diabetic rats caused a significant increase in both enzymatic and nonenzymatic antioxidants (Punitha *et al.,* 2006). Berberine also reduced body weight and caused a significant improvement in glucose tolerance without altering food intake in db/db mice. It seems that glucose lowering effects of berberine are achieved at least in part via stimulation of AMP-activated protein kinase activity as berberine treatment resulted in its increased activity in 3T3-L1 adipocytes (Lee *et al.,* 2006; Zhou *et al.*, 2007).

Antidiabetic efficacy of berberine was also clinically confirmed (Yin *et al.,* 2008). In a 3-month trial with 36 adults with newly diagnosed type 2 diabetes mellitus, berberine showed hypoglycemic effect similar to that of antidiabetic metformin. Significant decreases in hemoglobin A1c, fasting blood glucose and postprandial blood glucose were observed in the berberine group. In an additional 3 month study on 48 adults with poorly controlled type 2 diabetes mellitus berberine also acted by lowering fasting blood glucose and postprandial blood glucose. Effects started week after the beginning and remained to the end of the trial. Hemoglobin A1c as well as fasting plasma insulin and homeostasis model assessment of insulin resistance index were reduced. Functional liver or kidney damages were not observed although one third of patients experienced transient gastrointestinal adverse effects during the trial.

Mechanism of glucose lowering effects of berberine is not clearly elucidated. Although there are some findings that berberine promotes insulin secretion through increasing expressions of insulin receptor (InsR) and insulin-like growth factor-1 receptor (IGF-1R) mRNA in islet β cells of transgenic mice (Yu *et al.,* 2007), most of the studies agree that antidiabetic activity of berberine is insulin independent (Yin *et al.,* 2002; Punitha *et al.,* 2006). However, it seems that berberine may improve insulin action in high-fat-fed Wistar rats (Lee *et al.,* 2006). It also seems that insulin resistance induced by free fatty acids can be improved by berberine through targeting IkB kinase beta as demonstrated in a study on 3T3-L1 adipocytes (Yi *et al.,* 2008).

HEPATOPROTECTIVE ACTIVITY

Berberine has a long history as a tonic remedy for liver. Experiments on enzymatically isolated rat hepatocytes demonstrated that berberine reduced delayed outward potassium currents in a concentration-dependent manner. Furthermore, berberine exhibited mild inhibitory effects on inward rectifier potassium currents in rat hepatocytes and inhibited Ca^{2+} release-activated Ca^{2+} currents in a concentration-dependent fashion. Those effects may be involved in hepatoprotective effect of berberine (Wang *et al.,* 2004). Another possible mechanism of hepatoprotection involves antioxidant activity (Zhang *et al.,* 2008). In Wistar rats the fibrotic models were established by introduction of multiple hepatotoxic factors (CCl_4, ethanol, high cholesterol). The activity of hepatic superoxide dismutase in rats receiving different amounts of berberine was significantly increased in a dose-dependent manner. In addition, histopathological changes, such as steatosis, necrosis and myofibroblast proliferation, were reduced and the expression of α-smooth muscle actin and transforming growth factor were significantly downregulated. These results suggest that berberine could be used to prevent experimental liver fibrosis through regulation of the anti-oxidant system and lipid peroxidation.

LIPID-LOWERING ACTIVITY

Many studies indicated cholesterol-lowering effect of berberine. For example, treatment of hyperlipidemic hamsters with berberine reduced serum cholesterol by 40% and LDL-cholesterol by 42%, with increase in hepatic low density lipoprotein receptor (LDLR) mRNA and hepatic LDLR protein (Kong *et al.,* 2004). Berberine reduced body weight and plasma triglycerides as well as improved insulin action in high-fat-fed Wistar rats (Lee *et al.,* 2006).

Hypolipemic efficacy of berberine was clinically investigated. Oral administration of berberine in 32 hypercholesterolemic patients for 3 months reduced serum cholesterol by 29%, triglycerides by 35% and LDL-cholesterol by 25% (Kong *et al.,* 2004). Berberine can also act hypolipemic in diabetes mellitus patients. A clinical study on 36 adults with newly diagnosed type 2

diabetes mellitus treated with berberine showed significant decreases in plasma triglycerides during the 3 month trial. In other study 48 adults with poorly controlled type 2 diabetes mellitus were supplemented with berberine during 3 months. Again, total cholesterol and low-density lipoprotein cholesterol decreased significantly (Yin *et al.,* 2008).

It seems that mechanism of action of berberine is different from that of statin drugs. Studies on human hepatoma cells model demonstrated that Berberine upregulates LDLR expression independent of sterol regulatory element binding proteins, but dependent on extracellular signal-regulated kinase (ERK) activation. Berberine elevates LDLR expression through a post-transcriptional mechanism that stabilizes the mRNA (Kong *et al.,* 2004). Further studies demonstrated that the berberine-induced stabilization of LDLR mRNA is mediated by the ERK signalling pathway through interactions of cis-regulatory sequences of 3'UTR and mRNA binding proteins that are downstream effectors of this signalling cascade (Abidi *et al.,* 2005). In addition to that, there are some studies that indicate that the activity of berberine is to some extent due to stimulation of AMP-activated protein kinase (AMPK) activity (Lee *et al.,* 2006).

EFFECTS ON ALZHEIMER'S DISEASE

Alzheimer's Disease (AD) is a progressive and degenerative neurological disorder characterized by loss of cognition and memory. However, the pathogenesis of AD is still not clearly known. Current research in drug discovery for treatment of AD involves various targets, being only symptomatic, with the main therapeutic strategies based on "cholinergic hypothesis" and "amyloid cascade hypothesis". Therefore, acetylcholinesterase (AChE) inhibitors have lately gained interest as potential drugs in the treatment of Alzheimer's disease.

Berberine and, accordingly, the extracts of plants that contain it, may act as inhibitors of acetylcholinesterase (AChE). Such examples include *Chelidonium majus* L. (Cho *et al.,* 2006), *Corydalis speciosa* Maxim. (Kim *et al.,* 2004) and many others. Acetylcholinesterase (AChE) inhibitory effect of 7 quartenary protoberberine alkaloids, including berberine, on isolated rat duodenum and guinea pig ileum tissue preparations was studied. The IC_{50} values of berberine were lowest among investigated protoberberines. The AChE inhibitory activity of berberin, as well as other quaternary alkaloids, depended on the cation pseudobase equilibrium of the studied alkaloids in solution (Ulrichova *et al.,* 1983). Inhibitory effect of berberine and other alkaloid on human erythrocyte AChE and serum butyrylcholinesterase (BchE) was assessed (Kuznetsova *et al.,* 2002). Again, berberine produced strong but reversible inhibitory effect on AChE while the activity on BChE was weaker. Berberine is a competitive-noncompetitive inhibitor of the both cholinesterases.

The usefulness of berberine in treatment of Alzheimer's disease may not be contributed only to AChE inhibitory activity. Intragastric administration of berberine chloride for 14 days significantly ameliorated the spatial memory impairment as assayed by Morris water maze test in the rat model of Alzheimer's disease. However, in the same dose it also influenced inflammation factors by increasing the expression of interleukin-1 beta and inducible nitric oxide synthase (Zhu & Qian, 2006). In addition to that, berberine enhanced proportion of neurite-bearing PC12 cells induced by nerve growth factor (NGF) in a dose-dependent manner without cytotoxicity. Although the inhibition of acetylcholinesterase activity caused by berberine was comparable to that of physostigmine, that inhibition was not responsible for the potentiation of NGF-induced neurite outgrowth (Shigeta *et al.,* 2002).

ANTITUMOR EFFECTS

There is a vast number of studies that investigated different antitumor activities of berberine or the plants that contain it. Berberine showed apoptotic activity, initiated differentiation and inhibited viability on many type of tumor cells. For example, berberine showed a strong inhibition on the proliferation of hepatoma (HepG2, Hep3B, SK-Hep1 and PLC/PRF/5) and leukemia cell lines (K562, U937, P3H1 and Raji) (Lin *et al.,* 2004). Berberine also inhibited proliferation on six types of oesophageal cancer cells lines (YES-1 to YES-6) (Iizuka *et al.,* 2000) and exhibited cytostatic/cytotoxic effect of human breast cancer cells (MDA-MB231) (Issat *et al.,* 2006). Activity of berberine depended on the type of cell investigated, and it was found that, *e.g.* murine melanoma B16 cells were much more sensitive to berberine treatment than human leukemic U937 cells (Letasiova *et al.,* 2006).

Furthermore, berberine inhibited effects of the tumor promoters 12-*O*-tetradecanoylphorbol-13-acetate and teleocidin, such as increased ^{32}P incorporation into phospholipids of cell membrane and hexose transport, as well as suppressed promoting effect of teleocidin on skin tumor formation in mice initiated with 7,12-dimethyl-benz[*a*]anthracene (Nishino *et al.,* 1986). Furthermore, berberine induced a pluripotent human teratocarcinoma cell clone, NT2/D1, derived from the Tera-2 cell line, to differentiate into cells with neuronal cell morphology. After a 24 h treatment of cells at a non-toxic dose of 0.1 mg/mL in culture medium, the cells started to show morphologic changes, developing into terminally differentiated neuronal cells with long, inter-connecting network-like cellular structures (Chang *et al.,* 1990).

In addition to that, berberine exhibited the ability to induce morphological changes and internucleosomal DNA fragmentation, characteristic of apoptosis in promyelocytic leukemia HL-60 cells. Number of cells that underwent apoptosis increased with time (Kuo *et al.,* 1995). It seems that those effects are associated with down-regulation of nucleophosmin/B23 and telomerase activity (Wu *et al.,* 1999). Some experiments on HL-60 and WEHI-

3 cells indicate that berberine induces apoptosis through the activation of caspase-3 (Lin *et al.,* 2006). Similar mechanism was observed on androgen-insensitive (DU145 and PC-3) and androgen-sensitive (LNCaP) prostate cancer cells (Mantena *et al.,* 2006) as well as human gastric carcinoma SNU-5 cells (Lin *et al.,* 2006). On the other hand, findings on KB cells suggest that berberine-induced apoptosis might be cyclooxygenase-2-dependent since berberine inhibits expression of that enzyme (Kuo *et al.,* 2005).

Berberine also inhibited the viability and arylamine *N*-acetyltransferase (NAT) activity in a human colon tumor cell line (Lin *et al.,* 1999) as well as NAT activity and 2-aminofluorene-DNA adduct formation in human leukaemia cells (Chung *et al.,* 2000) in a dose-dependent manner. It seems that these effects are achieved by inhibition of expression of mRNA NAT1 in treated cells (Lin *et al.,* 2005). Some of the antitumor effects of berberine might also be attributed to DNA repair inhibition (Szeto *et al.,* 2002). Newer studies on oral cancer HSC-3 cells indicate that induction of reactive oxygen species (ROS) and Ca^{2+} production, as well as the dysfunction of mitochondrial membrane potential (MMP) caused by berberine correlated with apoptosis. Prolonged exposure of the cells to berberine caused increased apoptosis through reduced levels of MMP, release of cytochrome c and activation of caspase-3 (Lin *et al.,* 2007; Lin *et al.,* 2007b). Possible mechanisms include also nonsteroidal anti-inflammatory drug-activated gene (NAG-1), and activating transcription factor 3 (ATF3) induction, as demonstrated in human colorectal cancer cells (Piyanuch *et al.,* 2007).

CARDIOVASCULAR EFFECTS

Berberine has positive inotropic, negative chronotropic, antiarrhythmic, and vasodilator properties. Some of cardiovascular effects of berberine and its derivatives are attributed to the blockade of K^+ channels (delayed rectifier and K_{ATP}) and stimulation of Na^+-Ca^{2+} exchanger. Berberine has been shown to prolong the duration of ventricular action potential. Its vasodilator activity has been attributed to multiple cellular mechanisms. (Lau *et al.,* 2001). Its positive inotropic effect is achieved by enhancing both the force-velocity relationship and the duration of the active state. The mechanisms for these actions may include an alteration in trans-sarcolemmal flux of calcium as well as inhibition of intracellular calcium sequestration systems (Shaffer, 1985). Results of experiments in isolated guinea pig right ventricular papillary muscles suggested that mechanism for antiarrhythmic action of berberine may be suppression of delayed after-depolarizations, which may be attributable in part to a decrease in Na^+ influx (Wang *et al.,* 1994).

Acute cardiovascular effects of berberine in humans were assessed on 12 patients with refractory congestive heart failure before and during berberine intravenous infusion at rate of 0.2 mg/kg per min for 30 min. In that dose berberine decreased systemic and pulmonary vascular resistance,

as well as left ventricular end-diastolic pressures. Berberine increased cardiac and stroke index as well as and LV ejection fraction measured by contrast angiography. Furthermore, it increased in hemodynamic and echocardiographic indices of LV performance: peak measured velocity of shortening, peak shortening velocity at zero load, rate of development of pressure at developed isovolumic pressure of 40 mmHg, percent fractional shortening, and the mean velocity of circumferential fiber shortening. Berberine also decreased arteriovenous oxygen difference with no changes in total body oxygen uptake, arterial oxygen tension, or hemoglobin dissociation properties (Marin-Neto *et al.,* 1988).

Efficacy and safety of berberine for chronic congestive heart failure (CHF) was investigated on 156 patients with CHF and >90 ventricular premature complexes (VPCs) and/or nonsustained ventricular tachycardia (VT) on 24 h Holter monitoring. During the study, all the patients were receiving conventional therapy for CHF, consisting of angiotensin-converting enzyme inhibitors, digoxin, diuretics, and nitrates. A part of the patients were given berberine 1.2 to 2.0 g/day while the remaining patients were given placebo. Symptoms, a 6-min walk test, left ventricular (LV) ejection fraction (EF), frequency and complexity of VPCs, and quality of life were assessed after 8 weeks of treatment and during a mean 24-month follow-up. After treatment with berberine, there was a significantly greater increase in left ventricular ejection fraction (LVEF), exercise capacity, improvement of the dyspnea-fatigue index, and a decrease of frequency and complexity of VPCs compared with the control group. There was a significant decrease in mortality in the berberine-treated patients during long-term follow-up. Proarrhythmia was not observed, and there were no apparent side effects (Zeng *et al.,* 2003).

ACKNOWLEDGEMENTS

This study was conducted as part of the scientific project "Micromorphological and chemotaxonomic researches on some species of family *Lamiaceae*" with the support of the Ministry of Science, Eduction and Sport of the Republic of Croatia.

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13

An Ethnopharmacological Review on *Calotropis* species

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ABSTRACT

The genus Calotropis R.Br. is a member of Asclepiadaceae family, having twelve tropical species. The review encompasses two species Calotropis procera (Ait.) R.Br. and Calotropis gigantea (L.) R. Br., found in India and extensively explored in the field of ethnopharmacology throughout the world. These plants have been reported in Indian literatures since 600 BC to AD 16th century to cure many human ailments. The present review attempts to provide a bird's eye view on the existing knowledge of ethnopharmacology of Calotropis, including review of the ancient Indian literature of Ayurveda (Indian System of Medicine-ISM) in addition to ethnomedical properties, details of investigated pharmacologically relevant compounds and pharmacological studies supporting its use as medicinal plant(s). Major constituents of these plants are α-amyrin, β-amyrin, teraxasterol, gigantin, giganteol, isogiganteol, b-sitosterol and a wax. Latex contains glycosides like calotropagenin, calotropin, uscharin, calotoxin, calactin and proceragenin. Stem contains cardenolides such as uzarigenone, uzarigenine, deglucouzarin and frugoside. Roots contain

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cardenolides glycosides frugoside, coroglaucegenin and 4-β-D-glucofrugoside. Leaves contain mudarine, principal active compound in addition to three glycosides calotropin, uscharin and calotoxin. Flowers contain major compounds like α-calotropeol, β-calotropeol and rutin. The pharmacologically proved medicinal properties described in the article are antiulcer, antipyretic, anti-diarrhoeal, anticoccidial, anti-inflammatory / analgesic, cytostatic / anticancer, wound healer, anthelmintic, hepatoprotective and schizonticidal properties. Many classical remedies of ancient literature as well as traditional preparations have been proved excellent on certain diseases. The collective knowledge from the ethnic sources combined with modern phytochemical and pharmacological analysis may lead to the development of useful galenical. The focused and concerted efforts may bring about the products of utmost medical and by thus of a commercial value. It has great potential to provide international market for pharmaceutical industries.

Key words : Ethnopharmacology, *calotropis*, ayurveda, phytochemistry

INTRODUCTION

Calotropis R.Br. is a well-known genus throughout the tropical and sub-tropical world, commonly called as Giant Milk Weed or Swallow-Wort, having twelve species named *Calotropis acia* Buch.-Ham., *Calotropis busseana* K. Schum., *Calotropis gigantea* (L.) R.Br., *Calotropis hamiltoni* Wight, *Calotropis herbacea* Wight, *Calotropis heterophylla* Wall., *Calotropis inflexa* Chiov., *Calotropis persica* Gand., *Calotropis procera* (Ait.) R.Br., *Calotropis sussuela* G. Don, *Calotropis syriaca* (S.G. Gmelin) Woodson and *Calotropis wallichii* Wight. India has only two species *viz. Calotropis gigantea* (L.) R.Br. and *Calotropis procera* (Ait.) R.Br. In India it is commonly called as *Akado.* In Greek *kalos* means beautiful- the corona of flower is very beautiful and *tropics* means ship, keel or turning- a curved structure of petals, on which the name is based, *Calotropis*. These species are rarely found in forest but abundantly grow on wastelands and therefore with the urbanization its population is gradually decreasing. It is interwoven with the traditional rituals of Indian culture *viz.* white flowers of *Calotropis gigantea* are offered to Lord *Hanuman* on Saturdays. *Calotropis* spp. possesses number of traditional medicinal properties out of which many are confirmed on animal models under pharmacological aspect. Leaves, flowers, root, root-bark and latex are used in various medicinal preparations.

Calotropis is highly appreciated in the Indian System of Medicine (ISM); Ayurveda, for its therapeutic potential; therefore all the *Sanskrit* names of the Sun were given to *Calotropis*. *Rishies* (Saints) were the ancient scientists of India and pioneer of different fields of sciences. The medical science was

perhaps the most extensively explored by them. Major contributors in Ayurveda were Sushruta (600 BC), Charaka (AD 1st century) Vaghbhata (AD 5th century), Vrunda (AD 9th century), Chakradatta (AD 11th century), Shodhal (AD 12th century), Sharangdhar (AD14th century), Bangsan (AD 15th century) and Bhavprakasha (AD 16th century). Recent contributors of 20th century were Pade (1931) and Beheramji (1952). The chronology of Indian *Rishies* (given in brackets) comprehends the heritage of ISM. There are two types of books found in Ayurvedic literatures; one is *Samhita* and another is *Nighantu* (Materia Medica). *Samhitas* are written in *Sanskrit Shlokas* in which the diagnosis and properties of plants are interwoven. Each *shloka* is assigned with number and known as *Sutra*. *Nighantu* has separate chapters on plants in which the medication methods are described with doses and duration. The details of organoleptic medicinal properties are derived from the different *Sutras* from the noted references and summarized in the text.

Leaves are recommended in Ayurveda for fever, constipation (as purgative and flatulence), leprosy, cold, asthma, bronchitis, earache, boils, joint pain due to melancholy (*Vat*), headache, oedema, ascitis, skin psoriasis, scabies and syphilis (Sushruta, 1964; Sharangdhar, 1955; Vrunda, 1894; Pade, 1931; Beheramji, 1952). Ash of the leaves was used to cure spleen dysfunction (Vrunda, 1894) and splenomegaly (Pade, 1931). Leaves and latex both were reported for wound healing (Sharangdhar, 1955; Charaka, 1952; Beheramji, 1952). Sushruta (1964) held *Calotropis* for its bitter principle and hot property and recommended it as rabies antidote. The combined preparation from tomentum (hairs of leaves) with latex is claimed to be the antidote for snake poison (Vaidya, 1965). Charaka (1952), Bhavprakasha (1981), Shodhal (1978) and Chakradatta (1928) have reported latex as antidote for scorpion bite. Charaka (1952) has highly held this plant for its purgative, laxative, sudorific, emitic and stimulatory properties (Charaka, 1952; Vaidya, 1965). Latex was mentioned to cure vaginal infection, skin diseases, otorrhoea, scabies, black spots of face, cough, tuberculosis, dandruff and *Tinea capitis* (Shodhal, 1978; Sharangdhar, 1955; Bangsan, 1893; Kalidas, 1913; Vaidya, 1965). Vaghbhata (1939) had used latex and stem with stem bark for dental caries. Brushing teeth with finger thick stem was also recommended by them. Leaves and flowers were used to treat tuberculosis. Flowers were used for haematoma, bronchitis, splenomegaly, colic, stomachache and ascitis. Fruits are anthelmintic and applied for cough, and asthma. Seed cotton was applied for enhancing blood clotting and fast wound healing. Stem and stem bark were used for hydrocoele, elephantiasis, gout, eye problems, hepatomegaly and splenomegaly (Chakradatta, 1928; Bangsan, 1893). Root and root bark were used for piles, boils, jaundice, elephantiasis, scorpion bite antidote, oedema, joint pain, bronchitis, sore throat, leprosy, syphilis, blood purification, renal dysfunction, delirium, epilepsy, tetanus, gas, severe cold, whooping cough after pregnancy, ulcer and oedema and to facilitate parturition. Inhalation

of root (smoke) was effective on migraine. There is a tremendous scope of research in reproving all the classical claims. Reports on revalidation of classical claiming are referred in the text.

The taxon is widely used by tribes and the villagers in India for varied ailments. The whole plant is useful for fever, rheumatism, indigestion, cough, cold, eczema, asthma, elephantiasis, blotches of the skin, as tonic, expectorant, depurative, anthelmintic, blood circulation enhancer and mucous membrane regeneration for ulcers (Das, 1996; Vaidyaratnam, 1994; Ghosh, 1988; Ferrington, 1990). Terminal leaves are diuretic, digestive, antidiarrhoeal, abortifcient, analgesic, antidysentric, anticongetant, antihepatitic and are recommended on headache, lice, jaundice, sore gums, toothache, swellings and ulcers (Anonymous, 2004). Flower is ethnomedicinally reported for analgesic activity (Mascola *et al.*, 1988). In India latex of this plant is used for treating severe skin disorders like ringworm, guinea worm, blisters, boils, dermatitis, infected wounds and parasitic skin infestations; moreover it is also used for asthma, anorexia, inflammations, tumors, cardioactive disorder, scorpion stings, venereal sores, ophthalmic disorders and depilatory (Bown, 2003; Behl *et al.*, 1966; Morton, 1962; Vaidyaratnam, 1994; Mascola *et al.*, 1988; Duke, 1999). In Malawi it is reported as rubefacient, strongly purgative, violent emetic, deodorant and caustic (Williamson, 1956). In West Tropical Africa it is used on the conjunctiva in conjunctivitis, epiphora and as local anaesthesia (Delziel, 1937). Stem bark is used for leprosy, elephantiasis and to stimulate lactation in cattle. Root and root bark are tonic, anthelmintic, antispasmodic, febrifuge, depurative, expectorant, laxative, purgative, to treat elephantiasis, leprosy, chronic eczema, asthma, bronchitis, dyspepsia, malaria fever, menorrhagia, snake bite and it promotes gastric secretion and in a large doses emetic (Grieve, 1971; Parrotta, 2001).

Homoeopathy has used *Calotropis* to treat tuberculosis, syphilis, elephantiasis, leprosy, acute dysentery, pneumonic phthisis and as a sudorific.

BOTANY

Calotropis procera (Ait.) R.Br. and *Calotropis gigantea* (L.) R.Br. are the species found in India (Shah, 1978). It is a magnificent shrub, reaching 6-15 feet (Fig 1a), with large silver-green leaves densely covered with white hairs. It has umbel inflorescence with clusters of waxy purple-tipped flowers, and inflated pale green seedpods-follicles. *C. gigantea* has ovoid corolla-lobes, shorter than the staminal column, with two obtuse auricles just below the apex. Flower colour varies from deep violet to white (Figs 1c, d) while *C. procera* has corolla-buds hemispherical, corona lobes equaling or longer than staminal column and without auricles below the apex, with flower colour only whitish violet (Fig 1b). The follicles dehisce with only one suture when ripe to release silk-tufted seeds (Fig 1e). Roots have characteristic ridges and furrows on bark (Fig 1f).

Fig 1. a. Whole plant of *Calotropis procera*, b. Flower of *C. procera* in violet-white colour, c. Flower of *Calotropis gigantia* in light violet-white colour, d. Flower of *Calotropis gigantia* in white colour; Inset; Flower of *Calotropis gigantia* in deep violet colour e. Fruits of *C. procera*; Inset: Flower of *Calotropis gigantia* in deep violet colour e. Fruits of *C. procera*; Inset: Ripen and dehisced fruit with the aril of seeds, f. Root of *C. procera*

PHARMACOLOGICALLY RELEVANT CONSTITUENTS

Constituents of Latex

Seiber *et al.* (1982) separated the cardiac glycosides from the latex (Fig 2) which are calotropagenin (1), calotropin (2), uscharin (3), calotoxin (4), calactin

(5), uscharidin and voruscharin. The glycoside calotopin are of different types *viz.* calotropin D I, calotrapin D II, calotropin F I and calotropin F II. The glycoside calactin is toxic and insecticidal. Brüschweiler *et al.* (1969a, b) isolated proceroside, uzarigenin and syriogenin. Latex contains a powerful bacteriolytic enzyme lyses *Micrococcus lysodekticus* (Shukla & Krishnamurthy, 1961). Thakur *et al.* (1984) isolated two new triterpene esters, *viz.* 3'-methylbutanoates of α-amyrin and Ψ-taraxasterol, besides the known 3'-methylbutanoates of three triterpene alcohols from the hexane and methanol soluble extract of the latex of *C. gigantea*. *C. gigantea* latex contains two proteinase containing carbohydrate- calotropain-F1 and calotropain-FII; their properties are like chymopapain and papain respectively. They both are homogeneous in nature (Abraham & Joshi, 1979a, b). Procerain, a stable cysteine protease was isolated from the latex of *C. procera*. Procerain contains 8 tryptophan, 20 tyrosine and 7 cysteine residues. (Kumar *et al.*, 2003). Two kinds of esterases, E61 and E62, were isolated from the latex of *C. procera*. They were monomers with molecular mass of 27.1 kDa and 18.3 kDa, respectively. Esterase is known as a useful enzyme in the field of dairy food processing, fat and oil industry, and related biotechnology industry (Yoon *et al.*, 2003).

Constituents of Stem

The four different cardenolides have been isolated from the stem of *C. procera viz.* uzarigenone (6), uzarigenine (7), deglucouzarin (8) and frugoside (9) (Fig 2) (Elgamal *et al.*, 1999).

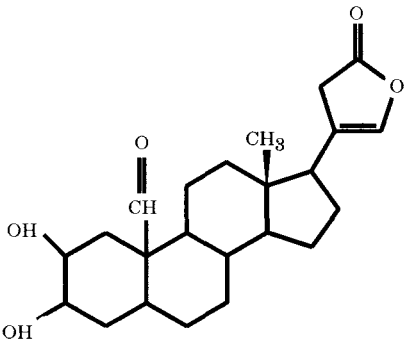
Constituents of Root

The major chemical constituents of the roots are mudarol, akundarol, uscharidin, calotropin, frugoside. and a yellow bitter resin, a black acid resin, a crystalline colourless substance (madaralban), amber-coloured viscid substance (madarfluavil), and caoutchouc (Anonymous, 1911). Three cardenolides glycosides named (Fig 2) frugoside (9), coroglaucegenin (10) and 4-β-D-glucofrugoside (11) were isolated from roots of *C. gigantean* (Kiuchi *et al.*, 1998). They are toxic to human cell lines but not for mouse cell line at 2 μg/mL level. Two new oxypregnane-oligoglycosides (Fig 2), calotroposides-A (12) and B (13), were isolated from root of *C. gigantea* (Kitagawa *et al.*, 1992). Isoursane pentacyclic triterpene C-18 (14) (Fig 2) was isolated from the root bark of *C. procera* (Bhutani *et al.*, 1992). Four new chemical constituents, one naphthalene derivative, named calotropnaphthalene, two terpene derivatives, namely calotropisesquiterpenol and calotropisesterterpenol and an aromatic product calotropbenzofuranone have been isolated from the roots of *C. gigantea* (Gupta, 2000).

Constituents of Aerial Part

Compounds isolated from aerial parts of *C. gigantea* are (Fig 2) isorhamnetin-3-O-rutinoside (15), isorhamnetin-3-O-glucoside (16) and a flavonols trisaccharide named isorhamnetin rhamnoglucoside (17) (Sen *et al.*, 1992). In general, both the species contain (Fig 2) β-sitosterol (18), taraxasterol (19), α-amyrin (20) and β-amyrin (21). *C. gigantea* contains additional gigantin, giganteol and isogiganteol. Akhtar *et al.* (1992) isolated a new cardenolide named proceragenin (22) having antibacterial activity, the structure shows three different moieties designated as 1, 1a and 1b in Fig 2. Asclepin was identified by Singh and Rastogi (1972).

In leaves, mudarine is isolated as principal active constituent with a yellow bitter acid, resin and glycosides calotropin, uscharin and calotoxin. Flower contains major compounds *viz.* α-calotropeol, β-calotropeol, rutin, β-amyrin and hyperoside. Procesterol (23), a new steroidal hydroxy ketone was isolated from the fresh and undried flowers of *C. procera* (Fig 2) (Khan & Malik, 1989). The seeds of *C. procera* contain frugoside, coroglaucigenin and corotoxigenin (Brüschweiler *et al.*, 1969a).



1. Calotropogenin

Structure No.	Nomenclature	R_1	R_2
2	Calotropin	α-OH, β-H	H
3	Uscharin		H
4	Calotoxin	S-CH2	H
5	Calactin	S-CH2	H

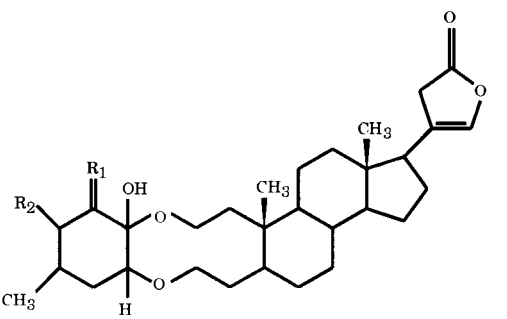
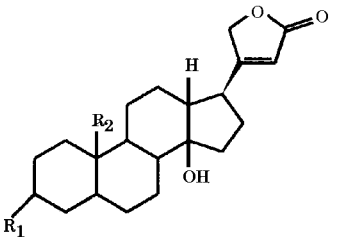


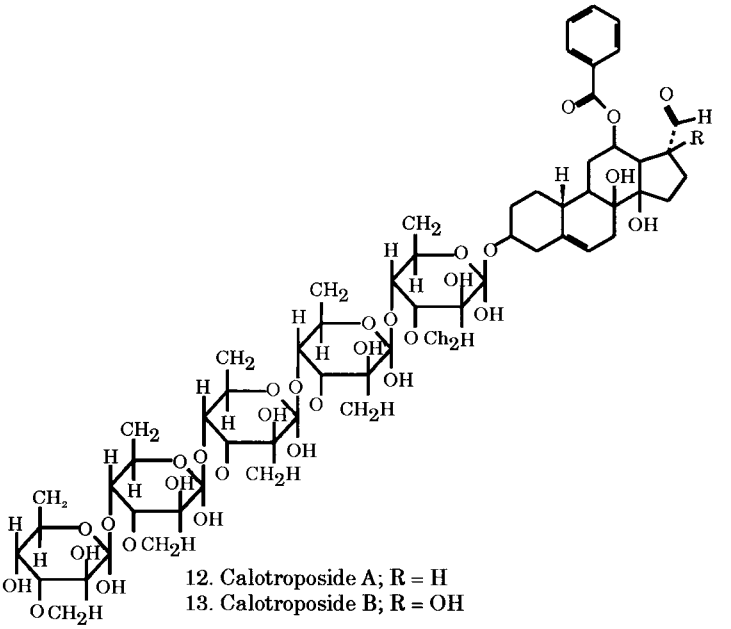
Fig 2. Cardenolides isolated from latex of *C. procera* (after Seiber *et al.*, 1982)

Structure of four cardenolides isolated from stem (6,7,8,9) of C. *procera* (After Elgamal *et al.*, 1999) and three from root (9,10,11) of *C. gigantea* (After Kiuchi *et al.*, 1998).



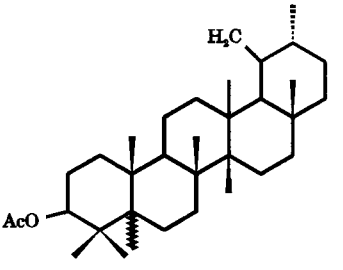
Structure No.	Nomenclature	R_1	R_2
6	Uzarigenone	=O	CH_3
7	Uzarigenine	OH	CH_3
8	Deglucouzarin	OGlc	CH_3
9	Frugoside	O-(6-desoxy allosyl)	CH_2OH
10	Coroglaucegenin	-OH	CH_2OH
11	4β-D-glucofrugoside	O-bis(6-desoxy allosyl)	CH_2OH

Calotroposides A (12) and B (13) isolated from root of *C. gigantea*



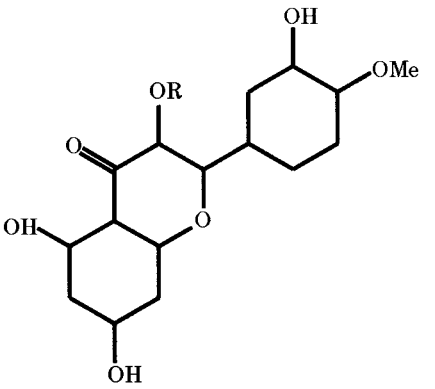
12. Calotroposide A; R = H
13. Calotroposide B; R = OH

Isoursane pentacyclic triterpene (14) isolated from the root bark of *C. procera* (after Bhutani *et al.*, 1992).

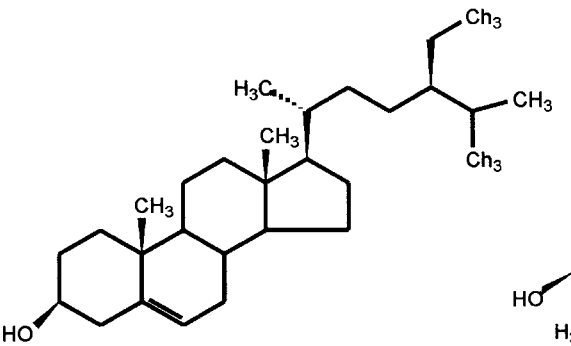


Compounds isolated from aerial parts of *C. gigantea* (after Sen *et al.*, 1992).

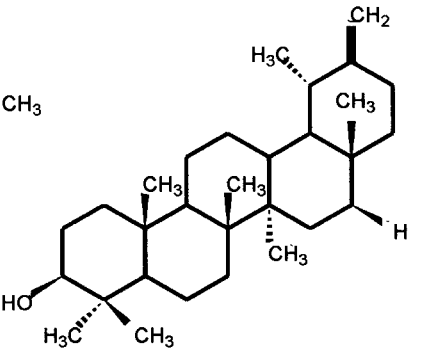
Structure No.	Nomenclature	R
15	Isorhamnetin-3-O-rutinoside	Glu-ORha O Gal
16	Isorhamnetin-3-O-glucoside	Glucose
17	isorhamnetin rhamnoglucoside	Glucose-O-Rhamnose



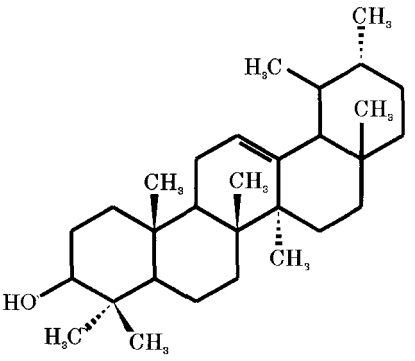
Compounds isolated from aerial part of *Calotorpis*: β-Sitosterol (18), taraxasterol (19), α-amyrin (20), β-amyrin (21).



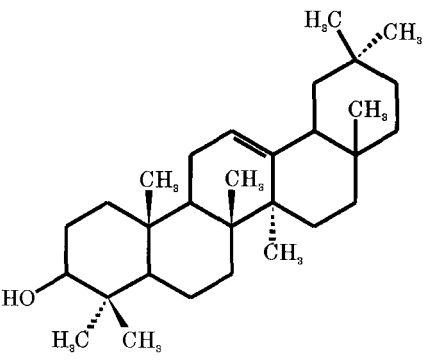
18. β-Sitosterol



19. Taraxasterol

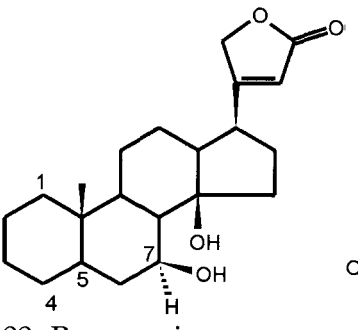


20. α-Amyrin

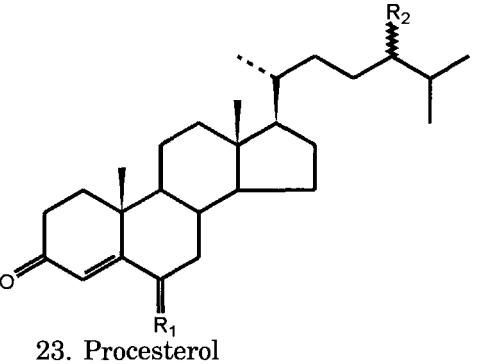


21. β-Amyrin

Proceragenin (22) (cardenolide) isolated from aerial part of *C. procera* having three different moieties designated as 1, 1a and 1b in fig (After Akhtar *et al.*, 1992). Procesterol (23), a steroidal hydroxy ketone, is isolated from the fresh flowers of *C. procera* with three moieties designated as 1,1a and 1b (After Khan & Malik, 1989).



22. Procergenin
1
1a C = O at 7
1a OAc at 7



23. Procesterol
1 R1 = a OH, 1H, αOH, βH, R_2 = (S) C_2H_5
1a R_1 = O, R_2 = (S) C_2H_5
1b R1 = βOH, αH, R_2 = (R) C_2H_5

PHARMACOLOGICAL PROFILE

Out of the above mentioned Ayurvedic and ethnopharmacological activities only few are pharmacologically evaluated.

Anti-ulcer Activity

The antiulcer activity of the chloroform fraction of *C. procera* root extract was reported on *in vivo* ulcer models named pyloric-ligated rats. It significantly inhibited aspirin, reserpine, absolute alcohol and serotonin-

induced gastric ulcerations in rats and also protected the gastric mucosa from aspirin-induced ulceration in rats. It gave significant protection in histamine-induced duodenal ulcers in guinea-pigs (Basu *et al.*, 1997). The use of root and root bark on ulcer is found in Ayurveda (Beheramji, 1952) and ethnopharmacology (Ghosh, 1988).

Anti-pyretic Activity

The antipyretic effect of dry latex was evaluated on male albino rats. The fever was induced by 20% Baker's yeast suspension. Administration of yeast increases rectal temperature from 97.32°F to maximum 100.02°F in 4 h. After 4 h of yeast administration, dry latex (250 or 500 mg/kg) was administered orally in saline solution. Aspirin (200 mg/kg) was used for comparison. Administration of dry latex 250 mg/kg and 500 mg/kg resulted in to decline of rectal temperature to 98.50°F and 98.45°F respectively in 2 h. Aspirin brought down the temperature to 96.9°F in 2 h (Kumar, 2000). The antipyretic activity of different extracts from *C. procera* was investigated on rats by Larhsini *et al.* (2002). All extracts have shown good effect in comparison to the standard drug acetylsalicylic acid. The roots of *C. gigantea* have also shown the same antipyretic effect (Chitme, 2005). In Ayurveda, leaves (Beheramji, 1952) and in ethnopharmacology the whole plant (Das, 1996) is used for fever.

Anti-diarrhoeal Activity

The anti-diarrhoeal effect of *C. gigantea* was evaluated against castor oil-induced diarrhoea model. The hydroalcoholic (50:50) extract of aerial part of *C. gigantea* was administered in 100, 200 and 400 mg/kg doses to rats. The plant extract in all tried doses significantly retarded the castor-oil induced enteropooling and intestinal transit. The effect is equivalent to atropine of 3 mg/kg (Chitme *et al.*, 2004). A single oral dose of dry latex (500 mg/kg) also produced a significant decrease in frequency of defecation, severity of diarrhoea and afforded protection from diarrhoea in 80% rats treated with castor oil. Dry latex produced a decrease in intestinal transit (27–37%) as compared to both normal and castor oil treated animals. Unlike atropine, dry latex significantly inhibited castor oil induced enteropooling (Kumar, 2001).

The normal saline soluble part of dry latex of *C. procera* was evaluated on smooth muscle function, both in *in vivo* and *in vitro* on rat and rabbit. Oral administration to rats (50–1000 mg/kg) produced a dose-dependent decrease in intestinal transit along with a decrease in intestinal content as compared to control group. At lower doses of dry latex saline fraction produced dose-dependent contractions of gastrointestinal smooth muscles in *in vitro* (rabbit ileum and fundus of rat stomach) that was followed by desensitization

at higher doses (Kumar & Shivkar, 2004). The anti-diarrhoeal effect is contradicted with report of latex as strongly purgative in Ayurveda (Charaka, 1952) and the ethnopharmacological uses (Williamson, 1956). However, flowers were mentioned for colic and root bark for renal dysfunction (Beheramji, 1952) in Ayurveda. Moreover, root bark (Grieve, 1971) and terminal leaves (Anonymous, 2004) were mentioned for diarrhoea and dysentery in ethnopharmacology. Jain *et al.* (1985) reported cure of the patients of diarrhoea and dysentery by root preparation. This indicates that the plant may have both types of compounds but in the experimental extract only anti-diarrhoeal compounds were extracted from latex while in whole latex administration the purgative compounds have shown to overcome the effect.

Anti-coccidial Activity

The anticoccidial activity of *C. procera* latex was examined in experimental *Eimeria ovinoidalis* infection in Najdi lambs. The symptoms observed for coccidiosis were bloody diarrhoea, tenesmus, anorexia, moderate dehydration, denudation of intestinal epithelium, presence of schizonts and cellular infiltration, decreases in the values of packed cell volume, haemoglobin and serum sodium concentration and increase in serum potassium levels in Najdi lambs. The lambs were treated with single oral doses of 0.02 mL/kg body weight of *C. procera* latex. Oocysts production was considerably suppressed for 4 days post-treatment and faeces were completely free from oocysts between 7 and 17 days after treatment with *C. procera* latex. These resulted into a return of normal appetite and activity, regular pelleted faeces and markedly reduced number of schizonts in intestinal cells. The concentration of serum sodium returned to normal but that of potassium remained high after therapy with *C. procera* latex, for that the dosing lambs with *C. procera* latex and sulfadimidine was suggested by the authors (Mahmoud *et al.*, 2001).

Anti-inflammatory and Analgesic Activity

Dry latex of *C. procera* was evaluated against various induced acute and chronic inflammations, like, carrageenan-induced oedema, Freund's adjuvant-induced oedema, cotton pellet granuloma, carrageenan air pouch inflammation, vascular permeability and UV-induced erythema. Oral administration of dry latex significantly inhibited oedema formation induced by carrageenan and Freund's adjuvant. It also prevented granuloma formation induced by cotton pellet and carrageenan. In addition to that it also inhibited fluid exudation, possibly due to its effect on vascular permeability. Moreover, it delayed the onset and intensity of UV induced erythema. The anti-inflammatory action of dry latex was also compared with standard antiinflammatory drugs (Sangraula *et al.*, 2001). A single dose of the aqueous suspension of the dried latex was also effective against the

acute inflammatory response in carrageenin and formalin induced rat paw oedema model (Kumar & Basu, 1994). Further purification from dry latex by methanol and aqueous extract was evaluated for anti-inflammatory action on the rat paw oedema model by Arya and Kumar (2005). The histological analysis revealed that the antiinflammatory effect of aqueous and methanolic extracts was more pronounced than phenylbutazone (PBZ) standard drug against carrageenin induced cellular infiltration and subcutaneous oedema. The effect was comparable to chlorpheniramine and PBZ standard drugs against histamine and prostaglandin (PGE_2) induced inflammation, respectively. Both extracts produced about 80%, 40%, and 30% inhibition of inflammation induced by bradykinin (BK), compound 48/80 and serotonin respectively. The extracts are effective mainly by inhibiting histamine and BK and partly by inhibiting PGE_2 (Arya & Kumar, 2005).

The dry latex of *C. procera* has significant analgesic effect against acetic acid induced writhings in mice by a single oral dose ranging from 165 to 830 mg/kg. The effect of dry latex at a dose of 415 mg/kg was more pronounced as compared to a 100 mg/kg oral dose of aspirin. Moreover, the oral dose of dry latex did not produce toxic effects in mice and the LD_{50} was found to be 3 g/kg (Dewan *et al.*, 2000). The anti-inflammatory effect of latex (Mascola *et al.*, 1988) and analgesic effect of latex, flowers (Mascola *et al.*, 1988) and terminal leaves (Annonymous, 2004) were reported in ethnopharmacological works.

Anti-cancer Activity

Ethanolic (70% v/v) flower extract of *C. procera* was tested for cytotoxicity on COLO 320 tumour cells responsible for cancer. This proved to have strongest cytotoxic effect with IC_{50}-values of 1.4 μg/mL. It's potential for cancer treatment compared with the standard anticancer drug cisplatin is more advantageous (Smit *et al.*, 1995). A wide variety of anti-cancer drugs exhibit cytotoxic effect by interfering with cell-cycle kinetics. These drugs are effective either by damaging the DNA during the S-phase of the cell cycle or by blocking the formation of the mitotic spindle in M-phase (Gali-Muhtasib & Bakkar, 2002). The cytotoxic and anti-mitotic activities of dry latex of *C. procera* were evaluated on root tip meristem of *Allium cepa*. Dry latex significantly inhibited the growth of roots and mitotic activity in comparison to standard cytotoxic drug cyclophosphamide and non-cytotoxic drugs cyproheptadine and aspirin, which served as controls (Sehgal *et al.*, 2006). *C. procera* contain characteristic cardioactive glycoside, *calotropine*, which has shown an antitumor effect *in vitro* on human epidermiod carcinoma cells of the rhinopharynx (Hansel *et al.*, 1993). It also acts as expectorant and diuretic. Latex is reported to cure tumours in ethnopharmacology (Vaidyaratnam, 1994).

Wound Healing Activity

The latex was partitioned with chloroform and water and the aqueous fraction was evaluated for wound healing activity after evaporating water. The excisional wounds of 8.0 mm diameter were inflicted on the back of male guinea pigs of Swiss strain bred as an experimental model. Topical application of 20 µl of 1.0% sterile solution of the residue of aqueous latex partition of *C. procera* (twice daily) was followed for 7 days. It significantly augmented the healing process by markedly increasing collagen, DNA and protein synthesis and epithelisation leading to reduction in wound area. Chloroform fraction did not show healing activity of wound. Wounds treated for 7 days with *C. procera* exhibited marked dryness and there was no visual sign of inflammation in the wounds. Further, there was no sign of any pathological fluid oozing out from the wound edges. Rasik *et al.* (1999) scientifically proved the traditional use of this plant in the management of wound healing. The latex was used for wound healing as mentioned in Ayurveda (Sharangdhar, 1955; Charaka, 1952) and ehtnopharmacology (Bown, 2003). Moreover, leaves and fruits (Beheramji, 1952) have also been reported as wound healer in Ayurveda.

Anthelmintic Activity

The crude latex of *C. procera* was evaluated for anthelmintic activity using adult earthworms. Both, fresh as well as aqueous extracts of dried latex exhibited a dose-dependent inhibition of spontaneous motility (paralysis) and evoked responses to pin-prick. With higher doses (100 mg/mL of aqueous extract of dry latex and 100% fresh latex) the effects were comparable with that of 3% piperazine. However, there was no final recovery in the case of worms treated with latex in contrast to piperazine in which the paralysis was reversible and the worms recovered completely within 6 h. The results show that latex possesses wormicidal activity and thus, may be useful as an anthelmintic (Shivkar & Kumar, 2003).

The anthelmintic activity is present in the latex of *C. procera*. Sheeps were infected by *Haemonchus contortus* larvae. They were treated with single oral doses of 0.01 mL or 0.02 mL/kg body weight with latex of *C. procera*, which resulted in to significant reduction of egg production, and fewer adult *Haemonchus* worms were found in the abomasum. The benefits found in symptoms were improved appetite but still reduced level of haemoglobin concentration in blood. *Calotropis* latex showed a concentration-dependent larvicidal activity *in vitro* within 20 min of application (Al-Qarawi *et al.*, 2001).

The cardiac glycosidal (cardenolide) extract of *C. procera* has contact and dipping LC_{50} values on adult stages of the camel tick, *Hyalomma dromedarii* Koch (Acari: Ixodidae) which were respectively 9.63 µg cm^{-2} and 1096 mg $litre^{-1}$. The risks and benefits both are associated with the use of cardiac

glycosides and to be considered in unison (Al-Rajhy *et al.*, 2003). This indicates that the anthelmintic property of the plant is due to the presence of cardiac glycosides.

Soil amended with the leaves of *C. procera* with *Paecilomyces lilacinus,* was most effective to reduce the multiplication of root-knot nematode-*Meloidogyne incognita* in soil. This treatment reduced root galling and improved plant growth (Ahmad & Khan, 2004). The anthelmintic property of flower (Beheramji, 1952) was reported in Ayurveda and that of whole plant (Vaidyaratnam, 1994), terminal leaves (Anonymous, 2004) and root/ root bark (Grieve, 1971) have been reported in ethnopharmacology.

Hepatoprotective and Antioxidant Activity

Efficacy of *C. procera* flower extract in the restoration of liver function after carbon tetrachloride induced hepatic injury in albino rats and mice has been evaluated. The ethanolic (70%) extract of flowers resulted in reduction of the biochemical markers of hepatic injury. The extract protected the animals from CCl_4 induced liver damage as revealed by histopathological observations. In addition, the extract showed free radical scavenging activity. The antioxidant activity was confirmed on *in vitro* models. The results indicate that flowers of *C. procera* possess hepatoprotective property possibly because of its anti-oxidant activity. This property may be attributed to the quercetin related flavonoids present in the flowers of *C. procera* (Qureshi *et al.*, 2007). The same hepatoprotective activity of *C. procera* root extract was also observed by Basu *et al.* (1992). However, in Ayurveda the ash of leaves and flowers were used for spleen dysfunction (Vrunda, 1894) and splenomegaly (Pade, 1931). The former application indicates the effect of elements in ash on splenomegaly.

Schizonticidal Activity

Sharma and Sharma (1999, 2000) have screened flower, bud and root of *C.procera* for *in vitro* antimalarial testing (schizonticidal activity) against Chloroquine sensitive (MRC 20) and Chloroquine resistance strain (MRC 76). The solvent used was ethanol and purified with column chromatography on Si gel (60–120 mesh) with ethylacetate, acetone and methanol. The highest activity was found in acetone fraction of bud on MRC 20 with 0.1 mg/mL IC_{50} value; where as the ethylacetate fraction of bud has shown maximum effect on MRC 76 with 0.3 mg/mL IC_{50} value.

Reversible Anti-ovulatory Activity

Effects of ethanolic (90%) extract and aqueous decoction of roots of *C. procera* were studied on oestrous cycle and oestrogenic functionality of female Wistar

rats. The extracts were administered orally for 5 days (one complete cycle) at the doses of 25, 50 and 100 mg/kg. The oestrous cycle showed temporary inhibition of ovulation. 80% and 70% rats showed ovulation inhibition in 100 mg/kg oral dose of ethanolic and water extract respectively. Aqueous extract was more active than ethanolic extract. A gradual normalization of the cycle started 10 days after the end of the treatment; at the fourth cycle all the animals showed a normal cycle. Milvane was used as a positive control. It is a commercially available oestro-progestin that prevent the normal oestrous cycle in 100% rats. The water decoction did not alter body, uterine weight, histoarchitecture of the uterus and the induced vaginal opening significantly in comparison to control. This suggested that inhibitory effect of *C. procera* on ovulation is not of oestrogenic nature and other mechanisms should be involved (Circosta *et al.*, 2001).

The effect of fresh leaf extract of *C. procera* on the reproductive organs of male Wistar rats was examined. The extract was orally given in 20 mg/g body weight daily for varying number of days resulted in to potentially deleterious effect on the testes and accessory sex organs (Akinloye *et al.*, 2002).

Larvicidal Activity

Aqueous phase of the latex of *C. procera* has 50% mortality at a dose of 28 ppm on larvae of *Anopheles labranchiae*. Ethanolic phase of latex also presented interesting larvicidal activity with LC_{50} 145 ppm. Ethanolic extract of roots was effective at LC_{50} of 215 ppm. DDT was used as reference with LC_{50} of 0.1 ppm for *Anopheles* larvae (Markouk *et al.*, 2000).

Molluscicidal Activity

The leaf and stem of *C. gigantea* were tried to control golden apple snail (*Pomacea canaliculata*) in lowland transplanted rice. It has consistent and very satisfactory molluscicidal activity in protecting the crop from snail damage. The leaves were more effective than the stems. The field requirement of leaf for snail control and crop protection was 200 kg/ha (Lobo & Llagas, 1991).

Antimicrobial Activity

C. procera as antibiotic against microorganisms was evaluated for apical twig and latex (Parabia *et al.*, 2007), leaves (Suresh & Chauhan, 1992), flowers (Larhsini *et al.*, 2001), root and stem bark (Jain *et al.*, 1996). Latex was reported for antifungal treatment of wheat seeds (Abdul-Fazal *et al.*, 1987). The solvents used in previously reported work of *C. procera* were water (Desta, 1993), n-butanol (Larhsini *et al.*, 2001) and ethanol (Nand Kishor *et al.*, 1997) for antimicrobial screening with significant inhibition in the growth of both gram-positive and gram-negative bacterial strains. In Ayurvada latex

and leaves were used for vaginal infection (Charaka, 1962), wound dressing (Beheramji, 1952), dental caries (Vaghbhata, 1939), dandruff (Shodhal, 1978) and *Tinea capitis* (Vrunda, 1894). These indicate its traditional application as antimicrobial agent.

TOXICITY

This plant is alleged as a poisonous plant but in permissible dose. It is harmless for humans and in large dose it may cause emesis and diarrhoea. Moreover, in experimental models the solvent extracts were used and injected in body which enhanced the adverse effect of plant, whereas in Ayurvedic or ethnotheraputic use the whole plant is orally administrated which proved harmless treatment at judicious dose.

Fresh leaves of *C. procera* (5 g/kg bodyweight for 30 days and 10 g/kg for another 30 days) were administered in sheep and cattle. There was no reporting of clinical or gross pathological sign of poisoning and no histological sign in liver or kidneys in sheep or cattle except it has shown weight loss in animals (Radunz *et al.*, 1983). No toxic symptoms were noted in sheeps fed with fresh leaves and flowers at 5 g/kg body weight daily for 30 days followed by 10 g/kg for a further 30 days. Three groups of cattle fed with 2.5, 5 or 10 g/kg for 84 days showed no toxic symptoms in sheeps and cattles (Radunz *et al.*, 1984).

The daily collected fresh latex of *C. procera* was orally administered to rats and changes in homological parameters on days 7 and 14 were observed. It was concluded that the latex of *C. procera* has no significant effects on blood parameters but it readily caused loss of weight (Dada *et al.*, 2002). The same feature was also noted in leaves administered in sheep and cattle by Radunz *et al.* (1983).

CONCLUSIONS

Calotropis procera is a treasure trove inheritance of Indian System of Medicine. It has properties to cure several diseases and many of them are evaluated through animals and human trials. Still just few products have emerged from it and there is an immense scope for scientific evaluation of its potential for tuberculosis and antidote for poison. People are using the crude plant to cure diseases by indigenous way. There is a tremendous scope for pharmaceutical industries to develop drug of choice for many disorders.

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Antisickling Activity and Thermodegradation of an Anthocyanin Fraction from *Ocimum basilicum* L. (Lamiaceae)

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ABSTRACT

The antisickling activity of anthocyanins extract from Ocimum basilicum L was evaluated using Emmel test. Chromatographic separations with methanol-toluene (3:1) provided three fractions with R_f: 0.66, 0.85 and 0.88. The most polar fraction exhibited the highest activity. Thermodegradation kinetics of this fraction at 373 K produced a first order rate constant of k (s^{-1}) = 0.34 10^4. Structural elucidation of isolated compounds is in progress.

Key words : *Ocimum basilicum L.*, anthocyanins, antisickling activity, thermodegradation

INTRODUCTION

Sickle cell anaemia also called drepanocytosis is among the most spread genetic diseases. In certain African regions, carriers of S haemoglobin can reach 20% of the population with prevalence in central Africa of 25% to 30% (Fattorusso & Ritter, 1994; Iyamu *et al.*, 2002; Kaplan & Delpech, 1993; Moody *et al.*, 2003; Voet & Voet, 1998).

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In Democratic Republic of Congo, more than one million habitants or almost 2% of the population are SS homozygotes. About 80% of children suffering from drepanocytosis that do not receive regular medical care die before their fifth birthday (Mpiana *et al.,* 2007a).

This hemoglobinopathy due to the substitution of glutaminic acid by valine in β^6 position of the haemoglobin is nowadays spread on the five continents because of population migrations (Gulbis *et al.,* 2005; Sofowora, 1975).

Up to date, no efficient therapy has been presented. Existing therapeutic schemes are expensive for the African poor population, toxic or present infection risk on VIH/SIDA or simply inefficient (Gulbis *et al.,* 2005; Mehanna, 2001; Mpiana *et al.,* 2007a; Voet & Voet, 1998). But these last years, phytotherapy is exploited as a therapeutic approach that can relieve this disease (Ibrahim *et al.,* 2007; Iyamu *et al.,* 2002; Moody *et al.,* 2003; Mpiana *et al.,* 2007a; Neuwinger, 2000).

Recently, we have released series of studies on the *in vitro* antisickling activity of some Congolese plants used to treat SS anaemia traditionally (Mpiana *et al.,* 2007a; Mpiana *et al.,* 2007b; *Mpiana et al.,* 2007c). *Ocimum basilicum L.* is one of these plants. We also showed that for certain of these plants, anthocyanins are among chemical groups that possess antisickling activity (Mpiana *et al.,* 2007b; Mpiana *et al.,* 2007c). It's well known that these natural pigments are unstable at heat, pH, light, ... but even so, decoction is the most preparation mode recommended by traditional practitioners.

In this study, we suggest to verify the antisickling activity of anthocyanins extracts of *Ocimum basilicum* L. and to fractionate it in order to identify the more actives fractions and study their thermal stability. A thermodegradation kinetics is then realized.

MATERIALS AND METHODS

Plant Material

Plant materials (leaf) used in this study were collected from a *O. basilicum* L. growing at the Université de Kinshasa site, Kinshasa (D. R. Congo) and were authenticated by Mr. B.L. Nlandu of the INERA (Institut National d'Etudes et Recherches Agronomiques/Faculty of Science, Université de Kinshasa).

Extraction

The dried and powdered leaf material (10 g) was repeatedly extracted by cold percolation with water (200 mL x 1) for 48 h. Fractions were filtered and the solvent was evaporated under reduced pressure using a rotary evaporator.

Extraction of anthocyanins was then done using 100 g of dried powdered plant material with distillated water and diethyl ether according to the universal procedures (Bruneton, 1999).

Biological Material

Blood samples used to evaluate the antisickling activity of the plant extracts in this study were taken from known drepanocitary adolescent patients attending the "Centre de Médecine Mixte et d'Anémie *SS*" and "Centre Hospitalier Monkole", both located in Kinshasa area, D. R. Congo. In order to confirm their *SS* nature, the above-mentioned blood samples were first characterized by Haemoglobin electrophoresis on cellulose acetate gel, as previously reported (Mpiana *et al.*, 2007a). They were found to be *SS* blood and were then stored at ± 4°C in a refrigerator.

Biological Activity

Blood sample is put in contact with plant extracts at different concentrations (with the physiologic solution as the dilution solvent) according to Emmel's test procedure (Courtejoie & Hartaing, 1992). In this study, Emmel's test was performed as previously reported (Mpiana *et al.*, 2007a).

Fractionnement

The thin layer chromatography (TLC) was run on Merck plate with methanol–toluene (3:1) mixture as eluting solvent. The resulting developed plates were visualized by irradiation with ultra-violet light at 254 nm and 365 nm. Separation was realised by preparative chromatography using silica gel with the same solvent system.

Thermal Degradation

Anthocyanin aqueous solutions were put into an oven (Telco model) at different temperatures during different time periods. Solution's absorbances were measured using a ZUZI UV-2200 spectrophotometer.

Mathematical Model and Data Analysis

Anthocyanin thermal degradation can be considered as a chemical reaction thereby an anthocyanin molecule A decomposes irreversibly into one or several molecules assigned as molecule B. This transformation can be schematically represented by the following equation:

$$A \xrightarrow[\Delta]{k} B \qquad [1]$$

This transformation is a first order decomposition for which rate equation is given by:

$$\frac{dC_A}{dt} = -kC_A \quad [2]$$

Where C_A, t and k are respectively: concentration of A, degradation time and kinetics constant.

The integration of [2] gives:

$$C_A = C_A^0 e^{-kt} \quad [3]$$

Where C_A^0 is the initial concentration of A

If A is the only compound that absorbed at a chosen wavelength, the Lambert-Beer relation for this case would be

$$E = l\varepsilon C_A \quad [4]$$

And

$$E_0 = l\varepsilon C_A^0 \quad [5]$$

Where E, E_0, l and ε are respectively the absorbance at time t, the absorbance at time t= 0 second, the optic pathway and the molar extinction coefficient.

The combination of equations [3], [4] and [5] gives:

$$E = E_0 e^{-kt} \quad [6]$$

If the compound resulting from degradation process absorbed simultaneously with A at the same wavelength, the Lambert-beer equation would be:

$$E = l(\varepsilon_A C_A + \varepsilon_B C_B) \quad [7]$$

Where ε_A, ε_B, C_A and C_B are respectively compounds molar extinction coefficients and concentrations.

Considering that

$$C_A^0 = C_A + C_B \quad [8]$$

And combining [3], [5], [7] and [8] provide

$$E = E_\infty + \frac{\varepsilon_A + \varepsilon_B}{\varepsilon_A} E_0 e^{-kt} \quad [9]$$

Where $E_\infty = l\varepsilon_A C_A^0$

If $\varepsilon_A > \varepsilon_B$, equation [9] gives the same exponential decreasing trend as does the equation [6].

The experimental result fitting with the two models (equations [6] and [9]) is carried out using Microsoft Origin 6.3 software package.

RESULTS AND DISCUSSION

Antisickling Activity of Anthocyanin Total Extracts

Figs 1,2 illustrate the morphology of *SS* blood erythrocytes (standard) and that of *SS* blood erythrocytes in the presence of anthocyanin total extract of *Ocimum basilicum* L.

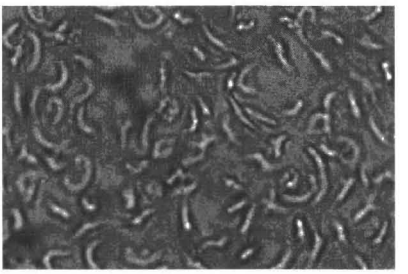

Fig 1. Morphology of drepanocytes none treated SS blood (Standard 500 X)

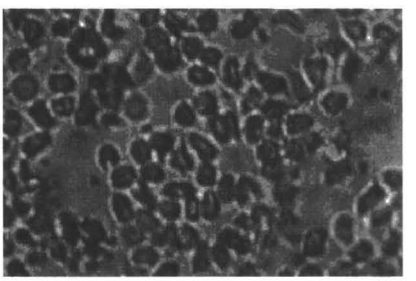

Fig 2. Morphology of drepanocytes treated with anthocyanins extracts (500 X)

As it can be noticed from these micrographies, the standard contains the majority of sickle-shaped erythrocytes, confirming the SS nature of the blood (Fig 1). Mixed together with anthocyanin extract (Fig 2), the majority of erythrocytes are reversed normal-shape. This fact illustrates antisickling activity of anthocyanins total extract and hence justifying the use of *Ocimum basilicum* L. in traditional medicine (Mpiana *et al.*, 2007a; Mpiana *et al.*, 2007b; Neuwinger, 2000).

Antisickling Activity of Isolated Anthocyanin Fraction

Methanol-Toluene (3:1) mixture was used to separate anthocyanins total extract into three different fractions which R_f are 0.66, 0.85 and 0.88. These

were temporarily coded fractions 1, 2 and 3 after fractionation using preparative chromatography. Antisickling activity of the fraction 1 was tested and was found to be active (Fig 3).

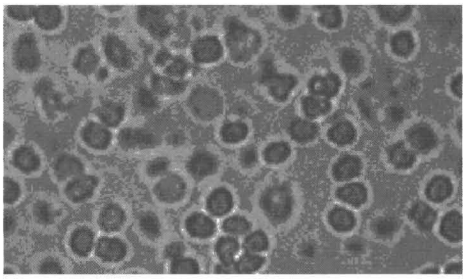

Fig 3. Morphology of SS erythrocytes treated by anthocyanin fraction 1 (500 X)

This Fig shows that the erythrocytes have been normalized compared to the standard (Fig 1). Therefore, this anthocyanin fraction is responsible of the antisickling activity of *Ocimum basilicum* L. The structural elucidation of this fraction is still in progress.

Thermodegradation of Isolated Fraction of Anthocyanin

It is known that anthocyanin compounds are unstable towards some physical and chemical parameters such as temperature, UV-visible radiation, pH, etc (Kahkoonen *et al.,* 2003). Since this plant is used in folk medicine either in infusion or decoction, we needed to check the behaviour of purified anthocyanin at high temperature. Fig 4 is UV-visible spectrum of fraction unexposed and exposed to heat.

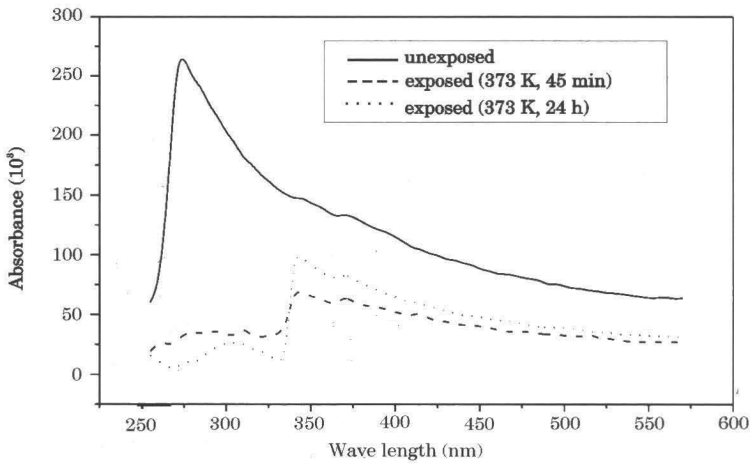

Fig 4. UV-visible spectra of anthocyanin fraction unexposed and exposed to heat at 373 K during 45 min and 24 h

The spectrum of anthocyanin fraction (unexposed) shows an absorption band at 274 nm assigned to π-π^* transition of flavylium ion which is the basic structure of anthocyanin (Kahkoonen *et al.*, 2003). When exposed to heat at 373 K, the spectrum is drastically modified. This indicates the degradation of the anthocyanin fraction when it is heated, with a total modification just after 45 min of heating.

It is interesting to follow the absorbance evolution of this anthocyanin fraction with the temperature at a constant exposure time.

The absorbance variation at 274 nm of this anthocyanin fraction with the temperature at constant exposure time is illustrated by Fig 5.

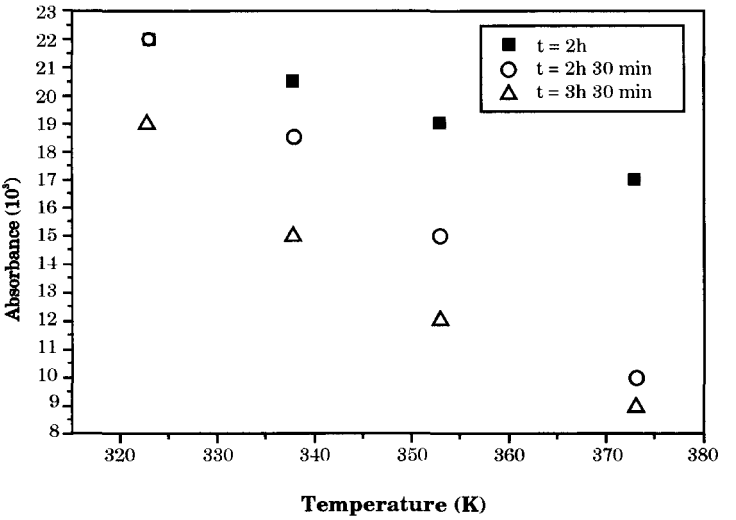

Fig 5. Absorbance variation (λ= 274 nm) of anthocyanin fraction with the temperature at constant exposure time.

This graph shows a decrease of the absorbance of the anthocyanin fraction with increase of temperature. This reveals the degradation of the anthocyanin fraction. The slope of the curve is more important for 2 h 30 min of exposure than that of 2 h, showing the influence of exposure time on the anthocyanin degradation.

The impact of exposure time on the separated anthocyanin at constant temperature is the kinetics of thermodegradation.

Kinetics of thermodegradation of anthocyanin fraction

This Fig shows an important absorbance decrease with exposure time. Before reaching a constant trend, the decrease is exponential. This exponential

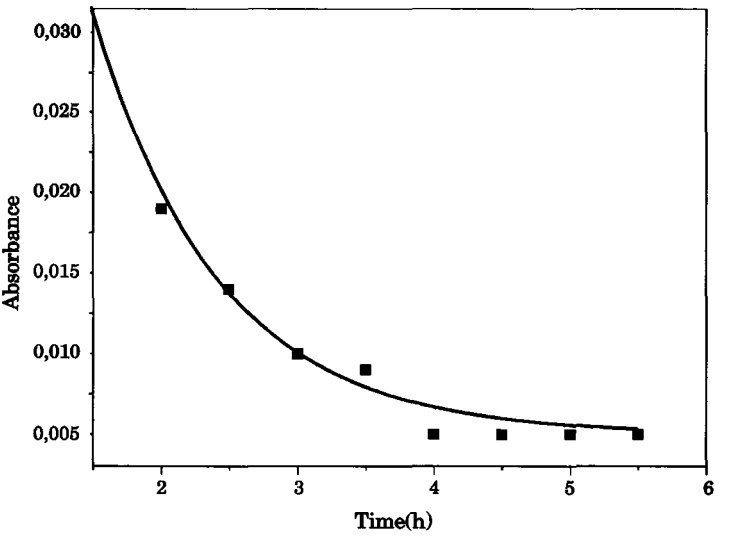

Fig 6. Shows the absorbance variation of anthocyanin fraction with time at 373 K

evolution is characteristic of concentration variation with the reaction time as in a first order reaction kinetics.

Although the molecular mass of isolated anthocyanin molecule is not yet known, a fitting of experimental values using the equation of the absorbance variation as a function of exposure time was done. The fitting of experimental data with equation [6] where only undegraded anthocyanin is supposed to absorb, does not give satisfactory results. However the fitting using the equation [9] model where the initial anthocyanin molecule A and its degraded form B absorb at that wave length, agrees with experimental results. Obtained parameter values are hereby provided.

ε_A (mol^{-1} cm^{-1}) = 2165.68

ε_B (mol^{-1} cm^{-1}) = 1680.80

k (s^{-1}) = 3.04 10^{-4}

It comes out of these results that kinetic constant at 373 K is low. But this value can increase at higher temperature.

ACKNOWLEDGEMENTS

The authors are indebted to Third World Academy of Science (TWAS) (Grant N° 08.030 *LDC/CHE/AF/AC-UNESCO FR*: **324020448**) for providing financial assistance.

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Pilot Clinical Trial of KJJ® in Children with Uncomplicated Respiratory Tract Infections

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ABSTRACT

The clinical efficacy of KJJ®, a proprietary combination prepared from Ocimum sanctum L., Hippophae rhamnoides L., Sambucus nigra L., Hyssopus officinalis L. Pelargonium sidoides L., was studied in a placebo-controlled, double-blind, randomised trial. Fifty-two children suffering from non-complicated acute upper respiratory tract infections were randomised into two groups, one of which was treated with 10 mL of KJJ 3-times a day, while the second received 10 mL of placebo 3-times a day, the treatment being continued for 6 days. Efficacy of treatment was evaluated from the results of examinations by a physician, including assessment of cough relief and reduction of irritation 30 min after administration of medication at the first visit to the doctor, and the daily responses to self-assessment questionnaires. Immediate cough relief and reduction of irritation was recorded 30 min after the first administration of medication in 60% of children who received KJJ but in only 12% of those receiving placebo. While significant improvements in inflammatory symptoms were observed in both groups following 2–3 days of treatment, the improvements in the severity and frequency of coughing, the efficacy of mucus discharge in the respiratory tract, the quality of sleep and the overall state of health were statistically significantly higher in the group receiving KJJ compared with the placebo group.

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Key words : Basil, sea buckthorn, elderberry, hyssop, household herbs, bronchitis, clinical trial

INTRODUCTION

A common approach in the folk medicine of cultures with a long written tradition involves treatment with complex combinations of plants. Such is the case regarding Ayurveda and Unani in India, and Kampo in Japan as well as for traditional Chinese and European medicines. This approach was employed by Hippocrates and Dioskorides in ancient Greece, the Galilees in Rome and Hildegard in Germany in the 12th century, with continuity to European phytotherapy and folk medicine. In many cases, it is the actual combination of constituents that establishes the efficacy of a preparation, the properties of which cannot be replicated by medications comprising one or two so-called active principles alone. A number of studies have demonstrated that, in the treatment of viral diseases, fixed combinations of plant extracts often show greater-than-expected medicinal benefits by virtue of a mix of constituents with synergistic effects acting upon different molecular targets (Vlietinck & Vanden Berghe, 1991; Nelson & Kursar, 1999; Wagner, 1999; Williamson, 2001)

In the present study, a pilot clinical trial was carried out in order to determine the efficacy of KJJ® (Kan Jang Junior) oral solution, a proprietary combination of commonly used household plants including Basil (*Ocimum sanctum* L.), Sea Buckthorn (*Hippophae rhamnoides* L.) Elderberry (*Sambucus nigra* L.), Hyssop (*Hyssopus officinalis* L.) and Geranium (*Pelargonium sidoides* L.), as a cough relief agent for the treatment of children with non-complicated respiratory tract infections (bronchitis). The novel preparation KJJ offers the specific advantage that its organoleptic properties are more acceptable to children than are those of the somewhat bitter Kan Jang® oral solution, which comprises a fixed combination of *Adhatoda vasica, Echinacea purpurea* and *Eleutherococcus senticosus* extracts.

METHODOLOGY

Study Design

This was a randomised, placebo-controlled, double-blind, parallel-group trial with positive and negative control, involving 52 children presenting non-complicated acute upper respiratory tract infections involving the nose, paranasal sinuses, pharynx, larynx, trachea, and bronchi. The study was carried out in accordance with the principles of Good Clinical Practice (GCP) at the Department of Clinical Pharmacology, Armenian National Institute of Health, Yerevan, Armenia, during the spring of 2005. Under the direction

of the investigator and the trial monitor, the study protocol has been reviewed and approved by the Independent Ethics Committee of the Ministry of Health of the Republic of Armenia.

Study Drugs

The test medications were manufactured in liquid form according to Good Manufacturing Practice (GMP) by the Swedish Herbal Institute (Gothenburg, Sweden). KJJ oral solution (batch KJJ-1) was quantified by HPLC with respect to the content of total polyphenols, eugenol, rosmarinic and caffeic acids. A liquid matrix, containing liquorice, nipagin, nipasol, sorbitol, polysorbate, and a natural aromatic mixture (comprising eucalyptus oil, peppermint oil and ginger, ethanol and water) was employed in the preparation of both KJJ and the placebo in order that both solutions would be organoleptically identical.

The medication, containing 15% of the proprietary combination, was provided in a dark glass bottle (500 mL) with a cap, sealing ring, and a measuring dosage cup (graduated at 5, 10, 15, 20 and 30 mL), and labelled "KJJ 500 mL: Batch Number ..., Clinical Study, Dose 10 mL – 3-times a Day, Patient Number...". The identification number on each of the bottles of verum or placebo was randomly encoded in a drug list so that the nature of the medication could be ascertained after completion of the study in order to perform the statistical analyses. The filling and labelling of medication bottles was performed in the presence of a pharmacist, and all drugs employed were stored separately at room temperature in a secure location so as to prevent their use for purposes other than that of the described study.

Patients

Boys and girls attending the Children's Department of Yerevan City Polyclinic No. 8 and showing symptoms of uncomplicated acute respiratory tract infections (IPCP code 400) were considered for inclusion in a trial designed to investigate tolerance to and efficacy of KJJ oral solution *versus* placebo. For the purposes of diagnosis, prior to consideration for inclusion in the study, the children initially underwent the following examinations and analyses: physical examination by a physician (compilation of anamnesis, detailed chest auscultation and percussion, thermometry), chest X-ray, and general blood and urine analysis. An investigator completed a baseline questionnaire for each patient in order to obtain details concerning age and history of illness. Using visual analogue scales (VAS) with marked end points (0 cm = no problems; 10 cm = pronounced problems), the investigator

gathered further information concerning the following parameters: (*i*) severity of illness as measured by degree of coughing, (*ii*) frequency of bouts of coughing, (*iii*), quality of sleep (as determined by the number of times the child awoke during the night), (*iv*) efficacy of mucus discharge in the respiratory tract, (*v*) degree of nasal congestion, (*vi*) soreness of throat, (*vii*) hoarseness or disturbance of voice, (*viii*) general feeling of sickness (malaise), and (*ix*) state of health. For the purposes of statistical evaluation, the VAS scores for each patient were recorded as the distance (in mm) from the zero-point to the indicated mark.

The criteria for inclusion in the study were: boys or girls from 5 to 12 years old suffering from uncomplicated upper respiratory tract infections in which, along with other symptoms, coughing presented a problem for which the indicated treatment would be a "cough remedy". According to ICPC classification, this indication includes the common cold, rhinitis, nasopharyngitis and pharyngitis. The criteria for exclusion were: children with asthma, pneumonia, an allergic reaction to herbal products or to bitterness, and children receiving medications (including anti-tussives, anti-histamines and decongestant medications) other than those involved in the study.

Following selection for inclusion in the trial, written informed consent was obtained from the parent or guardian of each child in accordance with the revised declaration of Helsinki (World Medical Association Declaration of Helsinki, 2000).

Methods

During their first contact with the project paediatrician, the selected children were divided into two groups (A and B) by a process of simple randomisation. Patients were allocated an identification number (1–52) in order of the beginning of their treatment, together with a bottle number that was randomly assigned by the investigator. The correlation between bottle number, medication and group was containined in a list in which the random (casual) distribution of labelled test samples (verum and/or placebo) had been encoded prior to the initiation of the trial. This list was not available to investigators, patients or care-providers, which were thus fully blinded with respect to treatment assignment until the statistical analyses had been completed at the end of the trial study. For each child, the casually-obtained identification number together with the code number of the randomly assigned bottle containing the medication were recorded in a protocol, in the self-assessment journal of the patient, and on a clinical report form.

After being grouped (study day 0), the children immediately commenced treatment with group A (27 patients) receiving orally 10 mL of KJJ 3-times

a day, and group B (25 patients) receiving orally 10 mL of placebo 3-times a day. The total duration of the study was 6 days, and control tests and assessments were conducted on the first study day and on each following day until the final evaluation on day 6.

Efficacy of treatment was evaluated through daily completion of a self-assessment questionnaire, the responses to which were recorded by the project paediatrician, with the assistance and collaboration of parents and care-providers as appropriate, in consideration of the age of the patients. The results of the examinations by the physician (including assessment of cough relief and reduction of irritation 30 min after administration of medication during the first visit to the doctor) and the duration of clinical manifestations of the acute phase of the disease were recorded daily by the paediatrician. Each patient was provided with an individual test-book, analysis log and a health diary for self-assessment by VAS scores of parameters (*i*) to (*ix*) as determined in the initial baseline questionnaire (see above). For patient assessment, the VAS end points were labelled as 0 cm – no improvement, and 10 cm - pronounced improvement, and the VAS score was defined as the distance (in mm) from the zero-point to the indicated mark. The results of these surveys were collected in the case report forms (CRFs).

Study Monitoring

The trial was monitored by an independent monitor in accordance with ICH and the Commission of the European Communities guidelines for GCP (ICH harmonised tripartite guideline, 1995; CPMP working party on efficacy of medicinal products, 1990).

The study site was visited by the monitor on Monday and Friday of each week of the study, and telephone contact was maintained every day. The aims of the monitor were to review the daily status of the study, to verify adherence to the study protocol, and to ensure completeness and accuracy of the CRFs. Adequate time was reserved for each visit of the monitor such that the CRFs could be examined on site and any necessary corrections attended to immediately.

Patient Compliance and Safety

Patient compliance was determined by questioning the parents/children and by collecting the bottles of medication and unused contents during the final visit to the doctor. The volume of unused liquid was measured and the lower limit for compliance was set at 98%.

Parents/children were informed at the start of the study that they should cease treatment immediately and seek urgent contact with the investigator should any concurrent illness or side-effects develop during the period of medication.

Statistical Methods

Each patient was identified by a number and by trial identification, and all data relevant to the study were entered into the CFR database (Microsoft™ Excel® 2000) patient by patient. The mean, standard error of the mean and standard deviation (SD) values for VAS scores were calculated according to standard methods of statistical analysis. In order to ascertain whether the mean values of parameters determined in the two groups of patients at the beginning of the treatment were statistically significant, unpaired t-tests (two-tailed) and Mann-Whitney tests were applied. For the comparison of data associated with the efficacy of the treatment collected from each group of patients on days 1 and 6 paired t-test was applied. In order to evaluate the difference between the efficacies of the treatments at the end of the study, the means of the differences between the beginning and the end of the treatment were compared for each variable by application of unpaired t-tests and Mann-Whitney tests. Data management and calculations were performed independently by two statisticians using GraphPad (San Diego, CA, USA) Prism software (version 3.03 for Windows) and Statistica 6.0 (Statsoft, Tulsa, OK, USA) statistical software.

RESULTS

The study population consisted of 52 children, 24 boys (46.2%) and 28 girls (53.8%), from 5 to 12 years-old (mean 8.22 years), who were randomly distributed between two groups, the KJJ-treated group (group A–27 patients) and the placebo-treated group (group B–25 patients). No statistically significant differences were observed between the two groups with respect to the baseline (day 0) VAS scores (Table 1) relating to (*i*) severity of illness, (*ii*) frequency of coughing, (*iii*) quality of sleep, (*iv*) efficacy of mucus discharge in the respiratory tract, (*v*) degree of nasal congestion, (*vi*) soreness of throat, (*vii*) hoarseness or disturbance of voice, (*viii*) general feeling of sickness (malaise), and (*ix*) state of health.

Table 1. The baseline characteristics and parameter values (day 0) of the study groups A and B

Characteristics	**Group A (KJJ-treated)**	**Group B (placebo-treated)**
Mean age (years ± SD)	8.29 ± 2.68	8.68 ± 2.78
Gender ratio (girls/boys)	16/11	12/13
Symptoms	**VAS scores**	
	Group A	**Group B**
(*i*) Degree (severity) of coughing	7.93 ± 0.54	8.04 ± 0.53
(*ii*) Frequency of coughing	7.89 ± 0.75	8.08 ± 0.57
(*iii*) Quality of sleep	7.71 ± 0.72	8.08 ± 0.70
(*iv*) Efficacy of mucus discharge	7.89 ± 0.80	8.16 ± 0.74

Table 1. *Contd.*

Table 1. *Contd.*

(*v*) Degree of nasal congestion	5.82 ± 1.44	6.52 ± 1.76
(*vi*) Soreness of throat	5.56 ± 1.36	6.20 ± 1.91
(*vii*) Hoarseness or disturbance of voice	5.19 ± 1.54	5.92 ± 1.89
(*viii*) General feeling of sickness (malaise)	5.30 ± 1.32	5.92 ± 1.75
(*ix*) State of health	5.48 ± 1.25	6.04 ± 1.51

VAS scores are means ± SD.
There were no significant differences between group means of characteristics or symptoms ($p>0.05$)

No children withdrew or dropped-out during the period of the trial, and at the end of the study all 52 children had complete health diary records. Only one adverse reaction was recorded during the trial, and this was reported as a burning sensation in the oesophagus after administration of KJJ. Nine patients in the placebo group commenced standard treatment at the end of the trial because there had been no improvement in their symptoms during the study.

At the conclusion of the study, the volume of medication remaining in the bottle returned by each patient was compared with the anticipated volume as calculated on the basis of the statement by the parent/child of the duration of treatment. The correlation between the amount of unused medication and the information revealed by each patient concerning the number of days that medication had been administered was extremely high, showing that all children fulfilled the compliance criterion by taking at least 98% of the recommended dose.

Assessment of the efficacy of KJJ in the relief of cough and reduction of irritation was based on the results of examinations made by a physician 30 min after the initial administration of medication during the first visit to

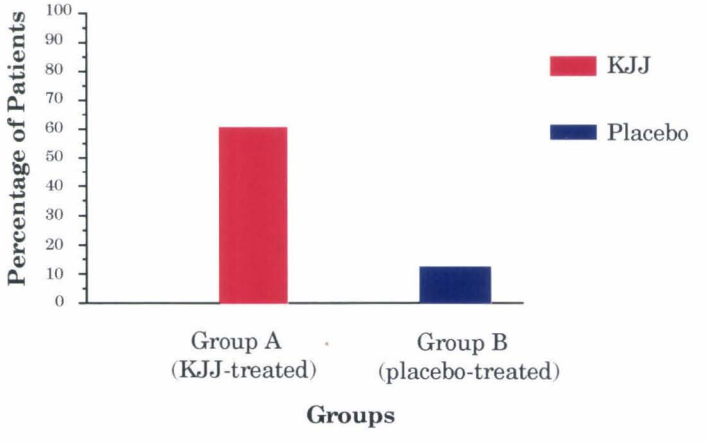

Fig 1. The percentage of patients in each group for which cough relief and reduction of irritation was recorded by a physician 30 min after first administration of medication

the doctor. Sixty percent of patients who had received KJJ, but only 12% of those who had received placebo, experienced immediate cough relief effect as indicated in Fig 1.

The overall efficacy of KJJ was assessed from statistical analyses of the differences in the means of each of the VAS scores relating to parameters (*i*) to (*ix*) determined on the first and the sixth days of the study for the treatment group A compared with the placebo group B (Table 2, Fig 2). Whilst noticeable improvements in the scores for all parameters were observed in both groups starting from days 2-3 of treatment, the improvements in VAS scores for (*i*) severity of illness, (*ii*) frequency of coughing, (*iii*) quality of sleep, (*iv*) efficacy of mucus discharge in the respiratory tract, and (*ix*) state of health were statistically significantly greater for the group receiving KJJ compared with the group treated with placebo. With respect to the degree of nasal congestion, soreness of throat, hoarseness, and malaise, whilst improvements in VAS scores were more marked in the group treated with KJJ, they were not significantly different from those determined for the placebo group (Table 2).

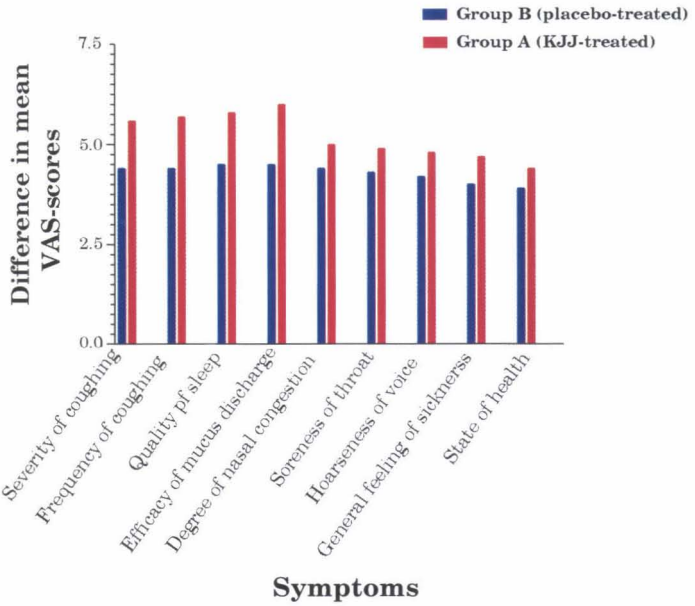

Fig 2. The differences between mean VAS scores for children in Groups A and B measured on the first and sixth day of the trial

DISCUSSION

There are numerous teas, juices, lozenges etc, derived both from individual plants as well as in the form of combinations used widely in Europe as household remedies for various colds and influenza-like ailments. Thus,

Table 2. The improvement in symptoms following treatment with KJJ compared with placebo as indicated by the difference in VAS scores determined on the first and the sixth day of the trial

	Symptoms	Improvement in VAS score		P value (between group comparison)
		Group A (KJJ-treated)	Group B (placebo-treated)	
(*i*)	Degree (severity) of coughing	5.64 ± 1.15	4.40 ± 1.04	0.00019**
(*ii*)	Frequency of coughing	5.74 ± 1.06	4.40 ± 1.04	0.000029***
(*iii*)	Quality of sleep	5.78 ± 1.05	4.48 ± 1.08	0.00006***
(*iv*)	Efficacy of mucus discharge	6.04 ± 1.19	4.52 ± 1.16	0.000025***
(*v*)	Degree of nasal congestion	5.04 ± 1.31	4.40 ± 1.12	0.066715
(*vi*)	Soreness of throat	4.93 ± 1.21	4.32 ± 1.18	0.07348
(*vii*)	Hoarseness or disturbance of voice	4.78 ± 1.25	4.16 ± 1.21	0.077
(*viii*)	General feeling of sickness (malaise)	4.70 ± 1.14	4.08 ± 1.22	0.06235
(*ix*)	State of health	4.44 ± 1.12	3.88 ± 0.83	0.04584*

VAS score differences are means ± SD.
Significant differences are indicated as : * – significant at $p<0.05$; ** – significant at $p<0.0001$

Sambucus nigra is used as a diaphoretic to increase bronchial secretion during a common cold (Blumenthal *et al.,* 1998) and in the treatment of influenza A and B viral infections (Vandenbussche *et al.,* 2004; Zakay-Rones *et al.*, 2004); products of *Hyssopus officinalis* are indicated for the treatment of colitis, colds, respiratory diseases, and chest and lung ailments (Kreis *et al.,* 1990; Gollapudi *et al.,* 1995; Blumenthal *et al.,* 1998); fruits and leaves of *Hyppophae rhamnoides* are used in antiviral medications for the treatment of influenza, adenovirus and viral infections of the upper respiratory tract (Zamkovaya, 2003); preparations of *Pelargonium* species are effective against acute bronchitis and tonsillopharyngitis (Kayser *et al.,* 2001; Bereznoy, 2003; Matthys *et al.,* 2003; Thäle *et al.,* 2008); and preparations of *Ocimum* species possess anti-inflammatory activity (Singh *et al.,* 1996; Kelm *et al.,* 2000).

In the present study, it has been demonstrated that treatment with the proprietary combination of extracts of these herbs present in KJJ oral solution significantly reduced the severity of cough, increased the efficacy of mucus discharge in the respiratory tract, and improved the quality of sleep and the general state of health compared with a placebo. Furthermore, it has been shown that treatment with KJJ can lead to a rapid improvement in the condition of a patient through relief of cough and irritation within 30 min of administration. The relatively high rate of placebo response is likely due to innate immunity, the psychological effect of receiving treatment, the traditional increase in the attentiveness of the relation of parents to ill children, and also by a positive attitude of the children in being able to participate in a test.

It is concluded that KJJ can be used safely in the treatment of uncomplicated acute upper respiratory tract infections (common cold, rhinitis, nasopharyngitis and pharyngitis) in children in order to relieve the severity and frequency of cough, and to reduce local irritation.

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Antidiabetic Effect of a Herbal Powder Consisting of *Curcuma longa*, *Emblica officinalis*, *Gymnema sylvestre* and *Tinospora cordifolia*

ANDALLU, B.[1], SUBHA, V.[1], VINAY KUMAR, A.V.[2] AND RAMA KUMAR, K.S.[3]

ABSTRACT

Administration of herbal powder consisting of rhizome of Curcuma longa, dry fruit of Emblica officinalis, dry roots of Gymnema sylvestre and Tinospora cordifolia in specific proportions has been found to regulate the abnormalities in type 2 diabetes. Herbal powder not only regulated glucose homeostasis but also ameliorated the changes in serum gluconeogenic enzymes following a dose of 10 g per day for a period of 60 days. The herbal powder effectively decreased the fasting blood glucose (16%) and glycosylated hemoglobin (HbA_{Ic}) levels (31%) thereby confirming antihyperglycemic action. Besides, the herbal powder decreased the activity of acid and alkaline phosphatases, gluconeogenic enzymes viz., lactate dehydrogenase, glucose-6-phosphatase, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase in serum indicating the mode of action through the regulation of glucose production via gluconeogenesis. The herbal powder also regulated the activities of erythrocyte glucose-6-phosphate dehydrogenase and lactate dehydrogenase. Thus, the multifactorial pathogenicity of diabetes could be effectively overcome by the multimodal therapeutic action of the herbal

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powder by virtue of bioactive principles present in Curcuma longa, Emblica officinalis, Gymnema sylvestre and Tinospora cordifolia.

Key words : Glycosylated hemoglobin, gluconeogenic enzymes, herbal powder, phosphatases, transaminases, type 2 diabetes

INTRODUCTION

Diabetes mellitus is one of the common metabolic disorders and India is the undoubted diabetes capital of the world with an estimated 35 million persons with diabetes (Clement, 2005). Insulin and oral hypoglycemic drugs like sulphonylureas and biguanides are still the major players in the management of the disease. However, long term usage of these drugs causes side effects in patients. Complete cure of the disease has been eluding physicians for centuries and the quest for the development of more effective antidiabetic agents is pursued relentlessly. In fact, many diabetics are now trying traditional medicines since they feel that it may help to alleviate personal worries and problems that are commonly believed to cause diabetes and it may be the only option for the poor (Pickup & Williams, 2003). Many herbal preparations contain combination of different herbs and have been described for the treatment of diabetes in ancient literature (Ghosh *et al.*, 2004). Compared to a single herb which may exhibit activity in a single direction, herbal preparations exhibit a multimodal activity owing to the presence of a number of bioactive principles that are needed to fight against the multifactorial pathogenicity of diabetes (Tiwari & Rao, 2002). Herbal medicines are being used by about 80% of the world population particularly in the developing countries for primary healthcare. These natural products are considered as the best in primary healthcare because of better cultural acceptability, safety, efficacy, potency, inexpensiveness and lesser side effects (Pullaiah & Naidu, 2003).

A number of plants have been used in healthcare for their health benefits and a few important ones are *Curcuma longa, Emblica officinalis, Gymnema sylvestre and Tinospora cordifolia. Curcuma longa* Linn. (turmeric, family-Zingiberaceae) is a medicinal plant extensively used in Ayurveda for the treatment of sprains and swelling caused by injuries (Ammon & Wahl, 1991) and is very rich in phenolics *i.e.* curcuminoids mainly, curcumin, dimethoxycurcumin and bisdimethoxycurcumin (Joe *et al.*, 2004). Curcumin, the primary active principle and pigment in turmeric has been claimed to possess antioxidant and anti-inflammatory and bioprotective properties (Balasubramaniam *et al.*, 2003). Fruits of *Emblica officinalis* Gaertn. (amla, family- Euphorbiaceae) contain 13 separable tannins (Chopra *et al.*, 1969) which have been reported to possess strong antioxidant property. Out of the hydrolysable tannins, Emblicannin A and B are galloellagitannins (Ghosal

et al., 1996). *Gymnema sylvestre* (meshasringi, family- Asclepidaceae) has the capability to regenerate the pancreatic β-cells thereby improving insulin secretion by virtue of its bioactive principles-the gymnemic acids which are a group of oleanane type triterpenoid saponins (Bone, 2002). *Tinospora cordifolia* (guduchi, family-Menispermaceae) is widely used for its antidiabetic properties (Nadkarni & Nadkarni, 1976) and the action is exerted mainly by its active principle, tinosporaside- a clerodane furan diterpene (Gopi *et al.*, 2004).

In view of the growing importance of traditional medicines as opposed to the modern medicines, the present study was undertaken to assess the effect of the herbal powder in ameliorating the abnormalities in diabetes.

MATERIALS AND METHODS

Procurement of Herbs and Preparation of Herbal Powder

Dry rhizomes of *Curcuma longa,* dry fruits of *Emblica officinalis*, dry roots of *Gymnema sylvestre* and *Tinospora cordifolia* were purchased in bulk from Ayurvedic shop, powdered separately and mixed in the following proportion as suggested by ayurvedic physician to get 10 g of powder containing 1.7 g *Curcuma longa*, 1.7 g *Emblica officinalis,* 4.9 g *Gymnema sylvestre* and 1.7 g *Tinospora cordifolia*. The sample was termed as herbal powder and was packed in clean polythene covers at 10 g/pack and used for the clinical trial.

Selection of Subjects and Treatment

After obtaining preliminary information pertaining to age, duration of diabetes, family history, diet and clinical complications with the help of a questionnaire, twenty male NIDDM patients between 40–60 years with mild diabetes and with no other complications were selected for the study from a local diabetic clinic, categorized into two groups– control (oral hypoglycemic drug-treated) and experimental (herbal powder-treated) comprising of ten subjects in each group. The consent of the subjects was obtained, the study was approved by ethical committee and the subjects were strictly under the supervision of diabetic expert.

The experimental group received 10 g herbal powder per day in two equal doses, mixed with water or buttermilk before breakfast and dinner, for a period of 60 days. The control subjects were given oral hypoglycemic drug (Glix MR) by the diabetologist during the experimental period. Fasting blood glucose levels were monitored at weekly intervals to assess the condition. At the commencement and at the end of the experimental period of 60 days, fasting blood samples were collected for biochemical analyses.

Biochemical Analyses

The parameters examined before and after the clinical trial were fasting blood glucose (Trinder, 1969), glycosylated hemoglobin (Trivelli *et al.*, 1971), enzymes *viz.* serum glutamate oxaloacetate transaminase(SGOT), serum glutamate pyruvate transaminase (SGPT), acid phosphatase (ACP), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and glucose-6-phosphatase (G-6-Pase) (Bergmeyer & Bernt, 1974) and erythrocyte enzymes *viz.* lactate dehydrogenase (Beutler, 1975), glucose-6-phosphate dehydrogenase (G6PDH) (Glock & McClean, 1953). The results of the biochemical parameters were analyzed statistically and the initial and final values were compared using paired difference t-test (Gupta, 1995).

RESULTS

A significant decrease of 16% ($p<0.005$) in the fasting plasma glucose levels (Fig 1) and that of 31% ($p<0.005$) in glycosylated hemoglobin levels (Fig 2) was seen in the experimental group treated with the herbal powder while no such decrease was noticed in the control group. With regards to the serum enzymes (Table 1), the activities of acid phosphatase, alkaline phosphatase and glucose-6-phosphatase in the experimental group decreased significantly by 9% ($p<0.01$), 12% ($p<0.005$) and 11% ($p<0.005$) respectively whereas, their activity in the control group was not much affected by the drug. Also, there was a significant decrease in the activities of lactate dehydrogenase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase (2%, $p<0.01$; 21%, $p<0.01$, and 16%, $p<0.005$ respectively) in serum in the experimental group while the levels in the control group were found to be almost maintained at around the initial values (Table 1).

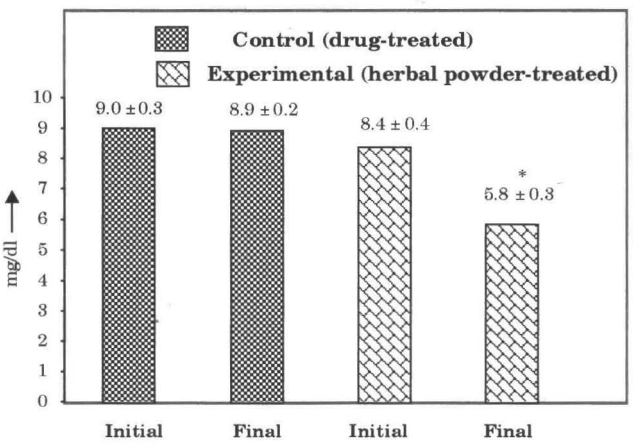

Fig 1. Fasting blood glucose levels in control and experimental diabetics. Values are in mean ± SEM of 10 subjects in each group. Comparison between initial and final values: *$p<0.005$

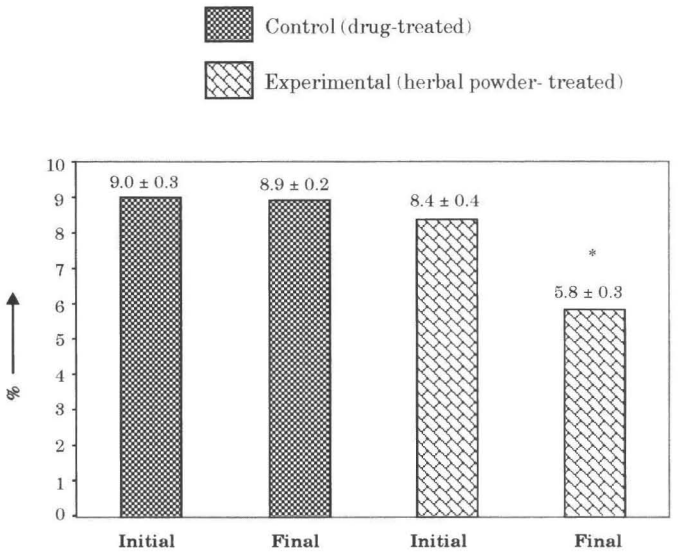

Fig 2. Values are in mean ± SEM of 10 subjects in each group. Figure in parenthesis indicate percent increase/decrease over respective initial comparision between initial and final values: *p<0.01;**p<0.005 values

Table 1. Activities of serum phosphatases and gluconeogenic enzymes in control and experimental diabetics

Parameter	Oral hypoglycemic drug		Herbal powder	
	Initial	Final	Initial	Final
Acid phsophatase (IU/L)	6.45 ± 0.03	6.40 ± 0.02 (1)	6.61 ± 0.21	6.03 ± 0.17* (9)
Alkaline phosphatase (IU/L)	97.69 ± 1.4	95.93 ± 1.4* (2)	109.98 ± 5.7	96.53 ± 1.7** (12)
Glucose-6-phosphatase (IU/L)	42.4 ± 0.7	42.3 ± 0.7	39.75 ± 1.1	35.35 ± 1.1** (11)
Lactate dehydrogenase (IU/L)	196.24 ± 0.4	195.71 ± 0.4	197.87 ± 0.8	195.21 ± 0.8* (2)
Glutamate-oxaloacetate transaminase (IU/L)	13.4 ± 1.1	13.0 ± 0.9 (3)	13.2 ± 1.0	10.4 ± 1.1* (21)
Glutamate-pyruvate transaminase (IU/L)	9.58 ± 0.5	9.35 ± 0.5* (2)	9.45 ± 0.6	7.98 ± 0.2** (16)

Values are in mean ± SEM of 10 subjects in each group. Figs in parentheses indicate percent increase/decrease over respective initial values. Comparison between initial and final values: * p<0.01; **p<0.005

The activities of LDH in the erythrocytes in experimental group showed a highly significant decrease (14%, $p<0.005$) whereas in the drug treated-group the decrease in LDH activity was insignificant. Also, a significant increase was seen in the activity of glucose-6-phosphate dehydrogenase (27%, $p<0.001$) in the herbal powder- treated group while no remarkable increase was observed in the drug treated-group with respect to its activity in erythrocytes (Table 2).

Table 2. Activities of erythrocyte enzymes in control and experimental diabetics

Parameter	Oral hypoglycemic drug		Herbal powder	
	Initial	Final	Initial	Final
Lactate dehydrogenase(IU/gHb)	200.54 ± 2.2	199.23 ± 2.1 (1)	226.09± 3.3	194.66 ± 1.4* (14)
Glucose-6-phosphate dehydrogenase (IU/gHb)	6.45 ± 0.2	6.90 ± 0.1	6.83± 0.1	8.66 ± 0.3** (27)

Values are in mean ± SEM of 10 subjects in each group. Figs in parentheses indicate percent increase/ decrease over respective initial values. Comparison between initial and final values: *$p<0.005$; **$p<0.001$

DISCUSSION

The decrease in blood glucose levels exhibited by the herbal powder is attributed to the bioactive principles present in the component herbs *i.e.* curcumin, a phenolic compound in *Curcuma longa* (Balasubramaniam *et al.*, 2003) which decreases blood glucose levels and lowers the formation of advanced glycation end products (Chattopadhyay *et al.*, 2004), gymnemic acid – a saponin in *Gymenema sylvestre* that inhibits intestinal absorption of glucose and enhances endogenous production of insulin through β-cell regeneration or repair (Bone, 2002) there by controls rise in blood glucose and tinosporaside – a clerodane furan diterpene in *Tinospora cordifolia* (Gopi *et al.*, 2004) which decreases the intestinal hydraulic permeability of nutrients including glucose (Upadhyay *et al.*, 2001) and improves glucose tolerance (Singh *et al.*, 2003) and tannoids present in *Emblica officinalis* which exhibit antioxidant property comparable to that of vitamin C (Ghosal *et al.*, 1996). Thus, the bioactive principles in the herbs have a direct or indirect action in lowering blood glucose levels in diabetics. The significant decrease in fasting blood glucose levels in experimental group is a result of the synergistic action/coordinated play of the bioactive compounds present in the component herbs of the herbal powder.

The decrease in HbA_{IC} levels exhibited by the herbal powder in experimental diabetic subjects is an interesting finding which resulted due

to significant fall in blood glucose levels caused by the components of the herbal powder as earlier researchers reported increased protein glycosylation due to sustained hyperglycemia (Brownlee & Cerami, 1981). The decrease in HbA_{IC} can also be due to HbA_{IC} lowering action of *Gymnema sylvestre* – a component of the herbal powder (Joffe & Freed, 2001). Another factor contributing to lowered HbA_{IC} is the high vitamin C content of *Emblica officinals* (Ghosal *et al.*, 1996) as vitamin C inhibits glycosylation of proteins by a competitive mechanism (Davie *et al.*, 1992).

Accelerated gluconeogenesis, negative nitrogen balance and muscle wasting are among the hallmarks of uncontrolled diabetes (Buse *et al.*, 1980). Consequently, there is an increased release of glutamine and glucose formation by the liver and kidney. This in turn means an increase in the activity of aminotransferases to produce intermediates in the glucose oxidation pathway (Garber *et al.*, 1976). Thus a decrease in the activity of the aminotransferases following herbal treatment in the experimental group also indicates control over gluconeogenesis.

The decrease in the activity of phosphatases (ALP and ACP) in the experimental group observed in the present study, can be attributed to the action of *Gymnema sylvestre* which enhances the endogenous insulin production, possibly by regeneration, as levels of C-peptide, a by–product of the conversion of pro-insulin to insulin were reported to apparently rise in earlier studies on *Gymnema sylvestre* (Bone, 2002). The decrease in the activity of serum phosphatases could also be due to the direct action of *Tinospora cordifolia* as earlier studies demonstrated lowered activities of serum G6Pase, ALP and ACP as well as LDH in diabetic rats treated with the root extracts of *T. cordifolia* thus showing hypoglycemic action (Sinha *et al.*, 2004).

The significant increase in the activity of LDH in diabetics is due to the excessive accumulation of pyruvate formed from alanine in the process of gluconeogenesis. The excessive pyruvate is then converted to lactate for which enzyme LDH is needed and therefore the activity of LDH may be increased due to less insulin availability in diabetics (Kamble *et al.*, 1998). It can thus be assumed that the active compounds in *Gymnema sylvestre*, specifically gymnemic acids may have an insulin-like action in correcting the levels of serum and erythrocyte LDH along with the control of hyperglycemia in the experimental group. Since there is an increase in the rate of gluconeogenesis in diabetics, there is a corresponding rise in the activity of G6Pase as G6Pase catalyses the common final enzymatic step in gluconeogenesis. Earlier studies reported an inhibitory effect of insulin on G6Pase activity (Gardner *et al.*, 1993). Our results with herbal powder had shown a significant decrease in the activity of G6Pase indicating once again that the action of components of herbal powder might be similar to that of insulin.

Activities of many glycolytic enzymes have been shown to be decreased in insulin deficient conditions. The activity of glucose-6-phosphate dehydrogenase (G6PDH) also showed a decreasing pattern in insulin deficiency. This may be due to glycation of proteins, stated to inactivate the enzyme by changing its conformational structure (Gupta *et al.*, 1997). Glucose-6-phosphate, the substrate for G6PDH may be diverted from the main pathway as a precursor for the glycoproteins thus decreasing the substrate availability for the enzyme. A decrease in the activity of G6PDH may also slow down the pentose phosphate pathway in diabetic conditions as it is the key enzyme of the pathway (Wolff *et al.*, 1991). Thus, due to the decreased production of NADPH the conversion of GSSG to GSH is decreased which plays an important role in strengthening the defense mechanism against oxidative stress (Murakami *et al.*, 1989). Herbal powder, by increasing the activity of G6PDH may lead to an elevation of GSH levels thus capable of countering the oxidative stress commonly seen in diabetics.

Our data showed that the herbal treatment effectively regulated the blood glucose levels but not below normal and decreased HbA_{IC} levels thereby confirming its antihyperglycemic action. The decreased activity of serum enzymes indicates the mode of action which is through the regulation of glucose production via gluconeogenesis. Also, the increased activity of glycolytic enzymes indicates the predominance of glycoslysis over gluconeogenesis in the experimental group after treatment with herbal powder.

Hence, from the present study, it can be concluded that the multifactorial pathogenicity of diabetes can be effectively overcome by the multimodal therapeutic action of the herbal powder containing a number of bioactive compounds reported in *Curcuma longa, Emblica officinalis, Gymnema sylvestre and Tinospora cordifolia.* With regards to this, it may also be stated that the revival of our ancient medical knowledge and its wide usage in the modern day together with the adoption of traditional dietary practices is the call of the hour.

ACKNOWLEDGEMENTS

Sri Sathya Sai University for the fund and facilities, the subjects for the cooperation, the ayurvedic physician and the diabetologist for supervision are acknowledged.

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Effect of *Aegle marmelos*, *Gymnema sylvester* and *Momordica charantia* on Blood Glucose and Lipid Profile in Alloxan Induced Diabetic Rabbits

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ABSTRACT

Aqueous extracts of Aegle marmelos (leaf), Gymnema sylvester (leaf), Momordica charantia (fruit) were evaluated for blood glucose level, lipid profile and change in body weight in alloxan induced diabetic rabbits and its effect on the above parameters in combination with conventional anti-diabetic drug. In diabetic rabbits Aegle marmelos, Gymnema sylvester, Momordica charantia produced significant reduction in blood glucose levels and also produced beneficial effects on lipid profile and change in body weight. Combination of Momordica charantia and Glibenclamide significantly enhanced the glucose lowering ability, improvement in lipid profile and body weight than any one of these drug alone. Aqueous extracts of Aegle marmelos, Gymnema sylvester, Momordica charantia, Glibenclamide and combination of Momordica charantia and Glibenclamide were effective in controlling blood glucose levels and improves the lipid profile and body weight in Alloxan induced rabbits.

Key words : Antidiabetic, diabetes mellitus, lipid profile, *Momordica charantia*

INTRODUCTION

Diabetes mellitus (DM) is a debilitating and often life threatening disease with increasing incidence in rural populations throughout the world (Kumar

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et al., 2007). Diabetes mellitus is a major metabolic syndrome characterized by derangement in carbohydrate metabolism associated with defect in insulin secretion or action (Szkuldeshi, 2001). Alloxan is widely used to induce diabetes mellitus in experimental animals, owing to its ability to destroy the ß-cells of pancreas possibly by generating excess reactive oxygen species. Overproduction of lipid peroxidation by-products and insufficient antioxidant potential have been reported in both experimental and human diabetes mellitus (Vander *et al.,* 2001). Patients are generally classified as either Type I (juvenile-onset diabetes) or Type II (maturity-onset diabetes) diabetics (Rang *et al.*, 2003). For both types of diabetes, control of blood glucose levels, lipid levels, blood pressure and weight will reduce the risk of vascular problems and associated disease (Williams, 1994). There is an increasing demand by patients to use the natural products with antidiabetic activity, due to the side effects associated with the use of insulin and oral hypoglycemic agents (Pourt, 1974; Kameswara *et al.,* 1997; Kameswara *et al.*, 2001). Available literature shows that there are more than 400 plant species showing hypoglycemic activity (Akhtar *et al.*, 1981; Mukherjee, 1981; Oliver, 1986; Ivorra *et al.*, 1989; Rai, 1995; Moumita *et al.,* 2007) and presently several laboratories are involved in isolating new herbal oral hypoglycemic agents.

DM does not only kill, but also is one of the major causes of adult blindness, kidney failure, gangrene, neuropathy, heart attack, and stroke (Kannel & McGee, 1979; Bopanna *et al.,* 1997). Large vessel atherosclerosis is the most common cause of death in diabetics. An ideal oral treatment for diabetes would be a drug that not only controls the glycemic levels but also prevents the development of atherosclerosis and other complications of diabetes (King *et al.,* 1998). Insulin and oral hypoglycemic agents like sulfonylureas and bigunides are still the major players in the management of the disease. More than 1200 species of organisms have been used ethnopharmacologically or experimentally to treat symptoms of DM, and several reviews on plants with known antidiabetic activity or with traditional use as antidiabetic remedies have been published (Ajganonkar, 1979; Upadhaya & Pandey, 1986; Oliver-Bever, 1986). It would thus appear that traditional antidiabetic plants might provide a useful source for developing new oral hypoglycemic compounds as pharmaceutical entities or simple dietary adjuncts to the exiting therapies. In accordance with the recommendation of the World Health Organisation expert committee on DM (Karpen *et al.,* 1982), investigation of hypoglycemic agents from plants, which have been used as traditional medicines, seems of paramount importance.

Extracts of various plant materials capable of decreasing blood sugar have been tested in experimental animal models and their effects confirmed

(Ajgaonkar, 1984; Upadhyay & Pandey, 1984). The present study was undertaken to explore the effect of aqueous extracts of *Aegle marmelos* (leaf), *Gymnema sylvester* (leaf), *Momordica charantia* (fruit) on the blood glucose level, lipid profile and change in body weight in alloxan induced diabetic rabbits.

The objective of present study was, to evaluate the effect of *Momordica charantia*, a well-documented hypoglycemic plant (Chatterjee, 1993; Narendhirakannan *et al.*, 2005), *Gymnema sylvester* (Kar *et al.*, 2003), *Aegle marmelos* (Ponnachan *et al.*, 1993; Kamalakkana, 2003) on blood glucose level, lipid profile and change in body weight in alloxan induced diabetic rabbits, its effect in combination with conventional anti-diabetic drug and to determine probable mechanism of action.

MATERIALS AND METHODS

Animals

Adult male albino rabbits (1–2 kgs), maintained at 25 ± °C in a well ventilated animal house under natural photoperiod conditions were used for the study. Animals were provided with standard diet and Water *ad libitum*. Animals described fasted were deprived of food for 18 h but had free access to water. Alloxan monohydrate was dissolved in normal saline and injected intraperitoneally after 18 h fasting to induce hyperglycemia (Sabu, 2000).

Alloxan (Sigma, Mumbai), glibenclamide (USV, LIMITED) were used for study. The dose 400 mg/kg, *p.o.* was standardized after a pilot study with different aqueous extracts doses of *Aegle marmelos* (AM), *Gymnema sylvester* (GS) *Momordica charantia* (MC) and to assess the antihyperglycemic effects in alloxan induced rabbits.

Study Design

Diabetes was induced in the rabbit by a single *i.v.* injection of alloxan monohydrate in distilled water at the rate of 140 mg/kg body weight. The animals were fasted 18 h before injection. Blood was collected from the marginal ear vein. After 72 h Alloxan treatment blood sugar 200–300 mg/dl were considered as diabetic and selected for study (Day 0) (Kameswara *et al.*, 2001; Ghosh, 1984). These rabbits further divided into seven groups of six animals each as mentioned below.

Group 1: control given on saline (1.5 mL/kg)

Group 2: diabetic given on saline (1.5 mL/kg)

Group 3: diabetic treated with AM (400 mg/kg, p. o.)

Group 4: diabetic treated with MC (400 mg/kg, p. o.)

Group 5: diabetic treated with GS (400 mg/kg, p. o.)

Group 6: diabetic treated with glibenclamide (0.5 mg/kg)

Group 7: diabetic treated with glibenclamide (0.5 mg/kg) and MC (400 mg/kg, p. o.)

At the time of grouping of the animals, fasting blood glucose (FBG) levels were measured. After two days of alloxan treatment, FBG was measured for confirmation of diabetic state in rabbits. There are several methods for estimation of blood glucose level (Rai, 1995).

Treatment with drug was started after 72 h the alloxan treatment (*i.e.* 0 Day) was continued for four weeks. All the drugs were given orally for four week twice a day in the morning. Blood glucose level measured before starting the treatment (Day 0) and weekly there after upto the end of the treatment period. Total cholesterol level (TCh), triglycerides (TG), high density lipoprotein level (HDL) (*i.e.* lipid profile) and changes in body weight of all rabbits were determined on Day 0 and after completion of the treatment at the end of the four week (Trinder, 1969). Blood glucose levels were determined using a Glucometer Monitor-GTM (One Touch Ultra blood glucose monitoring system from Zydus Pathline (Cadila Health Care) and lipid profile were estimated by enzymetic method using reagent kit (Span diagnostic ltd., Surat, India) (Carman, 1993).

Statistical Analysis

The results were expressed as mean ± SEM. Data on blood glucose level and lipid profile were analysed by one way ANOVA followed by Dunnet's't' test. Value of p less than 5% ($p<0.005$) were considered statistically significant.

RESULTS AND DISCUSSION

Effect on Fasting Blood Glucose Level

In alloxan diabetic rabbits there was a significant ($p<0.005$) increase in fasting blood glucose level. Treatment with *Aegle marmelos, Gymnema sylvester, Momordica charantia,* glibenclamide, produced significant reduction in fasting blood glucose level. Combination therapy of glibenclamide (0.25 mg/kg) and *Momordica charantia* (400 mg/kg) produced greater reduction in FBG as compared to all the other groups (Table 1).

Table 1. Effects of *Aegle marmelos, Gymnema sylvester, Momordica charantia* (400 mg/kg, p. o. twice a day for four weeks), Glibenclamide (0.5 mg/kg) and in combination on fasting blood glucose level in alloxan induced diabetic rabbits

Treatment groups	Fasting blood glucose (FBG) (mg/100 mL)				
	O Day	1st week	2nd week	3rd week	4th week
Control	87 ± 2.6	88 ±3.2	88.57±2.2	84.73± 1.9	86 ± 1.9
Diabetic control (Alloxan, 140 mg/kg)	220 ± 3.8*	227 ±2.8*	220 ± 4.9*	223.3 ± 1.3*	223 ± 3.3*
Diabetic treated with AM (400 mg/kg)	223 ± 2.4	221 ±1.4	187.1 ± 2.8	157.3 ± 4.2#	130 ±2.71#
Diabetic treated with GS (400 mg/kg)	215 ± 4.8	210 ±4.8	197 ± 2.8	163.3 ± 1.6	141 ±3.07#
Diabetic treated with MC (400 mg/kg)	225 ± 3.8	220 ±1.8	187.1 ± 2.8	145 ± 1.05#	135.8± 1.9#
Diabetic treated with glibenclamide (0.5 mg/kg)	220 ± 2.8	220 ±4.0	190 ± 3.8	131.7 ± 2.9#	129 ±3.16#
Diabetic treated with glibenclamide (0.5 mg /kg) & MC (400 mg/kg)	230 ± 3.0	220 ±4.0	172.9 ± 4.2	123.3 ± 2.1#	113.3± 2.1#
F (6, 36)	119.23	110.3	1130.4	182.69	95.4

N=6, the observations are mean ± SEM. *p<0.05, as compared to normal control group and #p<0.05, as compared to Alloxan group. (ANOVA followed by Dunnet's 't' test) AM: *Aegle marmelos,* GS: *Gymnema sylvester* MC: *Momordica charantia*

Effect on Total Cholesterol, Triglycerides and High Density Lipoprotein Level (HDL)

In alloxan diabetic rabbits there were a significant (p<0.005) increased in total cholesterol, triglycerides and decrease in HDL level. Treatment with *Aegle marmelos, Gymnema sylvester, Momordica charantia,* Glibenclamide and combination of glibenclamide with *Momordica charantia* produced significantly decreased total cholesterol level, triglyceride level and increased HDL level (Table 2).

Table 2. Effect of *Aegle marmelos, Gymnema sylvester, Momordica charantia* (400 mg/kg, p.o. twice a day for four weeks), Glibenclamide (0.5 mg/kg) and in combination on lipid profile in alloxan induced diabetic rabbits

Treatment groups	Total cholesterol (mg/100 mL)	Triglycerides (mg/100 mL)	HDL (mg/100 mL)
Control	88.23 ± 2.1	70 ± 2.5	36.5 ± 0.56
Diabetic control (Alloxan, 140 mg/kg)	116.7 ± 4.2*	93.33 ± 3.07*	30 ± 1.1*
Diabetic treated with AM (400 mg/kg)	85.33 ± 1.6#	81.67 ± 2.4#	29.83 ± 0.16
Diabetic treated with GS (400 mg/kg)	100.3 ± 3.7	78.33 ± 2.4#	35 ± 0.3#
Diabetic treated with MC (400 mg/kg)	81.5 ± 0.5#	78.33 ± 2.4#	35 ± 0.3#
Diabetic treated with glibenclamide (0. 5 mg/kg)	85.17 ± 1.3#	67.5 ± 2.1#	33.5 ± 1.14#
Diabetic treated with glibenclamide (0. 5 mg/kg) & MC (400 mg/kg)	75.17 ± 1.8#	69.77 ± 3.0#	36 ± 1.2#
F (6, 35)	41.08	12.60	20.30

N=6, the observations are mean ± SEM. *$p<0.05$, as compared to normal control group and #$p<0.05$, as compared to Alloxan group. (ANOVA followed by Dunnet's 't' test)
AM: *Aegle marmelos,* GS: *Gymnema sylvester* MC: *Momordica charantia*

Effect on Body Weight

A steady decrease in the body weight was observed in the alloxan treated rabbits, which was significant after 4th week of alloxan treatment. Treatment with *Aegle marmelos, Momordica charantia* and Glibenclamide with *Momordica charantia* produced significant increase in body weight after 4th week in alloxan induced rabbits. At the same time, there were no significant difference in the body weights in *Gymnema sylvester* and glibenclamide treated diabetic rabbits.

The ethnopharmacological use of herbal remedies for the treatment of diabetes mellitus is an area of study ripe with potential as a starting point in the development of alternative, inexpensive therapies for treating the disease. Several reviews on plants like *Taraxacum officinale* (dandelion), *Gymnema sylvester* (gymnema), *Glycyrrhiza glabra* (licorice), *Syzygium cumini* (jambul), *Opuntia streptacantha* (prickly pear), and *Panax ginseng/P. quinquefolium* (ginseng), *Fenugreek* (Ivorra, 1989; Brown, 1998; Marles, 1995) with known antidiabetic activity or with traditional use as antidiabetic remedies have been published. *Aegle marmelos, Gymnema sylvester, Momordica charantia*

are well known for its traditional use as an antidiabetic plant (Akhtar *et al.,* 1981; Kameswara *et al.,* 1997; Kinghorn & Compadre, 1991). It contains several hypoglycemic and hypolipidemic constituents and has been the object of clinical trials confirming its beneficial action in diabetes (Marles, 1995; Brown, 1998). In the present study, treatment with *Aegle marmelos* (400 mg/kg), *Gymnema sylvester* (400 mg/kg), *Momordica charantia* (400 mg/kg), glibenclamide (0.5 mg/kg) produced significant reduction in fasting blood glucose level. Combination therapy of glibenclamide (0.5 mg/kg) and *Momordica charantia* (400 mg/kg) produced greater reduction in FBG as compared to all the other groups. Alloxan, a beta cytotoxin induces a "chemical diabetes" (alloxan diabetes) in a wide variety of animal species through damage of the insulin secreting cells (Ajgaonkar, 1970). Induction of diabetes in these animals was confirmed by a significant rise in blood glucose and fall in the liver glycogen level (Upadhyay *et al.,* 1986) as shown in other animal species.

Present study indicates that aqueous extract of *Aegle marmelos, Gymnema sylvester, Momordica charantia* (400 mg/kg) and Glibenclamide (0.5 mg/kg) significantly decreased serum glucose level in hyperglycemic animals. *Momordica charantia* is a potential herbal alternative for blood sugar management, particularly in non-insulin dependent diabetes. *Momordica charantia* (bitter melon) is a proven hypoglycemic agent. At least three different groups of constituents in bitter melon have been reported to have hypoglycemic (blood-sugar lowering) actions of potential benefit in diabetes mellitus. These include a mixture of steroidal saponins known as charantin, insulin-like peptides, and alkaloids (Chatterjee, 1991). It is still unclear which of these is most effective, or if all three work together. While it is still being studied, and the effects of the herb are not entirely known, the herb has been shown to reduce blood sugar levels when used for an extended period of time.

Additionally, *Gymnema* reduces the taste of sugar when it is placed in the mouth, thus some use it to fight sugar cravings. From extract of the leaves were isolated glycosides known as Gymnemic acids, which exhibit anti-sweet activity (Kinghorn & Compadre, 1991).

Alloxan administration produced, elevated level of lipid peroxidation, hydroperooxides and conjugated diene that is a clear manifestation of excessive formation of free radicals and activation of lipid peroxidation system resulting in tissue damage. Karpen *et al.* (1982) observed an elevated level of lipid peroxides in the plasma of alloxan diabetic rabbits and lipid peroxidation is one of the characteristic features of chronic diabetes.

The significant decline in the concentration of these constituents in the liver tissue and serum of *Aegle marmelos, Momordica charantia, Gymnema*

sylvester (400 mg/kg) treated diabetic animals indicate that these extract effectively increased antioxidant potential *in vivo* (Karpen *et al.,* 1982).

Hypercholesterolemia is one of the most important risk factors for atherosclerosis, which promotes structural and functional vascular injury (Nileman *et al.,* 2005). Excess of fatty acid in plasma produced by the alloxan induced diabetes promotes the liver conversion of some fatty acids into phospholipids and cholesterol. These two substances along with excess triglycerides formed at the same time in the liver may be discharged into the blood in the lipoproteins.

It is observed by Krauss-Friedman's that the plasma lipoproteins increase as much as three fold in alloxan induced diabetes giving a total concentration of plasma lipids of several percent rather than normal 0.6%. This high lipid concentration may lead to the rapid development of atherosclerosis in diabetic patients (Lewis, 1978).

Aegle marmelos, Gymnema sylvester, Momordica charantia or in combination increased HDL level, decreased total cholesterol level, triglyceride level and this would be useful in diseases like diabetes mellitus and coronary heart disease because of their inverse relationship (King *et al.,* 1998). It is known that defense mechanism in liver, kidney, endothelium and heart are resistant to the diabetic conditions. Alterations of fatty acid composition by increased lipid levels may contribute to lowering the resistance of tissues and higher rate of oxidative stress. The change of body weight shows that rabbits given both extracts have a significant effect in controlling the loss of body weight, which is caused during diabetes. The activities were dose dependent (Kameswara *et al.,* 2001).The above results suggest that hypoglycemic effect was found at 400 mg/kg of aqueous extract that may be the optimum dose for hypoglycemic and was used in all the experiments of the present study. Further pharmacological and biochemical investigations are underway to elucidate the mechanism of the antidiabetic effect of *Aegle marmelos, Gymnema sylvester and Momordica charantia.*

It can be concluded from the data that *Aegle marmelos, Gymnema sylvester, Momordica charantia,* Glibenclamide alone or in combination significantly reduces the serum blood glucose level which are actually raised in alloxan diabetic rabbits. *Aegle marmelos, Gymnema sylvester, Momordica charantia* alone or in combination with glibenclamide has significantly decreased total cholesterol, triglycerides level, and increases HDL level. Moreover its antihyperlipidemic effect could represent a protective mechanism against the development of atherosclerosis. It is well known that hyperlipidemic has an association with atherosclerosis and the incidence of atherosclerosis is vastly increased in diabetics. *Aegle marmelos, Gymnema sylvester, Momordica charantia* or in combination with

glibenclamide may be utilized for the prevention or management of diabetic induced atherosclerosis in diabetes mellitus patients.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. S.B. Kasture, Dr. Mrs. V.S. Kasture, Principal A.P. Pingale, our management for their continuous support and encouragement.

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18

Chemical Constituents from Anti-inflammatory Fraction of *Taraxacum mongolicum* and Their Inhibition of AChE

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ABSTRACT

Bioactivity-guided separation over reverse-phase chromatography of the water extract of *Taraxacum mongolicum* Hand.-Mazz with the xylene-induced ear oedema test in mice showed that 50% MeOH eluent (Fr II) inhibited mouse ear oedema dose-dependently at 50, 100 and 200 mg/kg dosages. Forty compounds, including four new ones (**1**–**4**), were thereafter purified from Fr II, while their structures were elucidated on the basis of 1D- and 2D-NMR analyses along with the chemical and physical characteristics. The inhibitory activities of the new compounds on AChE were also tested.

Key words : AChE inhibition, anti-inflammation, biodiversity, flavone glycoside, herbal medicine, lignan, sesquiterpene, *Taraxacum mongolicum*

INTRODUCTION

Of the 70 species of the *Taraxacum* genus (Asteraceae) scattered in China, *Taraxacum mongolicum* Hand.-Mazz is the one most commonly used by

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local Chinese herbal physicians. Listed in Pharmacopoeia Chinensis, *T. mongolicum* is employed as a folk medicine for the treatment of inflammatory disorders and viral infectious diseases (Chinese National Pharmacopoeia Committee, 2000). *T. mongolicum* was given wide attention due to its remarkable curative effect on, among other inflammatory disorders, mastopathy, faucitis, furuncles, pneumonia, appendicitis, jaundice, gonorrhea, tonsillitis, and nephritis (Song *et al.,* 2001). However, there is still a flaw with regards to in-depth researches on the chemical constituents of this species. The reported phytochemical investigations of the title species described the isolation of only seven components, such as β-sitosterol, caffeic acid, chlorogenic acid, quercetin-7-*O*-β-glucoside, luteolin-7-*O*-β-glucoside, isoquercitrin, and hyperin (Ling *et al.,* 1997, 1999). As a part of our ongoing search for phytomedicines in Chinese medicinal plants (Wu *et al.,* 2006), we have examined the anti-inflammatory activity exhibited by different chromatographed fractions of *T. mongolicum* obtained from reverse-phase chromatography.

Inflammation is viewed as one of the characteristic of Alzheimer's disease (AD), non-steroidal anti-inflammatory drugs (NSAIDs) have therefore been accepted as an option to arrest the progression of AD or to delay the onset of the disease (Klegeris *et al.,* 2005). Meanwhile, Acetylcholinesterase (AChE) inhibitors are widely prescribed as initial medicines for the symptomatic treatment of AD (Holmes *et al.,* 2000). The new compounds isolated from the most anti-inflammatory active part, Fr II of *T. mongolicum* were assayed with the *in vitro* model of AChE inhibitory effects. The pharmacological screening of the extracts, isolation of pure components, as well as the structural elucidation and biological evaluation of the new compounds are described herein.

MATERIALS AND METHODS

General Experimental Procedures

1D and 2D-NMR spectra were obtained at 400 and 100 MHz for ^{1}H and ^{13}C, respectively, on an INOVA NMR spectrometer with TMS as the internal standard, and chemical shifts were given in δ values relative to that of the solvent (DMSO-d_6, $CDCl_3$, pyridine-d_5) on a tetramethylsilane scale. MS data were measured on a Bruker Esquire 3000 + instrument. Melting points were obtained in an X-4 digital melting point instrument and were uncorrected. IR spectra were recorded as KBr disks on a Bruker Vector-22 spectrometer. Column chromatography was performed with RP-18 (Pharmacia), Si gel (200–300 mesh, Qingdao Marine Chemical Facotory, China), Sephadex LH-20 (Pharmacia), and polyamide (30–60 mesh) (Huang Yan, China). Chemicals such as dexamethasone (Xian Ju, China) and xylene

were purchased from Sijiqing Firma at Hangzhou, China. TLC was carried out with precoated Si gel GF_{254} plates (Merck). Spots were visualized under UV light or by spraying with either 5% H_2SO_4 in EtOH or 3% $FeCl_3$ followed by heating, if not specified.

Animals

Male standard ICR strain mice weighing 20–25 g were bred in a standard animal house. The animals were kept in a room maintained at 22 ± 2°C and at a relative humidity level between 40% and 70%. The animals had free access to food and water. The experimental protocol was approved by the Animal Ethics Committee of Zhejiang University in accordance with "Principles of Laboratory Animal Care and Use in Research" (Ministry of Health, Beijing, China).

Materials

The whole plants of *T. mongolicum* Hand.-Mazz were collected from Bozhou, Anhui province in January 2001. The plant was identified by Prof. Liurong Chen from the College of Pharmaceutical Sciences at Zheijang University. A voucher specimen (C01-Jan-2006-ZJDX) was deposited at the College of Pharmaceutical Sciences of Zhejiang University.

Extraction

The pulverized material of *T. mongolicum* (10.0 kg) was extracted with hot water under reflux for 8 h and concentrated under reduced pressure to produce a brown syrup (1268 g).

Systematic Chromatography

Part of the above-mentioned syrup (400 g) was suspended in H_2O and was subjected to column chromatography over reversed-phase material RP-18 (4.0 kg) and eluted with MeOH-H_2O step gradients. Distilled water (100 L) was utilized to elute the column, and the solution was evaporated under reduced pressure (<70°C) to produce a dark brown extract F I (140.8 g). A 50% MeOH aqueous solution (100 L) was subsequently used to elute the column, and the elution was subsequently evaporated under reduced pressure (<70°C) to afford a brown slurry F II (129.4 g). The column was, after the same procedure as mentioned above, further subjected to a wash with MeOH (100 L) to give a brown extract F III (63.2 g).

Xylene-induced Ear Oedema in Mice

The method described by Vogel was employed (Vogel *et al.*, 1997). Male ICR mice were divided into the following seven groups: group A, normal saline; group B, dexamethasone (2.5 mg/kg); group C, F I-100 (100 mg/kg); group D,

F II-100 (100 mg/kg); group E, F III-100 (100 mg/kg); group F, F II-50 (50 mg/kg); and group G, F II-200 (200 mg/kg).

The vehicle and drugs were administered orally to the groups of mice, respectively, once per day for 3 days. Group A received orally the same volume of normal saline as the vehicle control. 1 h after the last administration of drugs, the ventral and dorsal sides of the right ears of the mice both received 10 µl of xylene by topical application. 1 h after xylene was applied to the right ears, the mice were sacrificed and 9 mm punches were made in the right and left ears by the borer. Each ear disc was weighed, and the differences in weights of the right and left ear discs of mice were recorded as oedema level.

Isolation of Components from the Anti-inflammatory Part F II.

The active fraction (F II, 100 g) was subjected to polyamide column chromatography with H_2O-MeOH mixtures of increasing MeOH percentage. Ten main fractions (F II-1 to F II-10) were obtained based on TLC monitoring. F II-2 (5.2 g) was re-chromatographed over silica gel with a $CHCl_3$-MeOH gradient (1:0→0:1). The major components **1** (19 mg) and **2** (16 mg) were thus isolated. F II-5 (16.0 g) was subjected to column chromatography over silica gel and eluted with $CHCl_3$-MeOH step gradients. Six fractions were collected, components **3** (17 mg) and **4** (25 mg) was purified by Sephadex LH-20 column ($CHCl_3$-MeOH, 1:1) from the fourth fraction. F II-10 fraction (0.5 g) was subjected to column chromatography over silica gel (200–300 mesh, 10 g) and eluted with PE-EtOAc (1:0→0:1). Stigmasterol and β-sitosterol (Chan, 1999) (12 mg) were obtained after recrystallization. F II-8 (10 g) was fractioned over a silica gel column (200–300 mesh, 200 g) using $CHCl_3$-MeOH (1:0→0:1) to give nine crude fractions (B_1–B_9). Fraction B_2 (1.1 g) was further column chromatographed over Sephadex LH-20 (100 g) eluting with MeOH to produce 11 mg of rufescidride (Silva *et al.,* 2004). Fraction B_3 (0.8 g) was subjected to column chromatography on a Sephadex LH-20 (100 g) and eluted with MeOH to give 9 mg of isodonsesquitin A (Ahmad *et al.,* 1992). Fractions B_4 and B_5 were combined (1.8 g) and further fractionated over an RP-C_{18} (100 g) column using MeOH-H_2O gradient (1:10→10:1) to give 17 mg of genkwanin (Bosabalidis *et al.,* 1998), 17 mg of 3',5,7-trihydroxy-4'-methoxyflavanone (Asztemborska *et al.,* 2003), 9 mg of artemitin (Ahmad *et al.,* 1995; Brown *et al.,* 2003), 12 mg of quercetin-3',4',7-trimethyl ether (Matsuda *et al.,* 2002), 24 mg of taraxasteryl acetate (Reynolds *et al.,* 1986), 21 mg of φ-taraxasteryl acetate (Khalilova *et al.,* 2004), 9 mg of lupenol acetate (Ageta *et al.,* 1981), and 45 mg of palmitic acid (Ming *et al.,* 1999), respectively. Furthermore, fractions B_6 and B_7 were combined (1.4 g) and further chromatographed over an RP-C_{18} (100 g) column eluting with MeOH-H_2O (1:9→9:1) to give 26 mg of furulic acid (Wu *et al.*, 2000), 19 mg of gallicin (Hossain *et al.,* 2006), 64 mg of luteolin (Ma *et al.,* 1999), 23 mg of caffeic acid (Chan *et al.,* 1999), 14 mg of *p*-coumaric acid (Blahova *et al.,* 2004), 26 mg of syringic acid (Sadtler Research

Laboratories, 1969), 93 mg of quercetin and 14 mg of isoetin (Gluchoff-Fiasson *et al.*, 1991).

F II-1, F II-3 and F II-4 fractions (25 g) were combined and purified on an RP-C_{18} column (200 g) using MeOH-H_2O (0:1→1:0) to give 15 mg of *p*-hydroxybenzoic acid (Song *et al.*, 2005), 9 mg of 3, 5-dihydroxybenzoic acid (Pouchert *et al.*, 1992), 24 mg of gallic acid (Hossain *et al.*, 2006), 8 mg of isoetin-7-*O*-β-D-glucopyranosyl-2'-*O*-β-D-xyloypyranoside (Gluchoff-Fiasson *et al.*, 1991), 9 mg of quercetin-7-*O*-[β-D-glucopyranosyl(1→6)-β-D-glucopyranoside] (Sun *et al.*, 2003), 6 mg of quercetin-3,7-*O*-β-D-diglucopyranoside (Park *et al.*, 2004), 18 mg of genkwanin-4'-*O*-β-D-lutinoside (Iwashina *et al.*, 1990), 7 mg of hesperidin (Dzhikirba, 1972). Further Sephadex LH-20 chromatography (200 g) of the 70% MeOH elute fraction followed by crystallization resulted in 9 mg of 1-hygroxymethyl-5-hydroxy-phenyl-2-*O*-β-D-glucopyranoside (Chou *et al.*, 1997), 15 mg of esculetin (Sadtler Research Laboratories, 1969), a mixture of luteolin-7-*O*-β-D-glucopyranoside (Ma *et al.*, 1999) and luteolin-7-*O*-β-D-galactopyranoside (46 mg) (Gao *et al.*, 1995) and a mixture composed of four phenolic acids (0.3 g). The mixture (46 mg) of glycosides, luteolin-7-*O*-β-D-glucopyranoside and luteolin-7-*O*-β-D-galactopyranoside was further separated by RP-HPLC [column: Waters Symmetry® RP_{18} 3.9 × 150 mm, 5 μm; mobile phase: MeOH: 0.1% AcOH = 35: 65; flow rate: 0.8 mL/min; detector: 254 nm; column temperature: 30°C] to give pure luteolin-7-*O*-β-D-glucopyranoside (21 mg) and luteolin-7-*O*-β-D-galactopyranoside (8 mg). Finally, the mixture of four phenolic acids (0.3 g) were thoroughly purified by RP-HPLC [column: Waters Symmetry® RP_{18} 3.9 × 150 mm, 5 μm; mobile phase: MeOH: 0.1% AcOH = 32: 68; flow rate: 0.8 mL/min; detector: 254 nm; column temperature: 30°C] to give pure 3-*O*-caffeoylquinic acid (5 mg) (Zhu, 2004), 3,5-di-*O*-caffeoylquinic acid (34 mg) (Reynolds, 1986), 3,4-di-*O*-caffeoylquinic acid (18 mg) (Iwai, 2004), and 4,5-di-*O*-caffeoylquinic acid (11 mg) (Iwai, 2004).

*Isoetin-7-O-β-D-glucopyranosyl-2'-O-α-L-arabinopyranoside (**1**):* $C_{26}H_{28}O_{16}$; Yellow amorphous powder; m.p.: 232–233 °C; UV (MeOH) λ_{max} 258, 357 nm; + $AlCl_3$ 272, 326, 425 nm; + $AlCl_3$/HCl 268, 318, 394 nm; ESI-MS *m/z* 597 $[M+H]^+$; ^1H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 2.

*Isoetin-7-O-β-D-glucopyranosyl-2'-O-α-D-glucopyranoside (**2**):* $C_{27}H_{30}O_{17}$; Yellow amorphous powder; m.p.: 223–224 °C; UV (MeOH) λ_{max} 257, 356 nm; + $AlCl_3$ 270, 326, 426 nm; + $AlCl_3$/HCl 267, 319, 393 nm; ESI-MS *m/z* 627 $[M+H]^+$; ^1H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 2.

*Acid Hydrolyses of **1** and **2**.* Glycosides **1** and **2** were hydrolyzed with HCl (2 M) at 100°C for 60 min. Hydrolysates were extracted with $CHCl_3$ and the organic layer was washed by 10% $NaHCO_3$ and distilled water, dried over Na_2SO_4, and was evaporated under reduced pressure to afford aglycones. Water layer was neutralized with Amberlite IRA-400 (Fluka) and was

evaporated to dryness. This dry residue (10 mg), and the authentic samples of D-glucose as well as L-arabinose were diluted in 1 mL of dry pyridine and were treated with 0.4 mL of hexamethyldisilazane as well as 0.2 mL of trimethylchlorosilane (Aldrich) at 0°C. The mixture was then incubated at 25°C for 5 h before the upper layer of the reaction solution was dropped out for GC analyses. AC-5 capillary column (30 m × Φ0.25 mm), column temperature: 180–250°C, column head pressure: 12 Pa, carrier gas: N_2.

HPLC separation of the sugar mixtures was performed under following conditions: the HPLC system was an Agilent 1100 series equipped with an Iso pump (G1310A), a manual injection (G1328A) with 20 μl of quantitative loop. Agilent Zorbax SB-C_{18} (4.6 × 250 mm, 5 μm) column was used with the temperature at 30°C, flow rate 1.0 mL •min^{-1}, MeCN-H_2O 75:25 as solvent system.

Mongolicumin A (3): $C_{18}H_{10}O_8$; Red amorphous powder; IP ν_{max} 3400, 1721, 1608, 1520 cm^{-1}; UV (MeOH) λ_{max} 196, 226, 276, 317, 373 nm; ESI-MS *m/z* 353 $[M-H]^-$; ^1H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 3.

Mongolicumin B (4). $C_{15}H_{16}O_4$; White crystals; m.p.: 272–274 °C; IP ν_{max} 3276, 1690, 1625, 1605, 990, 868 cm^{-1}; UV (MeOH) λ_{max} 246, 259, 312 nm; ESI-MS *m/z* 261 $[M+H]^+$; ^1H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 3.

AChE Inhibitory Activities. AChE activity was measured spectrophotometrically according to Ellman (Ellman *et al.*, 1961). Briefly, to 3 mL of 0.1 M phosphate buffer at pH 8.0, 20 μl of 75 mm acetylthiocholine iodide (Sigma, U.S.A.), or alternatively acetylthiocholine chloride was added, followed by 100 μl of 0.1 m 5, 5′ dithio-bis (2-nitrobenzoic acid) and 10–100 μl of the samples to be tested. At 25°C, the reaction was started by addition of 20 μl of an AChE (Sigma, U.S.A.) stock soln. (5 units/mL) and the time course of the enzyme activity was monitored for 3 min. Huperzine A (Sigma, U.S.A.) was applied as positive control agent.

RESULTS AND DISCUSSION

The most effective anti-inflammatory fraction was chosen through the bioactivity-guided separation of *T. mongolicum* with application of xylene-induced ear oedema test in mice. The results showed that the most effective fraction, Fr II, inhibited xylene-induced mouse ear oedema dose-dependently at 50, 100 and 200 mg/kg dosages. Thorough phytochemical examination of the pure components that existed in Fr II was subsequently performed. Forty compounds were purified and structurally characterized mainly by means of comprehensive spectral analyses, and four of them were determined as compounds with new structures. These new natural products include two glycosides of a rare flavone isoetin, isoetin-7-*O*-β-D-glucopyranosyl-2'-*O*-α-L-arabinopyranoside (**1**) and isoetin-7-*O*-β-D-glucopyranosyl-2'-*O*-α-D-glucopyranoside (**2**), a new lignan, mongolicumin A (**3**), and a new guaianolide,

mongolicumin B (**4**) (Fig 1). To our knowledge, this is the first report of lignan presented in *Taraxacum* species.

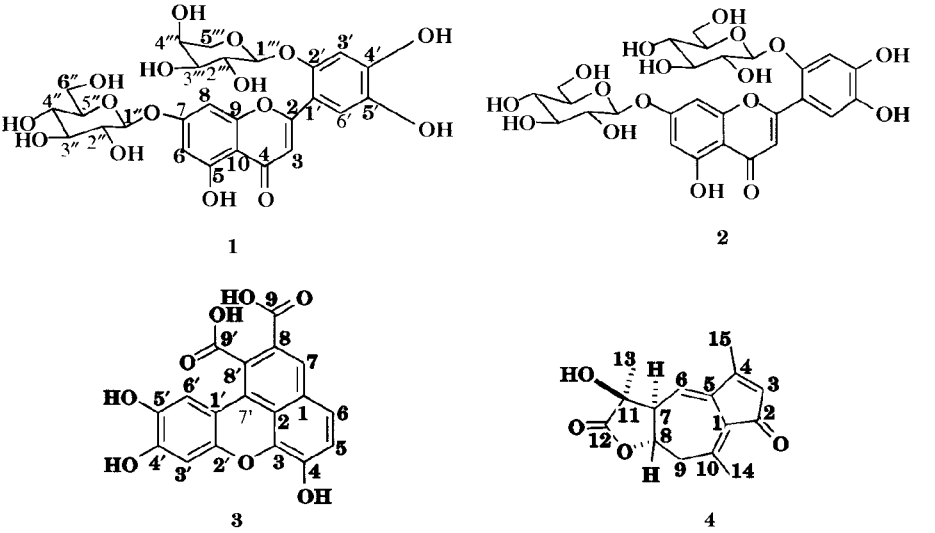


Fig 1. New compounds from *T. mongolicum*

Anti-inflammatory Effects of Fractions againt Xylene-induced Ear Oedema in Mice. The bioassay model of xylene-induced mouse ear is regarded as properly reflecting the early stages of acute inflammation (Wu *et al.*, 2006; Matsuda *et al.*, 1992). In our pilot test, the oral administration of various samples with the same dosage of 100 mg/kg suppressed xylene-induced ear oedema in mice with different efficacies. As shown in Table 1, the oedema inhibitory rates of F I-100, F II-100, and F III-100 were 28.7%, 50.1%, and 33.0%, respectively; whereas the reference drug, dexamethasone (2.5 mg/kg) produced the inhibitory rate of 74.8% (Table 1). F II was further investigated with different dosages, and the dose-dependent manner of inhibition was detected (Fig 2).

Table 1. Anti-inflammatory effects of fractionated parts from water extract of *T. mongolicum* and dexamethasone (DEX) on xylene-induced ear oedema in mice

Group	Dose (mg/kg, p.o.)	Oedema degree (mg)[a,b]	Inhibition rate (%)
Vehicle	-	23.77 ± 2.65	-
DEX	2.5	5.99 ± 1.39 ***	74.8
F I-100	100	16.95 ± 1.97 ***	28.7
F II-100	100	11.86 ± 1.84 ***	50.1
F III-100	100	15.93 ± 1.91 ***	33.0

[a]Values are expressed as mean ± S.D. of differences in weights between right and left ears of mice (n=10).

[b]*** $p<0.001$ compared with vehicle control group (one way ANOVA, Dunnett's t-test as the post hoc test).

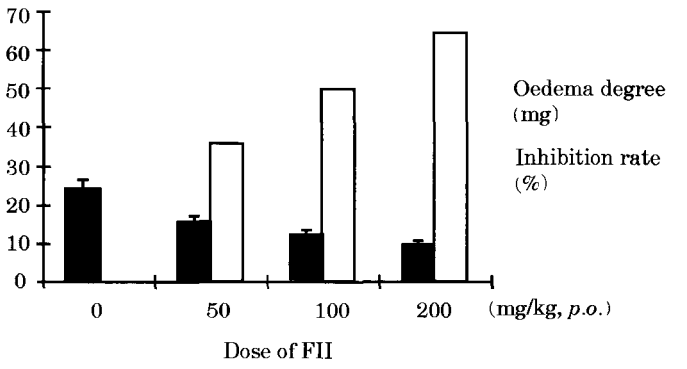


Fig 2. Anti-inflammatory effects of various doses of FII from water extract of *T. mongolicum*

Structural Elucidation of the Isolated Compounds.

Compound **1** was obtained as yellow amorphous powder. The ESI-MS of **1** showed a quasimolecular ion $[M+H]^+$ at *m/z* 597, indicating a molecular formula of $C_{26}H_{28}O_{16}$. Detailed analyses of the ^1H and ^{13}C NMR (Table 2) as well as DEPT spectra allowed a full characterization of the structure of **1**. The UV absorptions at 258 and 357 nm were typical of a flavonoid. Based on the experimental UV shift reagents as described (Mabry *et al.,* 1970), it was inferred that **1** was a flavonoid with a substituted hydroxyl group at C-5 and adjacent hydroxyl groups in ring B. Furthermore, complete acid hydrolysis and HPLC separation of both sugar and aglycone fractions enabled the identification of D-glucose and L-arabinose as the glycosidic parts of **1**, which was evidenced by comparison with trade samples run on GC and the acid hydrolysate. The aglycone, isoetin (5,7,2',4',5'-pentahydroxyflavone) could be identified by UV and ^1H NMR data with literature (Voirin *et al.,* 1975). Moreover, the ^1H NMR spectrum of **1** showed a pair of doublets at δ 6.44 and δ 6.72 for H-6 and H-8, respectively, as well as three other singlets at δ 7.31, δ 7.16 and δ 6.77 attributable to aromatic rings. In addition, two doublets at δ 5.08 (*J*=8.0 Hz) and δ 4.87 (*J*=6.0 Hz) were assigned to be the anomeric protons of the sugar moieties, which correlated respectively with the resonances at δ 100.0 and δ 101.8 in the HSQC spectra. The other glycosidic protons, appearing as broad multiplets, could be observed in a range of δ 3.2-3.6. Furthermore, the downfield shifts of H-6, H-8 and H-3' of **1**, when compared with the aglycone isoetin after glycosylation suggested these resonances came from protons *ortho* to the glycosylated hydroxyls, which pointed out the presence of the sugar residues at C-7 and C-2' of **1**, respectively. This deduction was further evidenced by the upfiled-shifted carbon resonances of **1** at C-7 (δ 163.1) and C-2' (δ 150.4) (Table 2) when compared with those of isoetin. Based on the coupling patterns and chemical

shifts of sugar resonances, the presences of a β-configurated glucose and an α-configurated arabinose could be evidenced (Markham *et al.*, 1987). Unequivocal assignments were in agreement with the results of the 2D-NMR spectra. The HMBC experiment showed diagnostic correlations between the combinations of δ5.08 (H-1″) and δ 163.1 (C-7) as well as δ 4.87 (H-1‴) and δ 150.4 (C-2') (Fig 2), which indicated that the β-glucose was located at the O-7 position while the α-arabinose was connected with the O-2' atom. Therefore, compound **1** could be identified as isoetin-7-*O*-β-D-glucopyranosyl-2'-*O*-α-L-arabinopyranoside.

The molecular formula of compound **2** was assigned as $C_{27}H_{30}O_{17}$ from its ESI-MS, ^1H and ^{13}C NMR data (Table 2). A comparison of the NMR spectral data of **2** with that of **1** indicated the close similarities of their aglycon parts, while the sugar residues of **2** and **1** were different. Acid hydrolysis of **2** was performed and the sugar was determined to be D-glucose based on GC comparison with the trade sample, while the aglycone was identified as isoetin on the basis of its NMR spectral data (Voirin *et al.*, 1975). The UV spectral data of **2** with diagnostic shift reagents (Mabry *et al.*, 1970) also indicated the presence of a free 5-OH and *ortho*-dihydroxy moiety at B ring. Furthermore, the ^1H NMR spectrum of **2** (Table 2) revealed the presence of two anomeric protons at δ 5.08 (d, *J*=8.0 Hz) and δ 4.91 (brs). A comparison of the ^1H and ^{13}C NMR data with those reported in literature allowed identification of the stereochemistry of the sugar substituents, one of which possessed a β-configuration and the other an α-configuration (Markham *et al.*, 1987). Meanwhile, the relative positions of the two glucopyranose units could be established from the diagnostic HMBC correlations between δ 5.08 (H-1″) and δ 163.0 (C-7), and between δ 4.91 (H-1‴) and δ 150.4 (C-2″). The structure of compound **2** was therefore elucidated as isoetin-7-*O*-β-D-glucopyranosyl-2'-*O*-α-D-glucopyranoside.

Mongolicumin A (**3**) was isolated as a red amorphous powder. The molecular formula $C_{18}H_{10}O_8$ for **3** was deduced by the analyses of its ESI-MS, ^1H and ^{13}C NMR spectra (Table 3). The typical IR absorption bands at 3400 and 1721 cm^{-1} suggested the presence of free carboxylic acid functionalities in the molecule, which was in accordance with its TLC characteristic of a shuttle-shape spot, as well as its positive reaction with bromocresol green (blue colour). Careful analysis of the NMR spectra led to the conclusion that **3** consisted of 18 sp^2 carbons, including five aromatic methines, five oxygen-bearing aromatic carbons, and two carboxyl groups. In the ^1H NMR spectrum of **3**, three singlets appeared at δ 8.05 (s, H-7), δ 7.40 (s, H-6'), and δ 6.62 (s, H-3'); furthermore, two *ortho*-coupled doublet resonances at δ7.46 (d, *J* = 8.8 Hz, H-6) and δ7.25 (d, *J*=8.8 Hz, H-5) could be assigned to the aromatic protons. No aliphatic protons were present in the spectral data of **3**. This highly conjugated aromatic features suggested the presence of an

Table 2. ^{1}H and ^{13}C NMR data of compounds **1** and **2** in DMSO-d_6

	1(mult, *J* in Hz)		**2**[a]	
Position	**δ_H (*J* in Hz)**	**δ_C, mult**	**δ_H (*J* in Hz)**	**δ_C, mult**
2		161.8, qC		161.8, qC
3	7.16 s	108.9, qC	7.11 s	108.9, qC
4		182.4, qC		182.2, qC
5		161.4, qC		161.3, qC
6	6.44 d (2.0)	99.5, CH	6.45 d (2.0)	99.5, qC
7		163.1, qC		163.0, qC
8	6.72 d (2.0)	94.7, CH	6.73 d (2.0)	94.7, qC
9		157.9, qC		157.2, qC
10	105.4, qC			105.4, qC
1′		110.6, qC		110.4, qC
2′		150.4, qC		150.4, qC
3′	6.77 s	104.4, CH	6.80 (1H, s)	104.7, qC
4′		150.4, qC		150.4, qC
5′		140.6, qC		140.5, qC
6′	7.31 s	114.8, CH	7.31 s	114.7, qC
1″	5.08 d (8.0)	100.0, CH	5.08 d (8.0)	100.1, qC
2″		73.3, CH		73.3, qC
3″		76.6, CH		76.5, qC
4″	3.20–3.60 m	69.7, CH	3.20–3.60 m	69.7, qC
5″		77.3, CH		77.3, qC
6″		60.8, CH_2		60.8, qC
1‴	4.87 d (6.0)	101.8, CH	4.91 brs	101.3, qC
2‴		72.5, CH		73.5, qC
3‴	3.20–3.60 m	70.7, CH		76.9, qC
4‴		67.3, CH	3.20–3.60 m	69.7, qC
5‴		65.4, CH_2		77.3, qC
6‴	—	—		60.8, CH_2

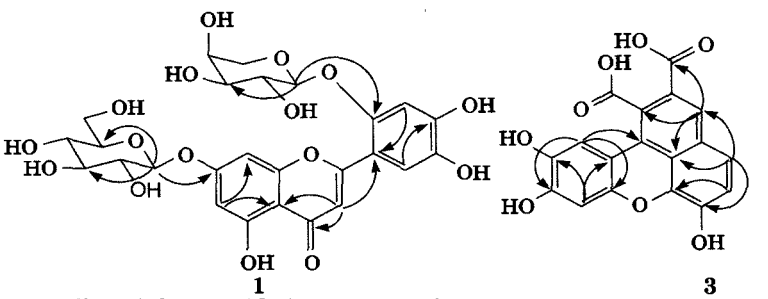
[a] Assignments were established by DEPT, COSY, HMQC and HMBC.

arylnaphthalene lignan, rufescidride (Tanaka *et al.*, 1997). Comparisons of the NMR data and the molecular weight of **3** with rufescidride suggested that **3** was a hydrated analogue of rufescidride, an arylnaphthalene lignan isolated from *Cordia rufescens* (Silva *et al.*, 2004). HSQC and HMBC experiments on **3** were also performed, and all of the 2D NMR data were in agreement with the assignment of **3** (Fig 3). On the basis of these results, it was concluded that **3** has the structure as shown and was named as mongolicumin A. An HPLC check of the extract revealed that compound **3** was a natural substance existing in the plant material.

Table 3. ^{1}H and ^{13}C NMR data of compounds 3 and 4 in DMSO-d_6

Position	3[a]		position	4[a]	
	δ_H (J in Hz)	δ_C, mult		δ_H (J in Hz)	δ_C, mult
1		125.8, qC	1		126.8, qC
2		123.3, qC	2		194.6, qC
3		136.7, qC	3	6.16 s	131.2, CH
4		141.8, qC	4		146.5, qC
5	7.25 d (8.8)	120.0, CH	5		162.9, qC
6	7.46 d (8.8)	121.4, CH	6	6.04 d (2.4)	118.3, CH
7	8.05 s	128.4, CH	7	2.99 dd (9.6, 2.4)	52.8, CH
8		126.2, qC	8	4.39 ddd (9.6, 9.6, 4.8)	74.9, CH
9		167.8, qC	9	3.20 d (11.2) 3.01 d (11.2)	43.9, CH2
1'		109.7, qC	10		139.5, qC
2'		146.4, qC	11		74.8, qC
3'	6.62 s	103.9, CH	12		178.4, qC
4'		148.6, qC	13	1.31 s	18.8, CH3
5'		142.1, qC	14	2.37 s	21.1, CH3
6'	7.40 s	112.5, CH	15	2.19 s	14.1, CH3
7'		123.0, qC			
8'		122.3, qC			
9'		171.6, qC			

[a]Assignments were established by DEPT, COSY, HMQC and HMBC.



1 3

Fig 3. Selected HMBC correlations for compounds **1** and **3** (H → C)

The ^{1}H and ^{13}C NMR spectral data of **4** showed similarities with those of taraxacin, a guainolide from *T. wallichi* (Ahmad *et al.,* 2000). However, it was observed that a tertiary-hydroxyl group at C-11 instead of the double bond between C-7 and C-11 of taraxacin was present in **4**, which indicated the occurrence of hydration at the double bond (Table 3). This made the Me-13 singlet of taraxacin at δ1.98 shift upfield to δ1.31 in the case of **4**. Furthermore, an additional double doublet due to the saturated H-7 at δ 2.99 (J=9.6, 2.4 Hz) was observed in the ^{1}H NMR spectrum of **4**. In respect of the biogenetic consideration of the guaiane-type sesquiterpene, C-11 usually adopted a β-orientation. Furthermore, the NOESY experiment of **4** exhibited significant NOEs between H-7 and Me-13, which indicated the α-orientation of Me-13. Moreover, the diagnostic large coupling constant between H-8 and H-7 ($J_{7,8}$=9.6 Hz) suggested the *trans* position of these two methines, therefore, the β-orientation of H-8 could be assigned. Thus, the stereochemistry of compound **4** was determined as shown and was named as mongolicumin B.

The other previously reported compounds were characterized based on the comparisons of their physical, mass and NMR spectral data with the literature. Some of them were directly compared with the authentic samples.

AChE Inhibitory Effects of Compounds 1–4. Huperzine A is a natural AChE inhibitor with great potential to be developed as an efficient agent for the treatment of AD. It was therefore adopted as a positive control for the bioassay in this presented study. Among the test compounds, mogulicumin A was found to possess an exciting inhibitory effect on AChE with an IC_{50} value of 8.47 μm, which is comparable with that of huperzine A (IC_{50}=8.31 μm). However, more detailed investigation are still needed to further explore the relationship between the anti-inflammatory activity and AChE inhibition. Pure samples of sufficient quantity for *in vivo* assay with xylene-induced ear oedema test in mice should also be prepared.

ACKNOWLEDGEMENTS

This work was financially supported by the Key Program of the Zhejiang Bereau of Traditional Chinese Medicine (04Z001), Key Project of Zhejiang Province (2005C13019) and the intramural foundation of Wenzhou Medical College. The authors are grateful to Dr. F. Guéritte (Gif-sur-Yvette), Prof. H.D. Sun (KIB, CAS) and Prof. L.H. Zhang (Beijing University) for the useful discussions.

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19

Inhibition of Hyaluronidase by Essential Oils and Other Natural Fragrant Extracts

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ABSTRACT

Hyaluronidase (EC 3.2.1.35) degrades hyaluronic acid, a major constituent of the skin which retains moisture. The influence of 20 essential oils and natural plant extracts were tested on its activity of hyaluronidase. Among them, Juniper berry absolute, Rosemary extract and Mimosa absolute had an inhibitory activity significantly higher than the benchmark, apigenin. Specifically, Juniper berry absolute was the most potent inhibitor of hyaluronidase. Indeed we have found its IC_{50} to be 3 times lower than apigenin. The use of natural fragrant extract in cosmetics is discussed.

Key words : Hyaluronidase, acid hyaluronic, inhibitors, essential oils, absolutes

INTRODUCTION

Hyaluronic acid (HA) is a major constituent of the extracellular matrix. It is copiously found in the dermis (0.5 mg/g wet tissue) and in the epidermis (0.1 mg/g wet tissue). It was isolated for the first time in 1934 from the vitreous humor of bovine eyes (Meyer *et al.*, 1934).

HA is a linear polymer of a disaccharide composed of *N*-acetylglucosamine and glucuronic acid linked together through alternating β-1,4 and β-1,3 glycosidic bonds.

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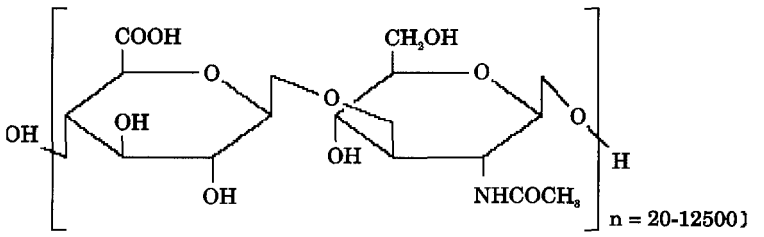

Fig 1. Structure of hyaluronic acid

This structure (Fig 1) gives to the molecule a high water-binding capacity and high viscoelasticity.

However, HA is decreasing in quantity and quality with age which contributes to give the skin a dry and wrinkled aspect. Given that HA retains moisture, it is used in cosmetics for skin hydration but an other approach would be to inhibit hyaluronidase, the enzyme degrading HA.

It has been published that some flavones and flavonoids were hyaluronidase inhibitors (Kuppusamy *et al.*, 1990). The purpose of this study was therefore to screen a number of essential oils and extracts of aromatic plants used in perfumery, to evaluate their capacity to inhibit *in vitro* the enzyme hyaluronidase.

MATERIALS AND METHODS

Reagents

Hyaluronidase type V from sheep testes (EC 3.2.1.35), Hyaluronic acid potassium from human ombelical cord, Ehrlich's reagent (4-dimethylaminobenzaldehyde) and Apigenin crystalline were purchased from Sigma-Aldrich (France).

Extracts from aromatic plants were either from our own production or from producers with certificates of authenticity.

Instruments

Thermomixercompact Eppendorf. Spectrophotometer UV/Visible Lambda 25 (Perkin-Elmer instruments). Data processing by UV Winlab software.

Principle

Hyaluronidase activity was determined by a modified colorimetric assay reported in detail by Sunnhild Salmen (2003) based on the method of Gacesa *et al.* (1981) and Reissig *et al.* (1955). The colorimetric assay (Reissig assay, Morgan- Elson assay) is based on the reaction of the *N*-acetyl-D-glucosamine (GlucNAc) at the reducing ends of hyaluronan and its fragments with *p*-dimethylaminobenzaldehyde resulting in a red coloured product. As shown

in Fig 2, the postulated main product of the degradation of hyaluronic acid by the bovine testicular hyaluronidase is a tetrasaccharide with *N*-acetyl-D-glucosamine at the reducing end. The chromogens I and II are formed under alkaline conditions (100°C, pH 9) of the Morgan-Elson reaction. The chromogen III, which is formed by elimination of water under acidic conditions (conc. HCl/glacial acetic acid) react in the final step with p-dimethylaminobenzaldehyde (Ehrlich's reagent) to give the instable red – coloured product, which can be photometrically measured at 586 nm.

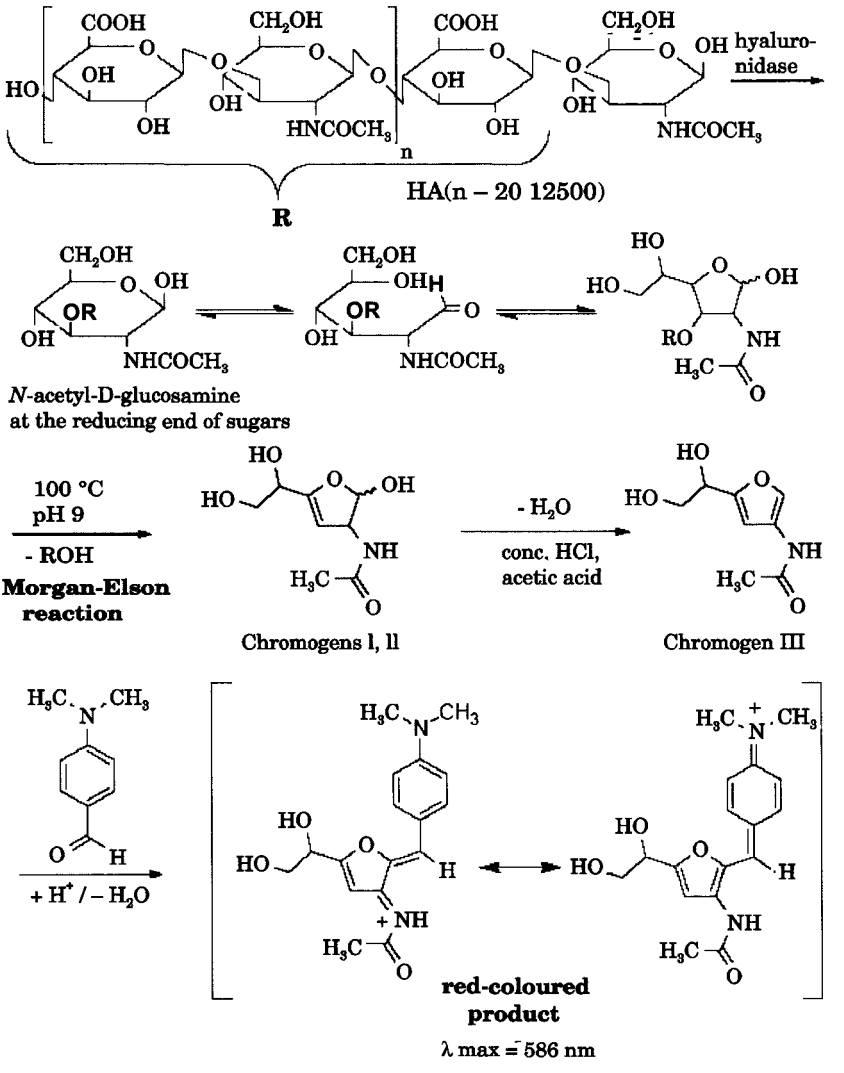

Fig 2. Mechanism of the Morgan-Elson reaction - reaction of *N*-acetyl- D-glucosamine with *p*-dimethylaminobenzaldehyde resulting in the red coloured product [2]

Essential oils, absolutes and extracts of aromatic plants are not soluble in water but in organic solvents. DMSO was selected because of its low hyaluronidase inhibiting capacity and its high capacity of solubilization of the potentially active products. Each test compound and the control were run in duplicate at 3 concentrations and the results averaged.

Enzymatic Assay

The test compounds (100 ppm-500 ppm) dissolved in DMSO (15 µl), were incubated 4 h at 37°C with agitation (Thermomixercompact Eppendorf) in an incubation mixture containing 85 µl of citrate-phosphate buffer pH 7.4, 80 µl hyaluronic acid solution (4 mg/mL water) and 30 µl enzyme solution (5 u/30 µl buffer).

The enzyme reaction was stopped by addition of 80µl of alkaline borate solution and subsequent heating for 4 min in a boiling water bath. After cooling on ice for 2 min, 1 mL of 4-dimethylaminobenzaldehyde solution (1 g dissolved in 1.25 mL concentrated hydrochloric acid and 3.75 mL glacial acetic acid; the solution was diluted with 4 volumes of glacial acetic acid immediately before use) was added and the mixture was incubated at room temperature for 10 min before the absorbance of the coloured product was photometrically measured at 590 nm.

Calculation

Enzyme activity was monitored by the formation of the red coloured product measured at 590 nm. The effect of the inhibitors on the enzyme activity was calculated according to the equation:

$$A\% = (B - C)/(D - E)$$

A%: calculated enzyme activity

B: absorbance of the incubation mixture containing inhibitor

C: absorbance of the incubation mixture containing inhibitor in absence of the enzyme (enzyme solution replaced with buffer)

D: absorbance of the incubation mixture in absence of the inhibitor (inhibitor solution replaced with DMSO)

E: absorbance of the incubation mixture in absence of both enzyme and inhibitor

(Enzyme solution replaced with buffer, inhibitor solution replaced with DMSO)

The Morgan-Elson assay is a useful method for the determination of hyaluronidase activity in the presence of inhibitors. However, not all compounds can be examined with this assay. For instance, this assay is not suitable for the investigation of furan ring derivatives since the furan ring reacts with the Ehrlich´s reagent (*p*-dimethylaminobenzaldehyde) to a coloured product which is also detectable at 590 nm and falsifies the quantification of the red coloured product of the Morgan-Elson reaction.

RESULTS

The concentration that gave 50% inhibition (IC_{50}) of the enzyme was then calculated and compared to a benchmark. The representative and widely accepted hyaluronidase inhibitors are apigenin and kaempferol. Apigenin was chosen as the benchmark in this study. Taken together the published results show that apigenin is suitable to inhibit hyaluronidase with a published IC_{50} in the range of 1 millimolar (~500 ppm).

With the above described protocol, the IC_{50} of apigenin was found to be 120 ± 10 ppm.

A scale of relative *in vitro* activity was defined as follows:

++++	$30 < IC_{50} \leq 100$ ppm
+++	$100 < IC_{50} \leq 200$ ppm
++	$200 < IC_{50} < 500$ ppm
+	$IC_{50} = 500$ ppm
-	Inactive above 500 ppm

Twenty essential oils, absolutes and a non fragrant rosemary extract were evaluated and the results are shown in Table 1.

Table 1. *In vitro* inhibition of hyaluronidase by aromatic extracts (by decreasing order of activity)

Extracts	***In vitro* activity**
Apigenin (reference)	+++
Juniperus communis	++++
Rosemary extract (*Rosmarinus officinalis*)	++++
Mimosa absolute (*Acacia decurrens*)	++++
Cypress absolute (*Cupressus sempervirens*)	+++
Elder tree absolute (*Sambucus nigra*)	+++
Lovage roots essential oil (*Levisticum officinalis*)	++
Copahu essential oil (*Copaiefera officinalis*)	++
Thym absolute (*Thymus zygis*)	++
Costus ess (*Saussurea lappa*)	++
Lovage roots absolute (*Levisticum officinalis*)	++
Coriander seeds essential oil (*Coriandrum sativum*)	++
Blue Camomile essential oil (*Matricaria recutital*)	+
Cananga essential oil (*Cananga odorata* var *macrophylla*)	+
Boletus absolute (*Boletus edulis*)	+
Karo Karounde absolute (*Leptactinia senegambica*)	+
Carnation absolute (*Dianthus caryophyllus*)	+
Rose absolute (*Rosa damascena*)	+
Tomato absolute (*Solanum lycopersicum*)	+
Gentian absolute (*Gentiana lutea*)	-
Popular buds absolute (*Populus nigra*)	-

DISCUSSION

Of the 20 products evaluated, 12 were absolutes, 7 were essential oils and one, a non odorous extract of rosemary.

Only a few essential oils showed inhibitory activity on the enzyme activity and at a relatively high concentration (IC_{50} > 200 ppm). On the other hand, 12 of the 13 extracts were active and 5 were as or more active than apigenin itself. More precisely it is interesting to note that Juniper berry absolute, rosemary extract and mimosa absolute had an inhibitory activity significantly higher than the benchmark. Among these extracts, Juniper berry absolute was Rosemary extract was the most potent inhibitor of hyaluronidase. Indeed we have found its IC_{50} to be 3 times lower than apigenin.

The volatile fragrant components of absolutes and resinoide can be identified by GC/MS but they represent only a fraction of the total extract. Unfortunately, the other components of higher molecular weights have been very seldom determined quantitatively and even qualitatively. For these reasons, it is difficult to explain the difference in inhibitory activity of the various absolutes that we have tested. Some general comments can still be made in view of the fact that the essential oils tested were in their majority totally inactive. Essential oils contain only low molecular weight volatile components (terpenes, sesquiterpenes, alcohols, aldehydes, etc.) whereas plant extracts like absolutes in addition to these volatiles contain higher molecular weight components of C_{20} and higher. From these experimental results, it seems more likely that good inhibitors of hyaluronidase are to be found in molecules of C_{20} and higher rather than in the "lighter" molecules found in essential oils (Baylac & Racine, 2004). This hypothesis is conforted by the difference between the exceptionally strong inhibitory activity of the Juniper berry absolute and the complete lack of activity of the essential oil corresponding on the hyaluronidase. In conclusion, this study shows that extracts of aromatic plants primarily used for perfumery purpose can also be used as actives for cosmetics. It opens the possibility to create fragances having the dual fonction of perfuming a cosmetic product and to contribute its activity.

ACKNOWLEDGEMENTS

We are grateful to Robertet management for allowing the publication of this work. Thanks are due to Mr. Ph. Racine, H. Brevard and Mrs. S. Baylac for helpful discussion and criticisms.

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Chemical, Biological and Pharmacological Studies of the Essential Oil of *Croton nepetaefolius* Baill

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ABSTRACT

The Brazilian flora of the semi-arid contains an impressive variety of vegetable species, which contains therapeutic compounds of interest for the pharmaceutical biotechnology. Inserted in this biodiversity, the genus Croton (Euphorbiaceae) contains a complex biochemical arsenal originated from the secondary metabolism of plant carbohydrates, composed of several constituents, specially monoterpene and sesquiterpene hydrocarbons and phenylpropanoids. C. nepetaefolius, largely used by local population for the relief of gastrointestinal pain, is an interesting species from the caatinga ecosystem which produces an essential oil with low toxicity, containing bicyclogermacrene, methyl-eugenol and 1,8 cineole as main components. The circadian analysis of the essential oil composition of wild plants in field shows significant variations that reflect important ecological functions. More than ten years of work with this oil created a significant accumulation of knowledge regarding their pharmacological properties. It is discussed several biological properties, such as antimicrobial and larvicidal activities and pharmacological effects at pre-clinical level, like antinociceptive, antihypertensive, myorelaxant and antispasmodic activity, besides blockade of neural excitability. All those studies support the popular use of the plant.

Key words : *Croton nepetaefolius*, essential oil, methyl-eugenol, 1,8-cineole, Brazilian caatinga flora, biological activities, pharmacological activities, pre-clinical assays

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INTRODUCTION

Brazil plays an important role as a country having a big biodiversity, with the highest vegetable genetic diversity of the world. It has approximately 55,000 known species in an estimated total between 550,000 and many of them present biological activity (Nodari & Guerra, 1999).

The ecosystem caatinga constitutes almost 1 million km^2 in the Northeast of Brazil. Its characteristic xerophylic vegetation is affected by the long and irregular drought, high temperatures and high ultraviolet radiation. The use of self-medication with medicinal plants is largely employed in the area due to families that cannot afford to buy drugs for their health problem medications (Desmarchelier *et al.*, 1999).

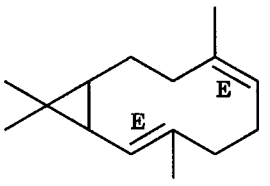
The Brazilian caatinga, or northeastern semi-arid region, is inhabited by a great diversity of plants that produce compounds with potential pharmaceutical applications. *Croton* is a large genus of Euphorbiaceae, comprising around 1,300 species of trees, shrubs and herbs distributed in tropical and subtropical regions of both hemispheres. Recently, an elegant review performed by Salatino *et al.* (2007) revealed that *Croton* species and their substances are among the highly studied themes in chemistry of natural products, pharmacology and ethnopharmacology, and new data are rapidly accumulating.

Widespread in the Brazilian northeastern flora, mainly in the caatinga region, *Croton nepetaefolius* species, popularly named marmeleiro sabiá and marmeleiro vermelho, is used as infusates and teas for several purposes in folk medicine, as stomachic, carminative and to relief intestinal pains (Abdon *et al.*, 2002; Craveiro *et al.*, 1981; Craveiro *et al.*, 1980; Leal-Cardoso & Fonteles, 1999). Various pharmacological actions of the essential oil of *C. nepetaefolius* (EOCN) have been demonstrated, such as antispasmodic, antimicrobial, antinociceptive, antihypertensive activities and the depressive effect on neural excitability (Magalhães *et al.*, 2004; Oliveira *et al.*, 2001; Lahlou *et al.*, 2000; Lima-Accioly *et al.*, 2006). In addition, various biological actions of the EOCN have also been demonstrated, like the antifungal (animal strains), and *in vitro* larvicidal activity against *Aedes aegypti* (Fontenelle *et al.*, 2008; Morais *et al.*, 2006a).

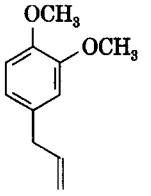
Essential oils are volatile organic compounds that are responsible for the fragrance of many plants. Essential oils, ethereal oils or essences are the names given to the complex mixtures of volatile substances, lipophylic, usually odorific and liquid, obtained from many parts of plants like flowers, leaves, fruits, seeds, roots, barks, rhizomes and stems. These oils are obtained from plant material mainly through steam distillation. Their constituents vary from terpenic hydrocarbons, terpenic and simple alcohols, aldehydes, ketones, phenols, esters, ethers, oxides, peroxides, furans, organic acids and sulfur compounds. These compounds are originated from the secondary metabolism of plant carbohydrates (Simões *et al*, 1999).

The main secondary metabolites present in the EOCN are monoterpene and sesquiterpene hidrocarbons and phenylpropanoids (Morais *et al.*, 2006b), such as 1,8-cineole, methyl-eugenol, and bicyclogermacrene.

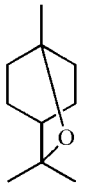
Bicyclogemacrene



Methyl-eugenol



1,8-cineole



CHEMICAL AND ECOLOGICAL CONSIDERATIONS OF *C. NEPETAEFOLIUS*

The chemical analysis of the bitch leaf EOCN was carried out with several specimens collected in Cocalzinho, a village that belongs to Vicosa muncipality of the state of Cear'*a*, Brazil. Viçosa is situated in Northwestern region of Cear'*a*, at Ibiapaba mountain, at 03° 33' 43" South latitude and 41° 05' 31" West longitude, 740 m height. Cocalzinho local temperatures oscillate between 22 and 34°C with annual pluviosity of 1349 mm, with the period of maximum precipitation occurring between the months of January and April, characterizing a climate change from sub-humid hot tropical to semi-arid hot tropical. The blooming begins, usually, in February and finishes in May, when the rains begin to be scarce. From August to November, coinciding with the period when the precipitation is smaller than 1% of the annual total, the plants lose their leaves (Lins, 1978).

To perform a circadian study of the chemical composition of EOCN, samples of wild plant leaves were collected in five different daytimes at 5:00, 9:00, 13:00, and 21:00 h. The samplings collection period ranged from 2004-2006. The results of the analysis of EOCN extracted from samples, presented on Table 1, showed an essential oil constituted by a variety of monoterpens, sesquiterpenes and phenylpropanoids, in agreement with the ecological plant needs, produced by a complex interaction of isomerase enzymes. Interestingly, the percentage of several compounds in EOCN composition fluctuates from sizable values in specific hours to zero at other hours of the day.

Table 1. Variation of the percentage yield of the main constituents of *C. nepetaefolius* collected in 4 different daytimes during three different years

Constituents[a]	IK[b]	06:00[c]	09:00	13:00	21:00
α-pinene	939	0.62 - 4.93	0.79 - 4.40	0.29 - 5.90	1.72 - 3.65
Camphene	954	0.0 - 1.35	0.2 - 2.05	0.55 - 2.24	1.54 - 1.72
β-pinene	979	0.31 - 4.05	0.39 - 1.75	0.86 - 2.78	1.32 - 1.93

Table 1. *Contd.*

Table 1. *Contd.*

Constituents[a]	IK[b]	06:00[c]	09:00	13:00	21:00
Sabinene	975	1.89 - 5.88	2.17 - 3.01	1.18 - 3.93	1.16 - 4.18
1.8-cineole	1031	0.83 - 24.98	1.03 - 14.26	5.35 - 14.5	13.22 - 15.4
Camphor	1146	0.0 - 3.53	1.13 - 5.48	1.88 - 5.94	0.0 - 4.56
α-terpineol	1189	2.58 - 2.80	2.43 - 2.84	0.75 - 3.54	2.58 - 3.02
ortho-vanilin	1298	0.0 - 4.45	0.0 - 3.63	0.0 - 5.04	0.0 - 3.61
α-cubebene	1351	1.18 - 1.27	0.0 - 1.49	1.15 - 1.43	0.0 - 1.13
β-elemene	1338	1.58 - 6.43	0.63 - 5.82	0.0 - 7.14	0.0 - 0.59
α-copaene	1377	0.4 - 5.0	0.3 - 4.5	0.5 - 5.4	0.5 - 3.5
β-cubebene	1388	1.3 - 4.37	0.95 - 7.85	3.07 - 4.15	0.0 - 3.69
β-elemene	1391	0.0 - 3.06	0.0 - 2.83	2.43 - 5.41	0.0 - 2.97
β-sesquiphelandrene	1524	0.0	0.34 - 1.98	0.57 - 1.2	0.0 - 0.4
Methyl-eugenol	1404	0.5 - 30.7	7.51 - 35.0	14.27 - 25.5	6.16 - 44.6
β-caryophyllene	1419	1.09 - 22.48	5.7 - 24.24	3.23 - 22.97	6.7 - 36.9
α-Z-bergamotene	1413	1.70 - 4.23	1.22 - 2.23	1.00 - 4.56	0.0 - 3.71
α-E-bergamotene	1435	0.0 - 3.22	1.12 - 6.33	0.0 - 9.09	0.0 - 1.69
Aromadendrene	1441	0.84 - 1.54	0.86 - 1.39	1.57 - 3.35	1.29 - 2.02
α-humulene	1455	0.0 - 2.66	1.15 - 2.82	0.88 - 5.90	0.0 - 3.57
Bicyclogermacrene	1500	5.03 - 15.40	2.45 - 10.74	7.90 - 16.17	1.19 - 7.35
Δ-cadinene	1523	0.35 - 4.44	0.0 - 3.96	0.4 - 4.08	0.0 - 4.6
Elemicin	1557	0.82 - 4.45	0.9 - 6.62	0.0 - 4.2	0.0 - 6.84
Cis-isoelemicin	1570	0.53 - 2.1	0.94 - 4.93	0.0 - 0.94	0.0 - 3.99
Espathulenol	1578	1.70 - 2.31	1.02 - 4.05	0.9 - 4.28	0.0 - 3.77
Caryophyllene oxide	1583	0.0 - 2.99	0.0 - 4.79	0.0 - 4.37	2.10 - 10.15

[a]The constituents are listed in order of elution in a apolar column of gas chromatograph/ mass spectrometer system.
[b]Time of retention in the gas chromatograph column
[c]Time of collection along the day
0.0 = Component was not detected

BIOLOGICAL ACTIVITIES OF *C. NEPETAEFOLIUS* ESSENTIAL OIL

Antifungal Activities

Many plants from Brazilian biomes have been used as natural medicines by local population for treatment of many diseases, including mycosis (Cruz *et al.*, 2007) and several plants have shown antimicrobial properties (Bertini *et al.*, 2005; Botelho *et al.*, 2007). Considering that the caatinga scrublands is a

biome with extreme diversity of medicinal plants, more phytochemical and pharmacological research is needed to establish the potential use of these plants as alternative treatments for dermatophitoses.

The antifungal activity of ECON was evaluated against *Candida albicans*, *Candida tropicalis* and *Microsporum canis* by the agar-well diffusion method and the minimum inhibitory concentration (MIC) by the broth microdilution method. Essential oil of this *Croton* species demonstrated better activity against *M. canis*. The acute administration of the essential oil up to 3 g/kg by the oral route to mice was devoid of overt toxicity. Due to its antifungal activity and low toxicity, the essential oils of studied *Croton* species is promising sources for new phytotherapeutic agents to treat dermatophytosis (Fontenelle *et al.*, 2008).

Larvicidal Activity against Aedes aegypti

Aedes aegypti L. is the major vector of dengue fever, an endemic disease in Brazil. In an effort to find effective and affordable ways of controlling this mosquito, the larvicidal activities of essential oils from several *Croton* species widely found in the Northeast of Brazil were analyzed and LC_{50} were calculated by linear regression. The main components for a sample of EOCN from plant collected at Fortaleza (LC_{50} 84 ppm) were methyleugenol and α-copaene. This LC_{50} of EOCN revealed a better action when compared to larvicidal LC_{50} of samples of other essential oils, such as essential oil of *C. argyrophyloides* (LC_{50} 102 ppm; main constituents: α-pinene and β-pinene) and essential oil of *C. sonderianus* (LC_{50} 104 ppm; main constituents: α-pinene, β-phelandrene and trans-caryophyllene) (Morais *et al.*, 2006b).

Supavarn *et al.* (1974) tested 36 vegetable extracts on *A. aegypti* larvae and found that 11.1% were capable of producing 50% mortality (LC_{50}) at a concentration of 500 ppm but only 2.8% produced the same effect at a concentration of 100 ppm. Evaluation of 16 Moroccan medicinal plant extracts for larvicidal activity, nine exhibited high larvicidal activity with LC_{50} (24 h) ranging from 28 to 325 ppm (Markouk *et al.*, 2000). Based on the above information one concludes that the essential oils of *C. nepetaefolius* presents high larvicidal activity.

To evaluate the larvicidal activity of essential oils, aqueous solutions (hydrolates) were obtained by steam distillation of stems and leaves of *C. argyrophylloides*, *C. nepetaefolius*, *C. sonderianus* and *C. zehntneri* against *Aedes aegypti*. The main constituents found in the essential oils present in hydrolates were β-*trans*-Guaiene, 1,8-cineole, *E*-caryophyllene (*C. argyrophylloides*); Methyl-eugenol, 1,8-cineole (*C. nepetaefolius*); *E*-caryophyllene (*C. sonderianus*); *trans*-anethole, estragole (*C. zehntneri*).

Twenty-five larvae of third instar were placed in plastic beakers, containing the hydrolates (50 mL), in a four repetitions scheme. Water was used as control and the number of dead larvae was counted after 24 h. The data obtained were submitted to Variance Analysis and Tukey test. It was observed significant differences among the hydrolates from different species and from different parts of each plant ($p<0.001$). The hydrolates of stem and leaf from *C. nepetaefolius* and *C. zehntneri* and leaf hydrolate of *C. argyrophylloides* presented 100% mortality against larvae. This study showed that all species analyzed presented compounds with larvicidal properties, with differences between each plant parts (Lima *et al.*, 2006).

Cardiovascular Effects of EOCN

Previous studies from our laboratory have shown that intravenous treatment of both pentobarbital-anesthetized and conscious normotensive rats with EOCN induced dose-dependent hypotension and bradycardia, two effects which occur independently (Lahlou *et al.*, 1999). Whilst EOCN-induced bradycardia appeared to depend upon the presence of an intact and functional parasympathetic nerve drive to the heart, EOCN-induced hypotension is mainly due to an active vascular relaxation rather than to a withdrawal of sympathetic tone (Lahlou *et al.*, 1999). Such a hypothesis is corroborated by *in vitro* experiments using rat isolated thoracic aorta and mesenteric vascular bed preparations from either deoxycorticosterone-acetate (DOCA)-salt hypertensive or normotensive rats. In conscious DOCA-salt hypertensive rats and their uninephrectomized controls, EOCN also decreased mean arterial pressure (MAP) and heart rate (HR) in a dose-related manner (Lahlou *et al.*, 2000). Treatment with DOCA-salt significantly enhanced EOCN-induced decreases in MAP without affecting bradycardia. In isolated thoracic aorta preparations from DOCA-salt hypertensive rats, EOCN induced a reduction of phenylephrine-induced contraction. Arteries from DOCA rats showed enhanced sensitivity to EOCN, as compared to uninephrectomized controls. This enhancement appeared to be related mainly to an increase in EOCN-induced vascular smooth muscle relaxation rather than to an enhanced sympathetic nervous system activity in this hypertensive model. These data showed an important anti-hypertensive property of EOCN, and supported the hypothesis that EOCN may be a direct vasorelaxant agent acting hypotensively by a mechanism, probably myogenic, that turns to be more active in hypertensive rats. Recently, it was reported that in both a resistance vascular (mesenteric) bed and a conduit artery (aorta) from normotensive rats, EOCN also showed vasorelaxant effects that are partially dependent upon the integrity of a functional vascular endothelium. Nevertheless, inhibition of other transduction pathways may be involved in the mediation of these effects (Magalhães *et al.*, 2008).

Cardiovascular responses to EOCN are partially attributed to the actions of its main constituents. In fact, intravenous treatment of conscious rats with either 1,8-cineole or methyl-eugenol dose-dependently decreased MAP and HR. The bradycadia is also of vagal origin whereas the hypotension is related to an active vascular relaxation as evidenced by the significant reduction of potassium-induced contraction caused by both 1,8-cineole and methyl-eugenol in rat isolated aorta preparations (Lahlou *et al.*, 2002, 2004). In mesenteric bed preparations, vasodilator responses to EOCN were also mimicked by methyl-eugenol and alpha-terpineol, and were also significantly reduced in the presence of L-NAME (Magalhães *et al.*, 2008).

Table 2. EC_{50} (μg/mL) for relaxant effect of EOCN on basal tone and IC_{50} (μg/mL) for inhibitory activity of EOCN on contractions induced K^+ (60 mm), Phenylephrine (PHEN), Acetylcholine ACh or Carbachol (CAR) or Histamine (HA)

Preparation	**Relaxation of basal tone**	**Blockade of K^+-induced contraction**	**Blockade of ACh- or CAR-induced contraction**	**Blockade of HA-induced contraction**
Ileum, guinea pig	15.7 ± 4.3 (17)*[a] microg/mL	18 ± 2.3 (6)[b] microg/mL	28 ± 4.9 (8)[b] microg/mL	21 ± 4.7 (6)[b] microg/mL
Cárdia sphincter	6.02 ± 2.15 (8)[a] microg/mL	-	-	-
Pilorus sphincter	0.90 ± 0.74 (4)[a] microg/mL	-	-	-
Ileocecal sphincter	6.56 ± 3.00 (7)[a] microg/mL	- -	- -	- -
Aorta, guinea pig		26.7 (14.7-48.2) (8)**,[d] microg/mL	- - -	- - -
Trachea, guinea pig	4.3 ± 0.78 (7)[c] microg/mL	128.8 ± 0.78 (8)[c] microg/mL	102.59 ± 17.82 (8)[c] microg/mL	123.9 ± 15.75 (8)[c] microg/mL

*, mean ± S.E.M. (number of experiments)

**, mean (95 % confidence interval) (number of experiments)

[a], Magalhães *et al.* (1998). *Phytother. Res.* **12:** 172–177.

[b], Magalhães *et al.* (2004). *Fundam Clin. Pharmacol.* **18:** 539-546.

[c], Magalhães *et al.* (2003). *Planta Med.* **69:** 874-877.

[d], Magalhães *et al.* (2008). *Fundam Clin Pharmacol* **22:** 169-177.

Antispasmodic Effects of EOCN

EOCN also demonstrated *in vitro* and *in vivo* myorelaxant and antispasmodic activities in non-vascular smooth muscle such as intestinal and tracheal muscles. These effects are concentration- and dose-dependents and are readily reversible. In guinea-pig isolated ileum preparation, EOCN reduces basal tone with the maximum amplitude of relaxation similar to that of the well characterized smooth muscle relaxant papaverine. Furthermore, EOCN reversibly relaxed the contraction induced by potassium in guinea-pig ileum preparations. This effect was shared by several of its main constituents including 1,8-cineole, methyl-eugenol and terpineol, but was probably due primarily to a methyl-eugenol contribution, since 1,8-cineole and terpineol are only a fraction of EOCN constitution and showed much smaller pharmacological potencies as compared to EOCN (see IC_{50} in Table 2) (Magalhães *et al.*, 1998; Lima *et al.*, 2000). Recently, we studied whether this antispasmodic effect is caused by indirect, neural or primarily by myogenic mechanism. Our results suggest that EOCN induces relaxation of guinea-pig ileum by a direct action on smooth muscle via a mechanism largely independent of alterations of transmembrane resting potential and Ca^{2+} influx, possibly at the level of the contractile apparatus (Magalhães *et al.*, 2004). EOCN also showed an interesting profile of intestinal myorelaxant and antispasmodic activity. In experiments *in vitro*, in stomach sphincters and small intestine, EOCN elicited a depressive action more potent on basal tone (EC_{50} for relaxation of cardia and pylorus basal tone: 6.02 and 0.90 microg/mL, respectively, Table 2) than on spontaneous motility and (EC_{50} for cardia and pylorus: 144.4 ± 37.4 (n=8) and 8.4 ± 1.7 n = 8 microg/mL, respectively). *In vivo,* EOCN preserved intestinal transit (or increased it, at 100 microg/mL) velocity while relaxing the viscera. This effect was interpreted to result from a greater inhibitory effect on the basal tone, as compared spontaneous motility (Magalhães *et al.*, 1998).

Another type of smooth muscle, the respiratory muscle, is reported to have important peculiarities concerning the excitation-contraction coupling (Janssen, 2002). We showed that EOCN relaxed the basal tone of the guinea-pig trachealis muscle *in vitro*, with an efficacy similar to that of the respiratory smooth muscle relaxant aminophylline and blocked an antigen-induced contraction that mimics human allergen-induced bronchoconstriction (Magalhães *et al.*, 2003). In these preparations, EOCN did not alter muscle resting potential neither the membrane depolarization induced by 60 mm [K+]. Also, EOCN's IC_{50} values for blockade of contractions induced by various agonists as well as for relaxation of preparations maintained in 60 mm [K+] did not differ between themselves (Table 2). This indicates that the EOCN antispasmodic activity did not depend on membrane receptors for

neurotransmitters and autacoids, but seems most likely mediated by a myogenic mechanism that may involves alteration of Ca^{2+} distribution through cell compartments (Rembold, 1996) or modification of sensitivity of contractile proteins to Ca^{2+} (Kamm & Grange, 1996). Further experiments are underway to test this possibility. However, differently from intestinal and vascular smooth muscle, it seems that a different mechanism account for EOCN's action on basal tone of trachealis, since the potency of this effect was smaller than that for the EOCN blockade of contractions induced by carbachol or histamine.

Antinociceptive Effects of EOCN

Administered orally in Swiss mice, EOCN was shown to induce an antinociceptive effect in several tests such as the acetic acid-induced writhing test, the hot-plat test and the formalin test. This antinociceptive effect occurred in a range of doses (30–300 microg/mL) 1 to 2 orders of magnitude smaller than the LD_{50}. In the formalin test, EOCN was more effective in both phases of the test at doses = 300 microg/mL, but was more potent in the second phase (efficacy at doses = 100 microg/mL). The mechanism of this effect remains to be elucidated. In the formalin test, the reduced paw licking by EOCN in the second phase is unrelated to an opioid mechanism as it remained unaffected by the pre-treatment with naloxone (Abdon *et al.*, 2002).

Neuronal Excitability Effects of EOCN

In rat sciatic nerve, EOCN and 1,8-cineole, its major constituent, blocked excitability (Lima-Accioly *et al.*, 2006). EOCN (500 and 1000 microg/mL) and 1,8-cineole (614, 920 and 1227 microg/mL (*i.e.* 4, 6 and 8 mmol/L)) concentration-dependently reduced peak-to-peak amplitude (at greatest concentrations blockade > 85%) and velocity of conduction of the compound action potential (CAP). EOCN effect was not explainable by its 1,8-cineole content (25% of essential oil weight) since 1000 microg/mL EOCN, which contains only 250 microg/mL 1,8-cineole, blocked almost completely CAP amplitude, and 307 microg/mL of pure 1,8-cineole showed no demonstrable effect (Lima-Accioly *et al*, 2006). Chronaxie and rheobase were significantly increased by 1000 microg/mL EOCN and 1227 microg/mL (*i.e.* mmol/L) 1,8-cineole, thus confirming that both drugs reduce nerve excitability. The effects of 1,8-cineole, but not of EOCN, were fully reversible upon 180 min drug wash, which is coherent with the conclusion that EOCN effects on nerve excitability are nor explainable by the content of 1,8-cineole on this essential oil. Lima-Accioly *et al.* (2006) investigation suggested that either a constituent other than and more potent than 1,8-cineole is present in OECN, or another constituent is amplifying the effect of 1,8-cineole. This remains not elucidated.

CONCLUSIONS

EOCN showed various useful biological activities such as antifungal and larvicidal and pharmacological efficacy in smooth muscle and nerve, with a greater potency in muscle, as compared to nerve. The comparison of the EOCN with other biological larvicides to *Aedes egypti* shows that this essential oil is a potent and affective larvicidal agent. Concerning pharmacological activity, among the various types of smooth muscle, it was more potent on intestinal and vascular than on tracheal smooth muscle. EOCN is thus better characterized as a smooth muscle relaxant corroborating the use of *C. nepetaefolius* as an antispasmodic remedy in folk medicine. Inhibitory actions of EOCN have an interesting pharmacological profile, as they seem mediated by intracellular mechanisms largely independent of alterations on transmembrane potential. Furthermore, its large efficacy as a myorelaxant agent associated with its low acute toxicity (its LD_{50} is >3 g/kg body weight, *per os*) makes it an agent of therapeutic potential.

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21

Pharmacokinetic Studies on Hepatic Uptake Mechanism of Tetrodotoxin in the Puffer Fish *Takifugu rubripes*

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ABSTRACT

In this study, we investigate the liver-specific uptake mechanism of tetrodotoxin (TTX) in the marine puffer fish Takifugu rubripes by the in vivo single administration and the in vitro uptake assay using the liver slices. After the single bolus injection of TTX into the hepatic portal vein under general anesthesia at 20°C, the blood concentration-time profiles of TTX showed multiple distinct phases, suggesting the TTX disposition in T. rubripes by the multiple compartments and the presence of the peripheral tissues that do not instantly achieve the concentration equilibrium of TTX with the central compartment. The area under blood concentration-time curves (AUCs) increased linearly after the single bolus injection at the dosage ranging from 0.25 to 0.75 mg TTX/kg body weight into the hepatic portal vein. The percentage of unbound fraction of TTX in the plasma was measured at around 60% throughout total TTX concentration from 1.4 to 22.5 μg TTX/mL plasma by the equilibrium dialysis, indicating that the plasma protein binding of TTX was not saturated in the range tested. After a single bolus injection of 0.25 mg TTX/kg body weight into the hepatic portal vein or hepatic vein, the AUCs were closely similar to each other regardless of the injection routes, indicating negligible hepatic first-pass effect of TTX in T. rubripes. After the gastrointestinal administration of 0.25, 0.50 or 1.00 mg TTX/kg body weight, the blood concentration-time profiles of TTX showed typical absorption curves with the first-order absorption process and the bioavailability was 62, 84 or 42%, respectively. In the case of a single administration of 0.25 mg TTX/kg

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body weight into hepatic accumulated in the liver 300 min after injection accounted for 84 ±6, 70 ± 9 or 49 ±17% of the administered dose, respectively. The results verified that TTX is well absorbed into the systemic circulation owing to relatively high gastrointestinal absorption by saturable mechanism and negligible hepatic first-pass effect, and finally accumulated in the liver within 300 min after injection. Consequently we estimated in detail the hepatic uptake clearance (CL_{uptake}) of TTX in T. rubripes by integration plot analysis after a single bolus administration of 0.25 mg TTX/kg body weight into the hepatic vein at 20°C. The hepatic concentration of TTX was gradually increased after the injection, reaching 1240 ± 90 ng/g liver at 60 min after injection, whereas the blood concentration of TTX decreased over time after the injection, from 1451 ±45 ng/mL at 10 min to 364 ± 59 ng/mL at 60 min. In contrast, TTX concentrations in spleen and kidney decreased in parallel with the blood concentrations, whereas those in the muscle and skin remained almost the same throughout the experiment at the low level. The amount of TTX that had accumulated in the liver 60 min after injection accounted for 63 ± 5% of the administered dose. The integration plot analysis indicated a CL_{uptake} of 3.1 mL/min/kg body weight in the liver for TTX, a rate far below that of the hepatic portal vein blood flow rate 36.0 ± 0.9 mL/min/kg body weight (at most, 9%). The finding is consistent with negligible extraction of TTX by the liver. These results demonstrated conclusively that the liver-specific distribution of TTX in T. rubripes is achieved by the removal from the systemic circulation, but by the hepatic first-pass effect. In the in vitro study using the liver slices, we examined the transmembrane transport process that is involved in the hepatic uptake mechanism of TTX in T. rubripes. When T. rubripes liver slices were incubated with 0–2000 μM TTX under the oxygenation ($95\%O_2$-$5\%CO_2$) at 20°C for 60 min, the uptake rates of TTX increased with the TTX concentration in the buffer but exhibited non-linearity, suggesting that the TTX uptake into the liver is carrier-mediated. Eadie-Hofstee plot indicated that the TTX uptake was composed of a saturable component (V_{max} 47.7 ± 5.9 pmol/min/mg protein and K_m 249 ±47 μM) and a non-saturable component (P_{dif} 0.0335 ± 0.0041 μL/min/mg protein). The TTX uptake was significantly decreased to 0.4 and 0.6 fold by the incubation at 5°C and the replacement of Na^+ by choline in the buffer, respectively, whereas it was not affected by the presence of 1 mM taurochoate, p-aminohippurate, tetraethylammonium or L-carnitine. These results revealed the involvement of carrier-mediated transport system in the TTX uptake by T. rubripes liver. Based on the above findings, the pharmacokinetics of TTX after the single gastrointestinal administration of 0.25 mg TTX/kg body weight was summarized as follows: the extent of bioavailability of TTX (F_{oral}) was 62% and the hepatic extraction of TTX, which was estimated by the ratio of CL_{uptake} to the hepatic portal vein blood flow rate, was at most 9%, and as the results, the product of the gastrointestinal absorption ratio and the avoidance rate of the metabolism on the gastrointestinal epithelium ($F_a \cdot F_g$) was estimated to be 68%. The preset study also suggests an involvement of saturable

mechanisms for relatively high gastrointestinal absorption of TTX. On these grounds, we concluded that TTX is stored in liver as a first step of the toxification in the puffer fish T. rubripes.

Key words : Bioavailability, bioaccumulation carrier-mediated transport/transporter, Fugu *Takifugu rubripes*, hepatic first-pass effect, pharmacokinetics, plasma protein binding, tetrodotoxin, toxification

INTRODUCTION

Marine puffer fish, members of the family Tetraodontidae, is among the most poisonous fish and contains a considerable amount of tetrodotoxin (TTX) that selectively binds to voltage-gated sodium channel in muscle and nerve tissues. As shown in Table 1, the concentration of TTX greatly varies among puffer fish species and shows tissue distribution in the fish, with the highest concentrations in the liver and ovary, and to a lesser extent in the gastrointestinal tract and skin (Halstead, 1988; Isbister & Kieman, 2005; Soong & Venkatesh, 2006; Hwang & Noguchi, 2007). However, it was found that an intraperitoneal injection of the minimum lethal dose of TTX for puffer fish was about 5 mg TTX/kg body weight (Saito *et al.*, 1985). Biological and physiological significance of TTX in puffer fish remains mysterious. Extensive studies on TTX toxification of marine puffer fish and the other toxic organisms have been conducted and led the hypothesis that two routes are implicated; symbiosis or parasitism with TTX-producing bacteria and the marine food chain with bioaccumulation.

Table 1. Toxicity of Japanese marine puffer fish

Scientific name	Ovary	Testis	Liver	Bile	Skin	Intestine	Muscle	Blood
Arothron firmamentum	◉	×	×	–	○	○	×	–
Canthigaster rivulata	×	–	○	–	◉	○	×	–
Lagocephalus lagocephalus oceanicus	×	×	×	–	×	–	×	–
L. lunaris spadiceus	×	×	×	–	×	×	×	–
L. inermis	×	×	◉	○	×	○	×	–
L. sceleratus	●	–	◉	◉	○	◉	○	–
Takifugu obscurus	●	×	◉	–	◉	◉	×	–
T. xanthopterus	◉	×	◉	–	×	○	×	–
T. rubripes	◉	×	●	–	×	○	×	×
T. chinensis	●	–	●	–	–	–	–	–
T. niphobles	●	○	●	–	◉	●	○	–
T. stictonotus	●	○	●	◉	◉	×	○	–
T. snyderi	●	○	●	–	◉	◉	○	–

Table 1. *Contd.*

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Scientific name	Ovary	Testis	Liver	Bile	Skin	Intestine	Muscle	Blood
T. vermicularis	●	○	◉	–	◉	○	○	–
T. porphyreus	●	×	●	–	◉	◉	×	–
T. poecilonotus	●	◉	●	–	◉	◉	○	–
T. exascurus	◉	×	◉	–	◉	○	×	–
T. pardalis	●	◉	●	●	◉	◉	◉	×
T. chrysops	●	×	◉	–	◉	○	×	×
T. flavidus	◉	×	◉	–	○	◉	×	–
T. pseudommus	◉	×	○	–	○	○	×	–
Tetraodon alboreticulatus	●	–	○	○	×	×	×	–
Sphoeroides pachygaster	×	×	×	–	×	×	×	–

Strongly toxic (●), >1000 MU/g; moderately toxic (◉), 100 ~ 999 MU/g; weakly toxic (○), 10 ~ 99 MU/g ; negative (×), < 10 MU/g; data not available (–), where 1 MU (mouse unit) is defined as the amount of toxin that kills a male mouse of ddY strain (20 g body weight, 4 weeks old) in 30 min after intraperitoneal administration. One MU is equivalent to about 0.2 μg of tetrodotoxin. [Excerpt from Shiomi & Nagashima, (2006), Japanese text book]

The former was reported by many researchers who identified the TTX-producing bacteria from the liver, ovary, skin and gastrointestinal tract of marine puffer fishes (Noguchi *et al.*, 1987; Yotsu *et al.*, 1987; Matsui *et al.*, 1989; Lee *et al.*, 2000; Yu *et al.*, 2004; Wu *et al.*, 2005), intestine of starfish (Narita *et al.*, 1987), sea urchin (Ritchie *et al.*, 2000), intestine of xanthid crab (Noguchi *et al.*, 1986a, b; Sugita *et al.*, 1987), posterior salivary gland and other soft parts of blue-ringed octopus (Hwang *et al.*, 1989), shellfish (Cheng *et al.*, 1995), venom of planktonic chaetognaths (Thuesen & Kogure, 1989), copepod (Maran *et al.*, 2007) and marine red calcareous alga (Yasumoto *et al.*, 1986). The latter was supported by the feeding tests of the cultured puffer fish in netcages or tanks. Puffer fish, the family Tetraodontidae, seems to have a special function to take up and accumulate TTX into the liver. Briefly, marine puffer fish shows non-toxicity in the case of the farming with the use of non-toxic artificial diets after hatching (Matsui *et al.*, 1981, 1982; Lin *et al.*, 1998; Noguchi *et al.*, 2006) and becomes toxic after the administration of TTX-containing diets (Matsui *et al.*, 1981; Yamamori *et al.*, 2004; Honda *et al.*, 2005; Kono *et al.*, 2008). Therefore, these findings strongly suggest that TTX is the exogenous substance for puffer fish.

However, it remains to be clarified the mechanisms of TTX toxification and TTX metabolism in marine puffer fish; how TTX is absorbed into the fish body, how TTX is transported in the body and how TTX is concentrated into the specific organs such as the liver and ovary. Furthermore, current studies including the feeding tests failed to estimate the kinetics interpretation of TTX in the body.

In general, the direct elimination, which is the first defense line for xenobiotics such as toxins and drugs, is regulated by drug transporters expressing in both the gastrointestinal tract and liver. Drug transporters reduce oral bioavailability by two mechanisms: direct inhibition of uptake from the gastrointestinal tract and rapid elimination of xenobiotics and their metabolites via bile. This step strongly reduces access of toxins and drugs to the systemic circulation (Elferink & Waart, 2007).

In this study, we examined the TTX toxification mechanism of the marine puffer fish *Takifugu rubripes* by *in vivo* and *in vitro* techniques from the standpoint of pharmacokinetics. Consequently, we demonstrated that TTX is well absorbed from the gastrointestinal tract into the systemic circulation owing to relatively high fraction of absorption, and the liver-specific distribution of TTX is achieved by the removal from the systemic circulation involving the carrier-mediated transport system, but not by the hepatic first-pass effect in the marine puffer fish *T. rubripes*. Our study also suggests an involvement of saturable mechanisms for relatively high intestinal absorption of TTX. On the basis of the above findings, we conclude that TTX is stored in the liver as a first step of the toxification in *T. rubripes*. Here we will give a review of our current findings about the pharmacokinetics of TTX in the marine puffer fish *T. rubripes* (Matsumoto *et al.*, 2007, 2008a, 2008b).

MATERIALS AND METHODS

Pharmacokinetics of TTX in *Takifugu rubripes*

Materials

Cultured marine puffer fish *Takifugu rubripes* specimens (0.75–1.35 kg body weight) were obtained alive from Tokyo central wholesale market (Tsukiji fish market) and transported to our laboratory. TTX was purified from the ovaries of the marine puffer fish *Takifugu pardalis* by ultrafiltration using YM1 membrane (Millipore, Bedford, MA, USA) and a series of column chromatography on a Bio-Gel P-2 column (Bio-Rad Laboratories, Hercules, CA, USA) and a Bio-Rex 70 column (Bio-Rad Laboratories) according to the method of Nagashima *et al.* (2003), and used in the administration assay. Crystalline TTX (Wako Pure Chemical Industries, Osaka, Japan) was used as a standard for LC/ESI-MS analysis. All other chemicals were of reagent grade.

TTX Administration

Prior to the TTX administration, the fish was anesthetized with the artificial seawater containing 0.03% 2-phenoxyethanol and placed on the experimental

workbench comprising a styrofoam storage tank and a stainless-steel basket after weighing. During experiments, the fish was swaddled by wet papers to prevent the body surface from xeransis, and breathed by the gill ventilation using the perfusion of the seawater treated with a bubble aeration at 20°C. After the laparotomy of the fish body, hepatic vein was cannulated with a PE50 polyethylene tube (Clay Adams, Parsippany, NJ, USA). TTX was dissolved in the modified Hank's balanced salt solution (160 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 10 mM HEPES, adjusted to pH7.4 with NaOH solution) and administered through three different routes. The TTX solution (0.25–1.00 mg TTX/500 μL/kg body weight) was bolus-injected into hepatic portal vein, hepatic vein or gastrointestinal tract using a 1 mL disposable syringe (Termo, Tokyo, Japan). Blood samples were obtained via the venous cannula using a heparinized 1 mL disposable syringe at 3, 5, 10, 20, 30, 45, 60, 90, 120, 150, 180, 240 and 300 min after the administration. The liver was dissected out at the end of each experiment and subjected to TTX determination below.

As for the integration plot analysis, TTX solution (0.25 mg TTX/500 μL/kg body weight) was given by a single bolus injection into the hepatic vein. Blood samples were obtained from the hepatic vein cannulated at designed time points (3, 5, 10, 20, 30, 45 and 60 min) after the administration. At 10, 20, 30, 45 and 60 min after the administration, spleen, kidney, skin, muscle and liver were separated and subjected immediately to TTX determination below.

TTX Determination in Blood and Tissue Samples

After the sampling, the blood was immediately mixed with an equal volume of modified Hank's balanced salt solution (pH7.4) containing 500 units/mL heparin and centrifuged at 3000 *g* for 5 min. The resulting supernatant was extracted with nine volumes of methanol containing 10vol% acetic acid and ultrafiltered through a Vivaspin 500 (MWCO 5,000, VivaScience AG, Hannover, Germany). The filtrate was lyophilized and re-dissolved in 20 mM heptafuluorobutylic acid in 10 mM ammonium formate (pH4.0). TTX was determined by the LC/ESI-MS analysis according to the method of Shoji *et al.* (2001) with some modification. Briefly, LC/ESI-MS analysis was performed on an alliance Zspray™ MS 4000 LC/ESI-MS system (Waters, Milford, MA, USA). The analytical column was a Develosil C30-UG-5 (1.5 × 250 mM, 5 μm particle size, Nomura chemical, Seto, Japan) and maintained at 25°C. The mobile phase consisted of 20 mM heptafuluorobutylic acid in 10 mM ammonium formate (pH4.0) containing 1vol% acetonitrile and was eluted at a flow rate of 0.10 mL/min. The eluate was induced into the ion source of ESI-MS and ionized by the positive ion mode with desolvation temperature at 350°C, ion source block temperature at 100°C and cone voltage at 45 kV. TTX detection at

m/z320 corresponding to the protonated molecular ion $(M+H)^+$ was achieved using the selected ion recording mode. For the quantitation of TTX, intact blood and liver extract were spiked with 0–1.0 μg of TTX standard. TTX in several organ tissues were extracted with 0.1% acetic acid by ultrasonication for 1 min and heating in a boiling water bath for 10 min on the basis of the standard assay for TTX (Kodama & Sato, 2005). The extract was defatted with dichloromethane and centrifuged at 1,370 g for 30 min at 5°C. The resulting supernatant was ultrafiltered through a Vivaspin 500 and the filtrate was analyzed for TTX by LC/ESI-MS.

Plasma Protein Binding Assay

Plasma protein binding of TTX was determined using an equilibrium dialysis unit (Sanplatec corp., Tokyo, Japan). Approximately 25 mL blood/kg body weight was collected from a puffer fish on the day of study. The blood was centrifuged at 1200 g for 10 min and the plasma was collected. The dialysis membrane (MWCO 3500, Spectrum Laboratories, CA, USA) was rinsed twice in MilliQ water, soaked in modified Hank's balanced salt solution (pH7.4) for 30 min and set into the dialysis unit to separate compartments of a dialysis chamber. A mixture of 50 μL TTX solution (0–20 ng) and 900 μL puffer fish plasma was put into the one side of the chamber (termed as the plasma sample), and 950 μL the buffer was put into the counter side of the chamber (termed as the buffer sample). Plasma protein binding of TTX was assessed after incubation for 24 h at 20°C. The plasma and buffer samples were collected from each side of the chamber. TTX amount was determined by LC/ESI-MS method as described above.

Determination of Hepatic Portal Vein Blood Flow Rate

Hepatic portal vein blood flow rate was measured with a bi-directional Doppler blood flowmeter (DVM-4300, Hadeco, Tokyo, Japan). As noted above, puffer fish was anesthetized and placed on the experimental workbench. After the laparotomy of fish body, the ultrasonic probe (10 MHz, type HP10M2N5A, Hadeco) was held on hepatic portal vein and output of the flowmeter was recorded. Next, TTX solution (0.25 mg TTX/500 μL/kg body weight) was bolus-injected into hepatic vein and output of the flowmeter was recorded at 10-min intervals for 60 min after the administration.

Pharmacokinetic Analyses on the In vivo Administration Test

The total body clearance (CL_{tot}), hepatic portal vein clearance (CL_{hp}), bioavailability (F_{oral}), hepatic availability (*viz.* avoidance ratio of the hepatic first-pass effect) (F_h), mean residence time (MRT) and steady-state volume of distribution (V_{ss}) were estimated from the following equations:

$$CL_{tot} = Dose_{hv}/AUC_{hv} \quad (1)$$

$$CL_{hp} = Dose_{hp}/AUC_{hp} \quad (2)$$

$$F_{oral} = \frac{AUC_{po} \bullet Dose_{hv}}{AUC_{hv} \bullet Dose_{po}} \quad (3)$$

$$F_{h} = \frac{AUC_{hp} \bullet Dose_{hv}}{AUC_{hv} \bullet Dose_{hp}} \quad (4)$$

$$MRT = \frac{AUMC}{AUC} \quad (5)$$

$$Vss = CL_{hp} \bullet MRT \quad (6)$$

where AUC_{hv}, AUC_{hp} and AUC_{po} are the area under blood concentration-time curve of TTX for up to infinity after the administrations into hepatic vein, hepatic portal vein and gastrointestinal tract, respectively. $Dose_{hv}$, $Dose_{hp}$ and $Dose_{po}$ are the applied dose of TTX via hepatic vein, hepatic portal vein and gastrointestinal tract, respectively. AUC and AUMC (area under the moment curve) were calculated according to linear or linear-logarithmic trapezoidal role using a moment analysis program (MOMENT) available on Microsoft Excel® (Tabata *et al.*, 1999).

For evaluation of the hepatic uptake clearance (CL_{uptake}) of TTX in *T. rubripes*, $AUC_{(0\text{-}t)}$ from zero to *t* min after the administration was calculated with the use of MOMENT program (Tabata *et al.*, 1999). The integration plot was obtained by plotting $C_{H(t)}/C_{B(t)}$ (on the ordinate) versus $AUC_{(0\text{-}t)}/C_{B(t)}$ (on the abscissa), the initial slope of this plot representing the CL_{uptake} (μL/min/g liver), where $C_{H(t)}$, $C_{B(t)}$ and $C_{H(0)}/C_{B(0)}$ represent the TTX concentration in liver (ng TTX/g liver) and blood (ngTTX/μL blood) at *t* min after the administration and the initial distribution volume of TTX in liver (μL/g liver), respectively (Kim *et al.*, 1988; Murata *et al.*, 1998; Okudaira *et al.*, 2001). The fitting to the equation (7) was performed by an iterative nonlinear least-squares method using a MULTI program and the algorithm of the Damping Gauss Newton method (Yamaoka *et al.*, 1981). The hepatic extraction rate of TTX (E_h) and the hepatic availability (F_h) were estimated from the following equations (8) and (9), respectively, where Q is the hepatic portal blood flow rate (36.0 ± 0.9 mL/kg body weight).

$$CH_{(t)}/C_{B(t)} = CL_{uptake} - AUC_{(0\text{-}t)}/C_{B(t)} + C_{H(0)}/C_{B(0)} \quad (7)$$

$$CL_{uptake} = Q \cdot E_h \quad (8)$$

$$F_h = 1 - E_h \quad (9)$$

2. *In vitro* Uptake Assay by the Liver Tissue Slices

Materials

Cultured marine puffer fish *T. rubripes* and wild black scraper *Thamnaconus modestus* were obtained alive from Tokyo central wholesale market and

transported to our laboratory. TTX purified from ovaries of *T. pardalis* as described above was used in the uptake assay for TTX concentration dependence. Crystalline TTX (Wako Pure Chemicals Industries) was used in the uptake assays for the time course and the inhibitory effect. *p*-Aminohippurate (PAH) and tetraethylammonium (TEA) were purchased from Sigma (St. Louis, MO, USA) and taurocholate (TCA) and L-carnitine from Wako Pure Chemicals Industries. All other chemicals were of reagent grade.

Preparation of Liver Tissue Slices

The liver was carefully removed from the fish body anaesthetized in ice-cold artificial seawater, and perfused through a hepatic portal vein with ice-cold oxgenarated (95% O_2-5% CO_2) perfusion buffer (160 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 12.5 mM HEPES and 5.6 mM D-glucose, adjusted to pH7.4 with NaOH solution) at a flow rate of 12 mL/min for 10 min. The liver was sliced (1 mM in thickness) with microtome blades (A22, Feather, Tokyo, Japan) and punched (8 mM in diameter) with a disposable biopsy punch (Kai Industries, Gifu, Japan).

Uptake of TTX into Liver Tissue Slices

Each round liver slice was pre-incubated statically in the transport buffer (160 mM NaCl, 4.8 mM KCl, 23.8 mM Na_2HPO_4, 0.96 mM KH_2PO_4, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 12.5 mM HEPES and 5.0 mM D-glucose, adjusted to pH7.4 with NaOH solution) at 20°C for 5 min. The uptake study was started by putting the liver slice into each well of a 24-well plate (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and adding 500 µL of the transport buffer containing 50 µM TTX. Incubation was carried out at 20°C for up to 90 min. At the time intervals, the liver slices were rapidly removed from the transport buffer and rinsed in the ice-cold transport buffer (TTX-free). In the experiments to examine the effect of TTX concentration on the uptake, the liver slices were incubated with 500 µL of the transport buffer containing 0-2000 µM TTX at 20°C for 60 min.

Inhibition Assay of TTX Uptake into Liver Tissue Slices

Inhibitory effect of substrates of the typical hepatic transporters on TTX uptake into the liver slices was investigated. The inhibitors were 1 mM L-carnitine, 1 mM PAH, 1 mM TCA or 1 mM TEA. After being pre-incubated as described above, the liver slices were incubated with 500 µL of the transport buffer containing 50 µM TTX and each inhibitor at 20°C for 60 min. To examine the effect of sodium-ion on the TTX uptake activity, NaCl and $NaHCO_3$ in the transport buffer were replaced by choline chloride and choline bicarbonate, respectively. Besides, the incubation temperature was lowered to 5°C to examine the temperature dependence of TTX uptake.

Determination of TTX

TTX accumulated in the liver slices was extracted with 0.1% acetic acid as described above. The resulting filtrate was analyzed for TTX by LC/ESI-MS previously described with minor changes. The analytical column was a Capcell Pak C18 AQ (1.5 × 250 mM, 3 μm particle size, Shiseido, Tokyo, Japan) and maintained at 25°C. The mobile phase consisted of 15 mM heptafuluorobutylic acid in 10 mM ammonium acetate (pH5.0) and was eluted at a flow rate of 0.10 mL/min.

Pharmacokinetic Analyses on the TTX Uptake Assay

Kinetic parameters for the TTX uptake were calculated by fitting the uptake rate to the following equation (Michaelis-Menten equation) with the use of a MULTI program (Yamaoka *et al.*, 1981) and the Damping Gauss Newton Method algorithm.

$$v = \frac{V_{\max} \cdot S}{K_m + S} + P_{dif} \cdot S$$

where v and S are the uptake rate of TTX (pmol/min/mg protein) and the TTX concentration in the transport buffer (μM), respectively. K_m, V_{max} and P_{dif} are Michaelis constant (μM), the maximum uptake rate of TTX into the liver slices (pmol/min/mg protein) and the non-specific uptake clearance (μL/min/mg protein) by the liver slices, respectively.

Determination of Protein

Protein in plasma or liver slices was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a reference standard protein.

Static Analyses

The determinations were carried out on at least three individual experiments in triplicate. Data were presented as the mean ± standard deviation (SD) or the mean ± standard error (SE), and Tukey's test and Student's *t*-test were used to analyze the significance of difference among the means at 5% significance level.

RESULTS

1. Pharmacokinetics of TTX in Takifugu rubripes after the Single Bolus Administration

The marine puffer fish *T. rubripes* specimens used in this study initially did not contain any detectable amount of TTX (<0.01 ng TTX/μL blood and <10 ng TTX/g tissue).

Pharmacokinetics of TTX in Blood after the Single Administration

Time courses of blood concentration of TTX after a single bolus injection of 0.25–0.75 mg TTX/kg body weight into the hepatic portal vein are shown in Fig 1. The pharmacokinetic parameters are summarized in Table 2. After the injection of 0.25 mg TTX/kg body weight, the blood concentration of TTX was 3.00 ± 0.58 ng/μL at 3 min and fell to 0.71 ± 0.02 ng/μL within 30 min, and then decreased gradually to 0.14 ± 0.06 ng/μL at 300 min (Fig 1). The dose-intensification from 0.25 to 0.75 mg TTX/kg body weight elevated the blood concentration of TTX. The area under blood concentration-time curves (AUCs) of TTX demonstrated a tendency to increase from 147 ± 33 to 348 ± 40 ng·min/μL with the dose-intensification from 0.25 to 0.75 mg TTX/kg body weight, although no significant differences were observed in AUCs at the dose ranging from 0.25 to 0.50 mg TTX/kg body weight ($p>0.05$) (Table 2). Meanwhile, the hepatic portal vein clearance (CL_{hp}), the mean residence time (MRT) and the steady-state volume of distribution (V_{ss}) were constant irrespective to the dose ($p>0.05$). These results indicate that the pharmacokinetics of TTX in the marine puffer fish after the single bolus injection of 0.25–0.75 mg TTX/kg body weight was not saturated.

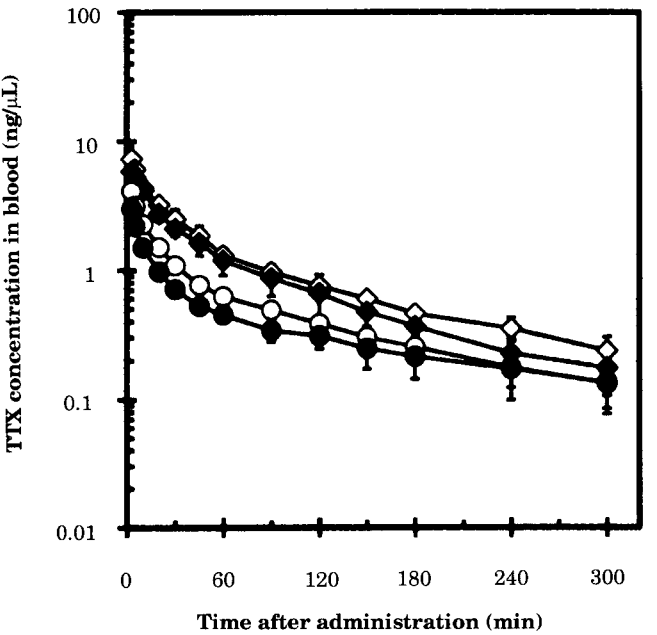

Fig 1. Time course of the blood concentration of TTX in *Takifugu rubripes* after the single bolus injection into hepatic portal vein. Puffer fish received a single bolus injection of 0.25 (●), 0.37 (○), 0.50 (◆) or 0.75 (◇) mg TTX/500 μL/kg body weight. Each point and vertical bar represent the mean ± SE of three individual experiments performed in triplicate.

Table 2. Pharmacokinetic parameters after the single bolus injection of TTX into the hepatic portal vein in *Takifugu rubripes*

Dose (mg/kg)	Body weight (kg)	AUC (ng*min/μL)	CL_{hp} (μL/min/kg)	MRT (min)	V_{ss} (mL/kg)
0.25	0.94 ± 0.10[a]	147 ± 33[a]	1848 ± 336[a]	173 ± 53[a]	284 ± 22[a]
0.37	1.16 ± 0.11[a]	176 ± 32[a]	2264 ± 447[a]	120 ± 23[a]	254 ± 13[a,b]
0.50	1.07 ± 0.06[a]	293 ± 64[a]	1890 ± 426[a]	103 ± 18[a]	182 ± 20[b]
0.75	1.02 ± 0.04[a]	348 ± 40[b]	2217 ± 281[a]	115 ± 19[a]	249 ± 29[a,b]

Values are the mean ± SE of three individual experiments performed in triplicate. Different superscripts in the same column indicate significant differences among the administrated groups ($p<0.05$ by Turkey's test). Abbreviations: CL_{hp} hepatic portal vein clearance; MRT, mean residence time; V_{ss} steady-state volume of distribution.

Bioavailability of TTX after the Direct Injection into Gastrointestinal Tract

TTX absorption through gastrointestinal tract was examined in *T. rubripes*, since it is thought that marine puffer fish accumulates the toxin mainly by the oral route (Matsui *et al.*, 1981; Yamamori *et al.*, 2004; Honda *et al.*, 2005; Kono *et al.*, 2008). Fig 2 shows the time courses of blood concentration of TTX after a gastrointestinal administration of 0.25, 0.50 or 1.00 mg TTX/kg body weight. After the administration of 0.25 mg TTX/kg body weight into gastrointestinal tract, the blood concentration of TTX was transiently increased to 0.46 ± 0.10 ng/μL until 30 min and thereafter decreased gradually to 0.09 ± 0.02 ng/μL at 300 min. Comparing the AUCs following direct injection into the gastrointestinal tract and hepatic vein elucidated that the bioavailability of TTX is 62%. In the cases of administration at the dosage of 0.50 and 1.00 mg TTX/kg body weight, the blood concentration of TTX increased to 0.45 ± 0.06 and 0.53 ± 0.11 ng/μL until 150 min after administration, respectively, and then modestly decreased to about 0.3 ng/μL at 300 min after administration in both cases. The AUCs were estimated to be 236 ± 41 and 234 ± 19 ng· min/μL and the bioavailability was 84 and 42% after the administration of 0.50 and 1.00 mg TTX/kg body weight, respectively (Table 3). These results suggest that the high gastrointestinal absorption of TTX by a saturable mechanism in *T. rubripes*.

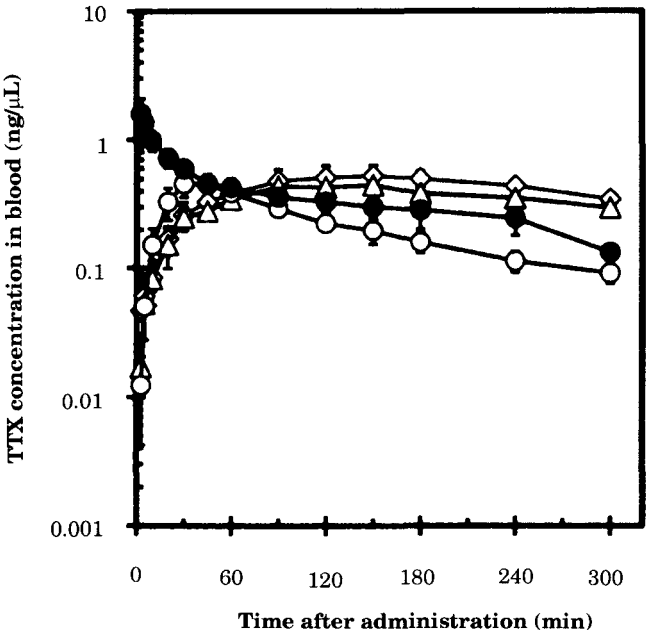

Fig 2. Time course of the blood concentration of TTX in *Takifugu rubripes* after the gastrointestinal administration. The fish received a single administration of 0.25 (○), 0.50 (△) or 1.00 (◇) mg TTX/500 μL/kg body weight into gastrointestinal tract. For comparison, the time course after a single bolus injection of 0.25 mg TTX/500 μL/kg body weight into hepatic vein (●) is also shown in the Fig. Each point and vertical bar represent the mean ± SE of three individual experiments performed in triplicate

Table 3. Bioavailability and hepatic availability of TTX after a single administration in *Takifugu rubripes*

Dose (mg/kg)	Body weight (kg)	AUC (ng·min/μL)	CL_{tot} (μL/min/kg)	MRT (min)	F_{oral} (%)	F_h (%)
0.25*hv*	1.02 ± 0.04[a]	141 ± 1[a]	1778 ± 17	223 ± 48[a]	-	-
0.25*hp*	0.94 ± 0.10[a]	147 ± 33[a]	-	173 ± 53[a]	-	104
0.25*po*	1.05 ± 0.05[a]	88 ± 4[b]	-	238 ± 5[a]	62	-
0.50*po*	104 ± .06[a]	236 ± 41[c]	-	498 ± 145[a]	-	-
1.00*po*	1.09 ± 0.03[a]	234 ± 19[c]	-	381 ± 75a	-	-

Values are the mean ± SE of three individual experiments performed in triplicate. Different superscripts in the same column indicate significant differences among the administrated groups ($p<0.05$ by Tukey'test). Abbreviations: *hv*, hepatic vein; *hp*, hepatic portal vein; *po*, per os (gastrointestinal tract); CL_{tot}, total body clearance; MRT, mean residence time; F_{oral}, bioavailability; F_h, hepatic availability.

Plasma Protein Binding of TTX by Equilibrium Dialysis

Plasma protein binding of TTX was determined by equilibrium dialysis. The concentrations of TTX in the chambers were determined after 24 h. TTX concentration of the bound form is obtained by subtracting TTX concentration in the buffer chamber from that in the plasma chamber. The saturation of the plasma protein with TTX was not observed at the concentrations ranging from 1.4 to 22.5 μg/mL (Fig 3A). The percentage of the unbound fraction of TTX was almost constant at around 60% within the initial concentration range (Fig 3B). This value is in a good agreement with the previous report obtained using the ultrafiltration method, in which the percentage of unbound fraction of TTX to *T. rubripes* plasma was at 64% where the total TTX concentration in plasma was 2.5 nmol/mL (equivalent to 0.80 μg TTX/mL) (Matsui *et al.* 2000). The protein concentration of plasma used in this experiment was measured to be 50.8 ± 4.1 mg/mL.

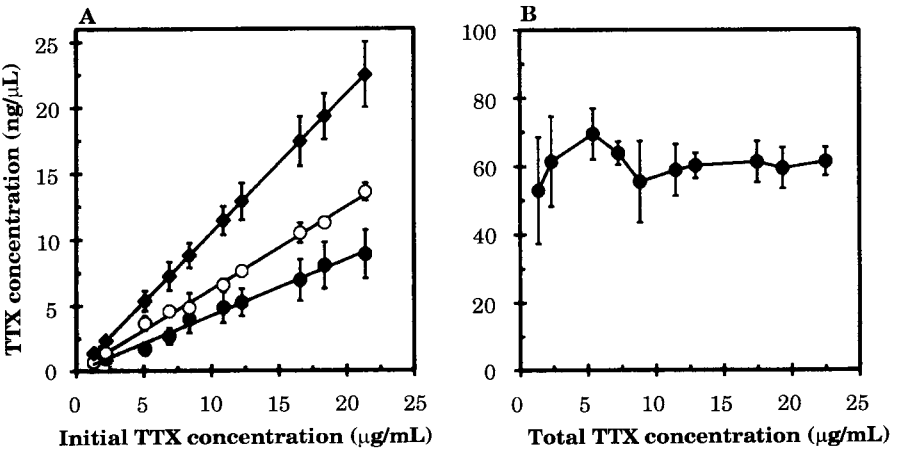

Fig 3. Binding property of TTX to the plasma protein of *Takifugu rubripes* by equilibrium dialysis. (A) TTX concentrations of the bound form (●), the unbound form (○) and the total (◆) as a function of the initial TTX concentration. TTX concentrations were estimated after the incubation at 20°C for 24 h. (B) Percentage of the unbound fraction of TTX. Each point and vertical bar represent the mean ± SE of three individual experiments performed in triplicate

Determination of Hepatic Portal Vein Blood Flow Rate

The hepatic portal vein blood flow rate in *T. rubripes* before the TTX administration was 35.8 ± 3.0 mL/min/kg body weight (n = 6, mean ± SE). After a single injection of 0.25 mg TTX/kg body weight into hepatic vein, the hepatic portal vein blood flow rate was 34.5 ± 2.6, 34.2 ± 3.3, 35.0 ± 3.5, 36.5 ± 3.6 and 40.2 ± 3.8 mL/min/kg body weight, respectively, at 10, 20, 30, 45 and 60 min after injection. Since the blood flow rate did not change significantly

in response to the administration ($p>0.05$), the average value was 36.0 ± 0.9 mL/min/kg body weight before and after the administration (Table 4).

Hepatic Availability of TTX

First of all, we estimated the hepatic first pass-effect of TTX in *T. rubripes* by comparing AUC following a bolus injection of 0.25 mg TTX/kg body weight into hepatic portal vein or hepatic vein for 300 min. As the result, time courses of the blood concentration of TTX by the two injection routes were closely similar to each other (Fig 4). As shown in Table 3, AUCs obtained by the injections into hepatic portal vein and hepatic vein were estimated to be 147 ± 33 and 141 ± 1 ng*min/μL, respectively, indicating negligible hepatic first pass-effect of TTX in *T. rubripes*.

Next, we investigated in detail the hepatic uptake clearance (CL_{uptake}) of TTX in *T. rubripes* by integration plot analysis after a single bolus injection of 0.25 mg TTX/kg body weight into hepatic vein at 20°C. The time courses of TTX concentrations in the blood and tissues after injection are shown in Fig 5. The pharmacokinetic parameters are summarized in Table 4.

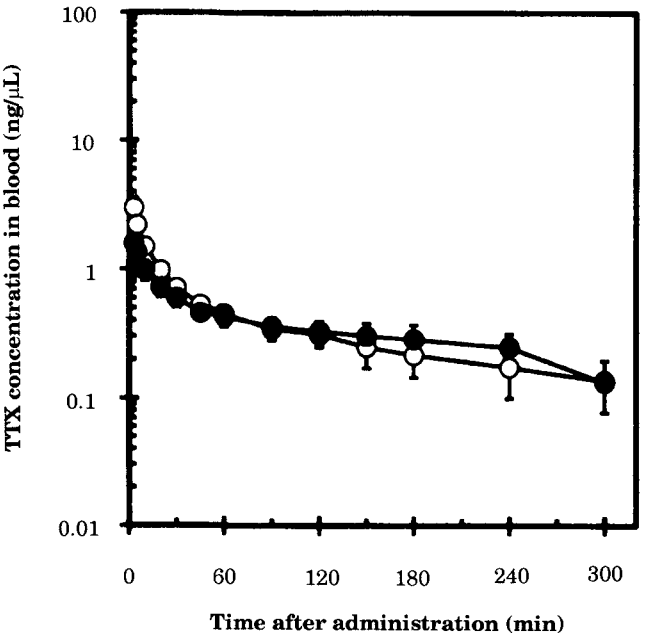

Fig 4. Effect of administration site on the time course of blood concentration of TTX in *Takifugu rubripes*. The time course was measured after a single bolus injection of 0.25 mg TTX/500 μL/kg body weight into hepatic portal vein (○) or hepatic vein (●). Each point and vertical bar represent the mean ± SE of three individual experiments performed in triplicate

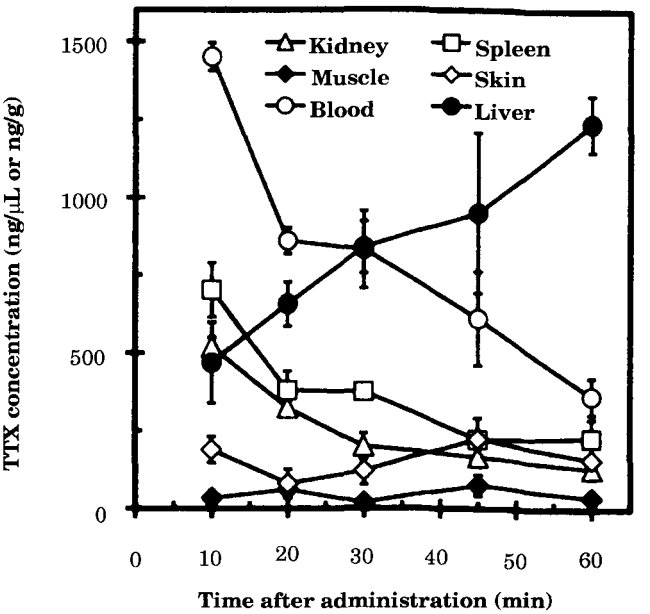

Fig 5. Time course of TTX concentration in the blood and tissues after a single bolus injection of 0.25 mg TTX/500 μL/kg body weight into hepatic vein in *Takifugu rubripes*. Each point and vertical bar represent the mean ± SE of four individual experiments performed in triplicate

Table 4. Pharmacokinetic parameters for the hepatic uptake of TTX in *Takifugu rubripes*

Pharmacokinetic parameters	**Values**
Hepatic portal vein blood flow (Q)	36.0 ± 0.9 mL/min/kg body weight
Hepatic uptake clearance (CL_{uptake})	3.1 mL/min/kg body weight
Hepatic extraction ratio (E_h)	9%
Hepatic availability (F_h)	91%

The blood concentration of TTX decreased over time after the injection, from 1450 ± 45 ng/mL at 10 min to 364 ± 59 ng/mL at 60 min. TTX concentrations in the spleen and kidney decreased in parallel with the blood concentrations. Briefly, TTX concentrations of the spleen and kidney were 702 ± 87 and 520 ± 31 ng/g at 10 min and decreased to 229 ± 61 and 129 ± 31 ng/g at 60 min after injection, respectively. However, TTX concentrations in the skin and muscle remained almost the same throughout the experiment at the low level. The TTX concentration in the skin varied from 79 ± 47 ng/g to 224 ± 68 ng/g, and that in the muscle varied from 19 ± 7 ng/g to 75 ± 34 ng/g ($p>0.05$). In contrast, the TTX concentration in the liver gradually increased after the injection, from 469 ± 130 ng/g at 10 min to 1240 ± 90 ng/g at 60 min.

The hepatic uptake clearance (CL_{uptake}) of TTX in *T. rubripes* was estimated by integration plot analysis (Table 4). As shown in Fig 6, the integration plot showed a good linearity (r=0.981) with the slope of the linear function corresponding to the hepatic uptake clearance (CL_{uptake}) and the slope was 22.7 μL/g liver. When the uptake clearance was converted from (μL/g liver) to (mL/kg body weight) using the average (n = 20) of the liver weight (142 ± 5.9 g liver) and body weight (1.03 ± 0.01 kg body weight), the value was calculated to be 3.1 mL/kg body weight. The hepatic extractability of TTX (E_h), estimated by dividing CL_{uptake} by the hepatic portal vein blood flow rate (36.0 ± 0.9 mL/kg body weight as described above) was at most 9%. Accordingly, the hepatic availability (*viz.* the avoidance rate of hepatic first-pass effect) (F_h) was calculated to be 91%.

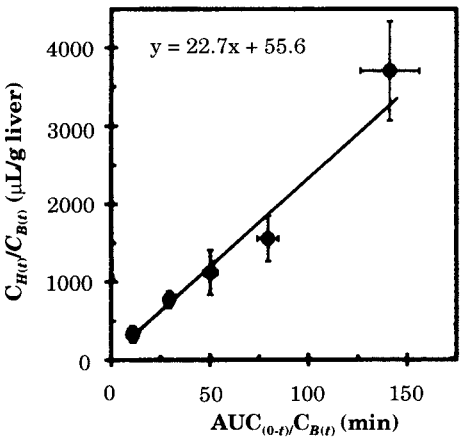

Fig 6. Integration plots for evaluation of the hepatic uptake clearance of TTX in *Takifugu rubripes*. Each point and vertical or cross bar represent the mean ± SE of four individual experiments performed in triplicate

Time Course of Hepatic Accumulation of TTX

At the end of each experiment, the amount of TTX that had accumulated in the liver after the injection was determined. When the fish received a single administration of 0.25 mg TTX/kg body weight into hepatic vein, hepatic portal vein or gastrointestinal tract, the amount of TTX that had accumulated in the liver 300 min after injection was 213 ± 7, 169 ± 37 or 131 ± 48 μg/liver, respectively, which accounted for 84 ± 6, 70 ± 9 or 49 ± 17% of the administered dose, respectively ($p>0.05$) (Fig 7A). There was a good correlation (r=0.84) between the administered dose and the hepatic amount of TTX that had accumulated for 300 min after a single bolus injection at the dosage ranging from 0.25 to 0.75 mg TTX/kg body weight into the hepatic portal vein (Fig 7B).

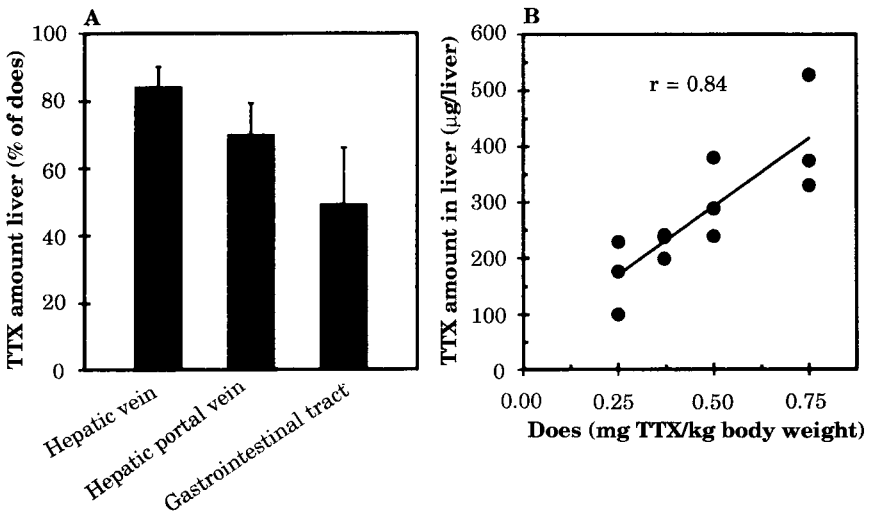

Fig 7. Amount of TTX in the liver of *Takifugu rubripes* after a single adminstration. (*A*) The ratio of the amount of TTX in the liver to the administered dose. The amount of TTX was determined 300 min after administration of 0.25 mg TTX/kg body weight into the hepatic vein, hepatic portal vein and gastrointestinal tract. Each column and vertical bar represent the mean ± SE of three individual experiments performed in triplicate. A comparison showed no significant differences among data ($p>0.05$). (*B*) Correlation between the amount of TTX in the liver 300 min after a single bolus injection and the administered dose of 0.25–0.75 mg TTX/kg body weight into the hepatic portal vein. Line is drawn by linear regression analysis

In another series of the administration test, changes in the TTX amount in several tissues were measured in shorter time (Table 5). TTX amount in the liver was increased over time after a single bolus injection of 0.25 mg TTX/kg body weight into hepatic vein and estimated to be 75 ± 19, 96 ± 16, 111 ± 10, 126 ± 28 and 166 ± 12 μg/tissue at 10, 20, 30, 45 and 60 min after injection, respectively. There was a significant difference between the value at 10 min and that at 60 min ($p<0.05$). In contrast, TTX amounts in spleen and kidney admittedly decreased with a decrease in the blood concentration of TTX (Fig 5 & Table 5). TTX amount in spleen was 1.1 ± 0.3 μg/tissue at 10 min and down by half at 60 min ($p<0.05$). TTX amount in kidney was 5.5 ± 0.4 μg/tissue at 10 min and also significantly decreased to be 1.5 ± 0.4 μg/tissue at 60 min ($p<0.05$). Intriguingly, the amounts of TTX in the muscle and skin did not show significant changes throughout the experiments ($p>0.05$). The ratio of the amount of TTX that had accumulated in the liver after injection to the administered dose increased with the course of time ($r=0.99$), and accounted for 28 ± 7, 38 ± 6, 44 ± 4, 49 ± 12 and 63 ± 5% at 10, 20, 30, 45 and 60 min after injection, respectively.

Table 5. The amounts of TTX in the tissues after the single bolus injection into hepatic vein in *Takifugu rubripes*

Time (min)	Body weight (kg)	Dose (μg/fish)	TTX amount (μg/tissue)						Total accumulation (% of dose)
			Spleen	Kidney	Skin	Muscle	Liver	Total	
10	1.05 ± 0.02[a]	263 ± 5.8[a]	1.1 ± 0.3[a,x]	5.5 ± 0.4[a,x]	27 ± 5.5[a]	06.4 ± 3.3[a]	75 ± 19[a]	115	44
20	0.99 ± 0.01[a]	248 ± 3.2[a]	0.6 ± 0.1[a,b]	3.6 ± 0.2[b,x]	11 ± 6.6[a]	16.± 5.1[a]	96 ± 16[a,b]	127	51
30	1.02 ± 0.02[a]	256 ± 3.9[a]	0.8 ± 0.2[a,b]	2.4 ± 0.4[b,c]	17 ± 5.9[a]	4.8 ± 1.9[a]	111 ± 10[a,b]	136	53
45	1.04 ± 0.04[a]	261 ± 8.8[a]	0.3 ± 0.2[b,x]	1.8 ± 0.5[c,x]	35 ± 12[a]	20 ± 8.2[a]	126 ± 28[a,b]	182	70
60	1.06 ± 0.02[a]	266 ± 6.2[a]	0.4 ± 0.1[a,b]	1.5 ± 0.4[c,x]	24 ± 8.8[a]	11.± 4.0[a]	166 ± 12[b]	202	76

Values are the mean ± SE of four individual experiments performed in triplicate. Different superscripts in the same column indicate significant differences among the groups ($p<0.05$ by Tukey's test).

In vitro Uptake Assay of TTX into Liver Slices

Time Course of TTX Accumulation in the Liver Slices

The time profile of TTX uptake by the liver tissue is shown in Fig 8. The liver slices obviously accumulated TTX as reported previously (Nagashima *et al.*, 2003; Matsumoto *et al.*, 2005). In the experiment where TTX concentration in the transport buffer was 50 μM, TTX content in the liver slices was 134 ± 14 pmol/mg protein even 5 min after incubation and significantly increased with the time to 977 ± 77 pmol/mg protein at 90 min ($p<0.05$).

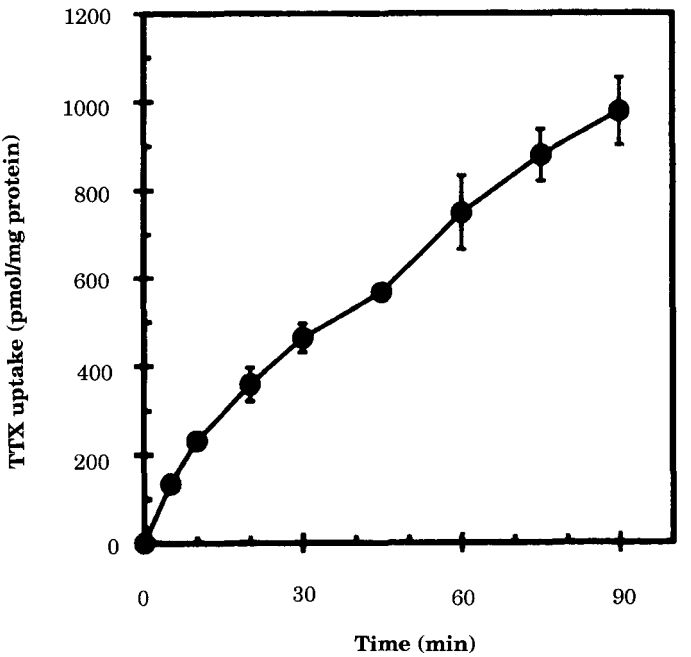

Fig 8. Time course of TTx uptake by the liver slices to *Takifugu rubripes*. The liver slices were incubated with the transport buffer containing 50 μM TTX at 20°C for up to 90 min. Values are the mean ± SE of three individual experiments performed in triplicate

Concentration Dependence of the TTX Uptake Rate by the Liver Slices

The TTX uptake rate (pmol/min/mg protein) into the puffer fish liver slices was assessed with various concentrations of TTX in the transport buffer at 20°C for 60 min. As shown in Fig 9A, the uptake rates were dependent on TTX concentration in the transport buffer and exhibited a non-linear curve, suggesting the involvement of a saturable process in the TTX uptake by the puffer fish liver slices. Eadie-Hofstee analysis revealed that the TTX uptake was composed of a saturable process and a non-saturable process, since the two-phase curve was obtained (Fig 9B). The apparent maximum velocity,

V_{max} and Michaelis constant, K_m of the saturable component were estimated to be 47.7 ± 5.9 pmol/min/mg protein and 249 ± 47 μM, respectively. The apparent uptake clearance,

P_{dif} of the non-saturable component was calculated to be 0.0335 ± 0.0041 μL/min/mg protein. These results reveal the involvement of saturable transport system in the uptake of TTX into the liver slices of *T. rubripes*.

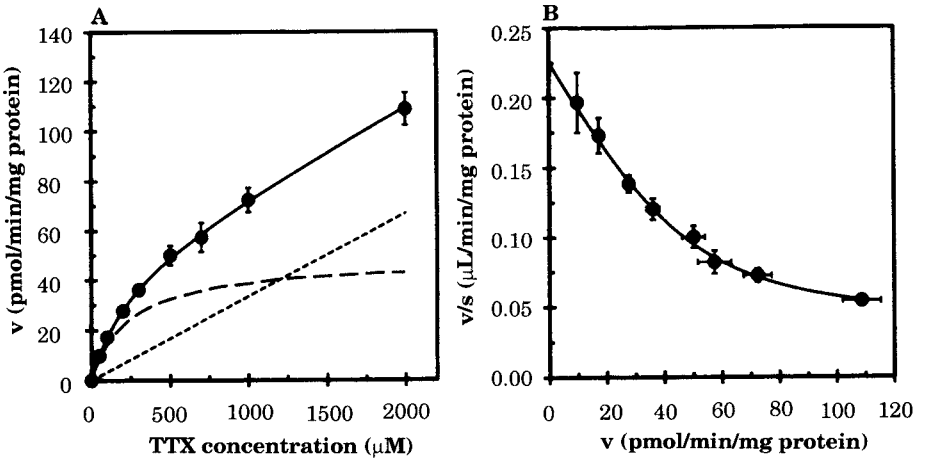

Fig 9. Effect of TTX concentration in the transport buffer on TTX uptake into the liver slices of *Takifugu rubripes*. (*A*) The liver slices were incubated with the transport buffer containing 0–2000 μM TTX at 20°C for min. Dashed and dotted lines indicate the saturable and the non-saturable uptake obtained from non-linear least-squares regression analysis. (*B*) Eadie-Hofstee polt of the uptake of TTX. Values are the mean ± SE of three individual experiments performed in triplicate

Inhibition Assay on the TTX Uptake into Liver Tissue Slices

It is likely that the TTX uptake by the liver slices was dependent on temperature, since the uptake rate at 5°C significantly decreased to 0.4 fold of that at 20°C ($p<0.05$) (Table 6). Furthermore, a replacement of sodium-ion by choline markedly reduced the TTX uptake to 0.6 fold of the control ($p<0.05$). Addition of 1 μM L-carnitine to the transport buffer slightly inhibited the TTX uptake, although the difference was not statically significant. None of other substrates tested such as TCA, PAH and TEA showed the inhibitory effect on TTX uptake by the liver slices.

Table 6. Effects of replacement of sodium with choline, temperature and transporter inhibitors on the TTX uptake into the liver slices of *Takifugu rubripes*

Compound	Uptake (% of control)
Control (20°C)	100 ± 14
Na^+ replacement	63 ± 4*
Low temperature (5°C)	40 ± 5*

Table 6. *Contd.*

Compound	Uptake (% of control)
1 μM TCA	100 ± 13
1 μM PAH	96 ± 5
1 μM TEA	102 ± 8
1 μM L-carnitine	85 ± 3

Values are the mean ± SE of three individual experiments performed in triplicate. *p<0.05 (Significantly different from control by Student's t-test)

Liver-Specific Uptake of TTX by the Puffer Fish

Another incubation experiment was carried out to investigate the variation among fish species in the TTX uptake by liver slices. In the experiment, the transport buffer was slightly modified; 200 μM NaCl and 10 μM HEPES. The uptake rate of TTX by the liver slices of puffer fish *T. rubripes* at 20°C increased in a TTX-concentration dependent manner as the same as in Fig 9A and attained to 127 ± 7 pmol/min/mg protein at 2000 μM TTX (Fig 10). As to black scraper liver slices, in contrast, the uptake rate at 20°C was 25 ± 3 pmol/min/mg protein (mean ± SD of one experiment) at 2000 μM TTX and almost the same as that at 5°C (data not shown). This result confirms the distinct uptake of TTX by the puffer fish liver and suggests the special function(s) to take up and accumulate TTX in the puffer fish liver.

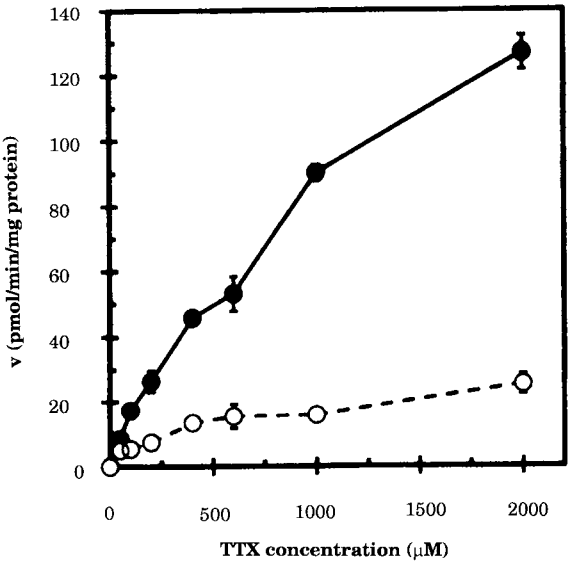

Fig 10. Comparison of the TTX uptake into the liver slices between puffer fish *Takifugu rubripes* and black scraper *Thamnaconus modestus*. The liver slices prepared from puffer fish (●) and black scraper (○) were incubated with the transport buffer containing 0–2000 μm TTX at 20°C for 60 min, respectively. Values are the mean ± SE of four individual experiments for puffer fish and the mean ± SD of an experiment for black scraper. Each determination was performed in triplicate.

DISCUSSION

We report here the pharmacokinetics of TTX in the puffer fish *T. rubripes* by the *in vivo* single administration and by *in vitro* incubation assay with the use of the liver slices.

In the *in vivo* study, the total body clearance (CL_{total}) and the hepatic portal clearance (CL_{hp}) of TTX in *T. rubripes* were quite small compared with the hepatic portal vein blood flow rate, even though these clearances were regarded as the hepatic clearance. Accordingly, it is reasonable that the hepatic first-pass effect of TTX in *T. rubripes* was negligible. Actually, the hepatic uptake clearance (CL_{uptake} = 3.1 mL/min/kg body weight) was significantly small as compared with the value of hepatic portal vein blood flow rate (36.0 ± 0.9 mL/min/kg body weight). On the basis of our data, it is likely that the membrane transport into hepatocyte from extracellular space is the rate limitation step for the hepatic uptake of TTX. Since the hepatic uptake clearance gives maximum value of hepatic clearance, this is in a good agreement with negligible hepatic extraction.

In addition, Nagashima *et al.* (1999) investigated the subcellular distribution of TTX and its analogs in the hepatocytes of the marine puffer fish *T. pardalis* and *T. snyderi* by the differential centrifugation method and revealed that the toxin amount was significantly higher in the cytosol fraction (82.2 ± 8.7%) than in the other fractions: blood cell fraction (5.6 ± 3.7%), nuclear fraction (5.4 ± 4.2%), mitochondrial fraction (5.4 ± 2.3%) and microsomal fraction (1.4 ± 0.5%). These investigations strongly supported that TTX in the systemic circulation is taken up into the cytosol fraction by carrier-mediated uptake system.

Fig 11 depicts the bioavailability of TTX after a single administration of 0.25 mg TTX/kg body weight into gastrointestinal tract in *Takifugu rubripes*.

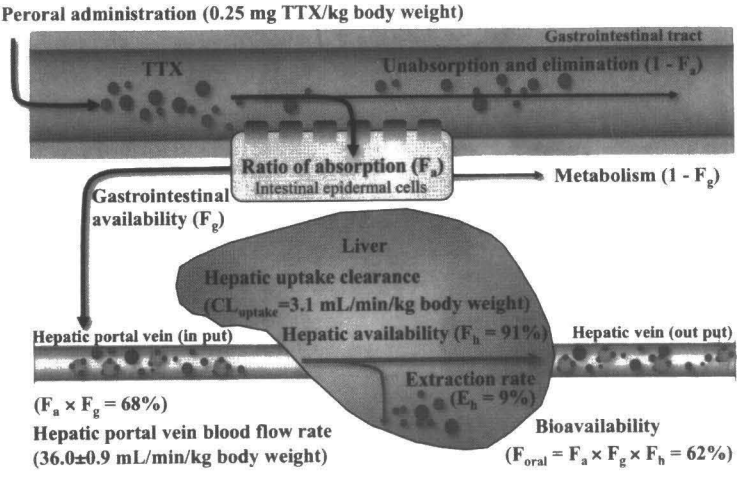

Fig 11. Bioavailability of TTX after a single administration of 0.25 mg TTX/kg body weight into gastrointestinal tract in *Takifugu rubripes*

Gastrointestinal absorption of TTX is also important for the toxification of puffer fish, since TTX orally taken up has to pass through the gastrointestinal tract. This study evidenced that TTX is transferred across gastrointestinal epithelia into the systemic circulation in *T. rubripes* (Fig 2). Considering negligible hepatic first-pass effect, the fraction of absorption was approximately 60% at 300 min after a gastrointestinal administration of 0.25 mg TTX/kg body weight. Furthermore, the time of maximum blood concentration of TTX was delayed from 30 min to 150 min after a gastrointestinal administration with the dose escalation from 0.25 to 1.00 mg TTX/kg body weight, and AUCs after the administration of 0.50 and 1.00 mg TTX/kg body weight were almost the same at around 230 ng•min/μL. Since the pharmacokinetics of TTX following an administration into the hepatic vein was not saturated at the doses examined, it is likely that these are ascribed to the saturation of gastrointestinal absorption. Accordingly, these data also suggests an involvement of saturable mechanisms for relatively high gastrointestinal absorption of TTX.

In the *in vitro* experiment, we examined the involvement of carrier-mediated transport system in uptake of TTX into the liver slices of *T. rubripes*. The uptake of TTX into the liver slices showed clear concentration dependence and comprised a saturable component (K_m 249 ± 47 μM and V_{max} 47.7 ± 5.9 pmol/min/mg protein) and a non-saturable component (P_{dif} 0.0335 ± 0.0041 μL/min/mg protein). In nature, it is thought that *T. rubripes* exclusively takes up TTX from systemic circulation to the liver using this saturable mechanism, since TTX concentration in the blood of wild puffer fish was negligible and less than 10 mouse unit/mL, equivalent to 6.3 μM TTX (Hashimoto, 1979; Halstead, 1988).

Liver selectively transports endogenous and exogenous compounds from the systemic circulation to accumulate, metabolize or detoxify them using various transport systems. Hepatic uptake of organic anions, organic cations, organic solutes and bile salts is mediated by transporter proteins such as organic anion transporting polypeptides (OATP), organic anion transporter (OAT), organic cation transporter (OCT), organic solute transporter (OST) and sodium taurocholate cotransporting protein (NTCP) (Faber *et al.*, 2003; Petzinger & Geyer, 2006; Shitara *et al.*, 2006). However, to our knowledge, there are no reports concerning the characterization of carrier-mediated uptake in puffer fish liver. The TTX uptake by *T. rubripes* liver slice was temperature-dependent and sodium-ion dependent (Table 6). Whereas the TTX uptake was barely affected by TCA, PAH and TEA, which are the typical substrates of NTCP, OAT and OCT, respectively (Inui *et al.*, 2000; Geyer *et al.*, 2006), it was slightly depressed by L-carnitine (Table 6). It is possible that organic cation/carnitine transporter is involved in TTX uptake, since TTX has a pKa8.76 and is amphoteric in a neutral pH (Goto *et al.*, 1965). Our

results indicate that TTX is taken up through cell membrane of the puffer fish liver by the active transport mechanism that is dependent on sodium-ion gradient. Unlike *T. rubripes*, the liver slices of black scraper *Thamnaconus modestus*, non-toxic fish, showed a low uptake activity (Fig 10) and independence of incubation temperature, suggesting that carrier-mediated uptake of TTX is limited to puffer fish. Further study is needed to identify and characterize the responsible TTX transporter and elucidates the distribution of TTX transporter among TTX-bearing animals.

Interestingly, it has been reported that puffer fish often contained paralytic shellfish toxins (PSTs) that are produced by marine dinoflagellates and fresh water cyanobacteria and have the selective blocking action against the voltage-gated sodium channel in the same manner as TTX (Briceji & Shumway, 1998). Kodama *et al.* (1983) first found a trace amount of saxitoxin (STX) in the liver of marine puffer fish *T. pardalis* in Japan, besides TTX as the major toxin component. Fresh water puffer fish *Tetraodon leiurus*, *Tetraodon suvatii* and *Tetraodon fangi* in Thailand (Kungsuwan *et al.*, 1997; Sato *et al.*, 1997), *Tetraodon cutcutia* in Bangladesh (Zaman *et al.*, 1997; 1998) and *Colomesus asellus* from the rivers of the Amazon in Brazil (Oliveira *et al.*, 2006) were determined to contain PSTs as the major toxic principle, being often toxic enough to cause food poisoning incidents including even death. Furthermore, seven species of tropical marine puffer fish, *Arothron mappa*, *Arothron manillensis*, *A. nigropunctatus*, *A. reticularis*, *A. hispidus*, *A. stellatus* and *Chelonodon patoca* collected in Philippines were reported to contain considerable amounts of PSTs along with TTX (Sato *et al.*, 2000). Nakashima *et al.* (2004) found STX as the major toxin component in the skin of marine puffer fish *A. firmamentum* caught along the coasts in Japan. Very recently, Landsberg *et al.* (2006) found STXs in marine puffer fish *Sphoeroides nephelus*, *S. testudineus* and *S. spengleri* from the coasts in Florida, U.S. Janga and Yotsu-Yamashita (2006) investigated in detail the toxin composition among tissues of marine puffer fish *T. pardalis* collected in Miyagi Prefecture, Japan, and reported that TTX and its analogs were the major toxins in ovary and liver, and STX were also detected in the tissues as the minor principles.

We demonstrated that the *T. rubripes* liver slices specifically accumulated TTX in preference to STXs (Matsumoto *et al.*, 2005). This finding indicates that puffer fishes, particularly genus *Arothron*, *Sphoeroides* and *Tetraodon* instead of *Takifugu* would be able to accumulate PSTs as the major toxin component in their bodies.

Nevertheless, puffer fish accumulates a high concentration of TTX in the liver (Halstead, 1988; Soong & Venkatesh, 2006; Hwang & Noguchi, 2007). Indeed, the accumulation of TTX in the liver reached 80% of the administered dose within 300 min following a bolus injection into the hepatic

vein, and TTX amount in the liver of *T. rubripes* showed a dose-dependent increase (Fig 7). In addition, this study reveals that the TTX pharmacokinetics was not saturated after a single bolus injection of 0.25–0.75 mg TTX/kg body weight into hepatic portal vein (Table 2). This is reasonable considering the fact that the unbound concentration of TTX reached at highest dose was at most 19 μM, which was considerably smaller than the K_m value (249 ± 47 μM) determined *in vitro* using liver slices.

In the integration plot study, the TTX concentration in the liver also increased over time after the injection of 0.25 mg TTX/kg body weight into hepatic vein and the amount of TTX that had accumulated in the liver 60 min after injection accounted for 63 ± 5% of the administered dose. In contrast, lienal and kidney concentrations of TTX were decreased in parallel with the blood concentrations. Unlike the liver, spleen and kidney, TTX concentrations in the muscle and skin remained almost the same throughout the experiment ($p>0.05$) (Fig 5). It seems that tissues are classified into at least three groups in terms of pharmacokinetics of TTX in *T. rubripes*; 1) kidney and spleen, tissues in which TTX concentration is instantaneously proportional to the blood concentration (the central compartment), 2) liver in which TTX is accumulated (the peripheral compartment operating as the major elimination process) and 3) muscle and skin, tissues in which TTX concentration is independent of the blood concentration (the peripheral compartment including the low distribution rate). Time course of the gonad concentration of TTX after the administration was not examined, since the puffer fish used in the present study was not matured.

In the present study, we demonstrated that TTX is absorbed from the gastrointestinal tract into systemic circulation and dominantly accumulated into the liver in *T. rubripes* within a relatively short time. Our study also suggests an involvement of saturable mechanism for the gastrointestinal absorption and liver-specific uptake of TTX in *T. rubripes*. Although it has been suggested that the liver-specific accumulation of TTX in *T. rubripes* is accounted for by the carrier-mediated uptake mechanism, the molecular characteristics of the TTX transporter have not be identified, yet. On the basis of the above findings, we concluded that TTX is stored in liver as a first step of the toxification in *T. rubripes*.

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Herbal Drugs - Applications in the Post Genomic Era

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ABSTRACT

Natural products are increasingly being used as an alternative to synthetic chemical compounds as effective therapeutic agents. In this post genomic era, the applications of genomic technologies in the field of herbal medicine and natural products are many. For instance, they may be used for the discovery of new diagnostic and therapeutic compounds; elucidation of molecular mechanism of action of phytochemical components and identification and validation of new molecular targets for herbal drug development. They may be used in pharmacogenomics for the prediction of potential side-effects and interactions of the herbal drug, in the identification of genes responsible for conferring drug sensitivity or resistance and prediction of patients most likely to benefit from the herbal drug. Additionally, these technologies find application in pharmacognosy for the standardization and quality control of plants and plant products. This article reviews the more recent developments and the future trends in the field of herbal medicine.

Key words : Pharmacogenomics, genomic technologies, drug discovery, adverse effects, herb-drug interactions, future trends

INTRODUCTION

Modern medicine has its roots in the indigenous or traditional systems of medicine, which relied heavily on natural products as therapeutic agents or

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remedies. Among these, plants are a valuable source of bioactive compounds that may potentially be used as drugs. Examples of plant products and derivatives used by the pharmaceutical industry include paclitaxel, vincristine, vinblastine, artemisinin, camptothecin, podophyllotoxin, etc. It seems very likely that natural products may play a very important role in the future of drug discovery and development.

As we enter the post-genomic era, the traditional pharmacology or chemistry based approach is giving way to the pharmacogenomic strategy. There has been a substantial increase in our knowledge of the genetic basis of disease and the genomic factors affecting its treatment in the past decade or so. Genomic technologies find several applications in new drug discovery and the characterization of older drug entities as well. Consequently, the fields of pharmacogenomics and pharmacogenetics have had a tremendous impact on our understanding of herbal drugs.

PHARMACOGENETICS AND PHARMACOGENOMICS

Pharmacogenetics is the study of the hereditary basis for differences in populations response to a drug. It is known that individuals tend to show variations in their response to specific drugs. The same dose of a drug will result in elevated plasma concentrations for some patients and low concentrations for others. Some patients will respond well to the drugs, while others may show no response at all. A drug might show adverse effects in some patients but not in others. These variations are a result of a multitude of complex factors including ethnicity, age, sex etc. Generally speaking, humans are classified into three major groups: The Negroid, Mongoloid, and Caucasoid, who are genetically 99.9% identical. The differences between these groups are due to single nucleotide polymorphisms (or SNPs) (Patwardhan *et al.*, 2004). These variations in genes cause different members of a population to have differences in the gene expression, resulting in different drug metabolizing enzymes and/or sites of drug action (McCarthy, 2001). This can lead to different responses to these drugs. Measuring the DNA differences can thus predict the variation in response to the medicine. Determining who will respond favourably to a particular therapy is clinically very significant as it helps in the selection of the most appropriate therapeutic regimen. This is especially important when different populations are known to be more prone to adverse effects of a particular drug. Pharmacogenomics, on the other hand deals specifically with genetic variability in drug response. It involves the assessment of drugs on specific tissues and is concerned with the overall evaluation of the expression pattern caused due to the action of a specific compound (Sadee, 1999). Unlike pharmacogenetics, it is a study of gene expression due to a drug without taking into account differences in individual responses. Thus, although these fields are interrelated, the scope of pharmacogenetics and pharmacogenomics are distinct: the former will

help in the clinical setting to find the most appropriate medicine for a patient, while the latter in the setting of pharmaceutical and herbal research and development to find the best drug candidate from a given series of compounds under evaluation (Lindpaintner, 2002).

The advent of genomic medicine has ensured that the concept of 'Personalized Medicine' may soon become a reality. It has the potential to revolutionize the way new drugs are discovered, characterized and studied for safety and efficacy. Success stories of agents like trastuzumab (for the treatment of HER-2 positive breast cancer) and abacavir (against HIV and AIDS) have demonstrated this fact. Herbal medicine too, stands to gain immensely from these technologies and pharmacogenomics has already begun to make its mark in the discovery and development of natural products as effective therapeutic agents.

MICROARRAY CHIP TECHNOLOGY

Novel technology in the form of DNA microarray chips is now making its presence felt in the field of traditional herbal medicine. This technology makes it possible to scan the entire human genome for relevant polymorphisms (Service, 1998) to identify those which influence the response to a particular botanical product. A DNA microarray (also commonly known as gene or genome chip, DNA chip, or gene array) is a collection of microscopic DNA spots, commonly representing single genes, arrayed on a solid surface by covalent attachment to a chemical matrix. This high-throughput methodology allows for the simultaneous screening of thousands of SNPs in a given sample and can be used for expression profiling or for comparative genomic hybridization. In fact, the sensitivity of the microarray technique has improved to such an extent that samples of even 10 to 50 nanograms can be analyzed (Russel, 2002; Relógio *et al.*, 2002). Genome-wide association studies are already being used in the discovery of susceptibility genes for diseases such as asthma (Grant & Hakonaeson, 2008) and prostate cancer (Nam *et al.*, 2008), but they are also equally suitable for determining the genes involved in drug response. Such a study was conducted at the Mayo Clinic (USA) for determining the genes involved in the response to the thiazide class of diuretics (Turner *et al.*, 2008). Similarly, the responses to botanical medicines can also be studied. The advantage of genome-wide scanning is that it can help to identify genes even if the mechanism of action of the drug in the body is unknown.

APPLICATIONS OF PHARMACOGENOMICS

Our knowledge of pharmacogenomics and the pharmacogenetics has several applications in the field of herbal medicine. It may be used for the discovery of novel bioactive compounds from natural sources, as well as for the prediction of the safety and efficacy of herbal drugs. Elucidation of their

molecular mechanisms of action is also possible. Additionally, genomic technologies may help to identify potential herb-drug interactions. Complementary DNA microarrays (or cDNA microarrays) are commonly used high-throughput screening technologies that allow for the screening of the entire genome in a relatively short period of time. A single microarray can now be used to screen 100,000 SNPs found in a patient's genome in a matter of hours. As DNA microarray technology is developed further, SNP screening may well become part of routine clinical practice to determine the most suitable therapy for individual patients.

HERBAL DRUG DISCOVERY AND DEVELOPMENT

Advances in high-throughput technologies have resulted in huge databases of genomic, proteomic and chemical data which in combination with efficient separation methods and powerful spectrometric methods for identification and structure elucidation can be used for identification of bioactives. Herbal products are usually whole herbs, their formulations or extracts consisting of several such compounds. With the increased demand for scientifically validated and standardized herbal product there is a need for better understanding of the molecular mechanisms underlying their biological activity. Genomic technologies are now being increasingly used in herbal medicine as an effective tool for drug discovery and elucidation of its molecular targets. This will allow for the investigation and the evaluation of bioactives in a manner comparable to chemically synthesized molecules. Research has determined that genomic technologies have the capacity to identify therapeutic efficacy on the basis of gene expression signatures *in vitro* has potential utility in drug discovery and drug target validation (Gunther *et al.*, 2003).

In the field of anti-cancer agents, microarray-based profiling has been shown to be helpful to discover possible targets of these agents and related upstream (including ATP-binding cassette (ABC) transporters, reduced folate carriers, and nucleoside transporters) and downstream mechanisms (apoptosis, for example), which also influence the response of tumor to the drug molecule (Efferth *et al.*, 2007).

The use of genomic technologies has been especially useful for the study of camptothecin and its derivatives. The microarray-based expression profiling of 149 genes significantly predicted response to camptothecin in a panel of 30 colon carcinoma cell lines (Mariadson *et al.*, 2003). In another study, KB cervical carcinoma cells selected for resistance to 9-nitrocamptothecin showed a dramatic overexpression of *ABCB1* (*MDR1*) and *ABCC2* (*MRP2*) in microarray hybridization, while glutathione S-transferase-π was downregulated (Annereau *et al.*, 2004). Allied genomic technologies, like comparative metabolomics, which can be used for monitoring disease development, drug metabolism and chemical toxicology, are particularly useful in phytomedicine research. Metabolomes of medicinal plants are a

valuable source for the development of new herbal drugs. The understanding of metabolite signatures in extracts of herbal remedies or traditional medical plants is therefore essential, since secondary metabolites have the ability to inhibit targets which imparts therapeutic value (Ahn & Wang, 2008).

Several botanical constituents in the herbal drug PC-SPES inhibit tumor growth through cell cycle arrest and apoptosis. LNCaP prostate carcinoma cells were treated with PC-SPES, and changes in gene expression were determined by complementary DNA (cDNA) microarray hybridization and northern blot analyses. mRNA levels of α-tubulin were observed to have decreased sevenfold. This indicated that PC-SPES might possibly be interfering with microtubule polymerization (Bonham *et al.*, 2002). This activity has implications for the clinical management of patients with advanced prostate cancer who may be taking PC-SPES concurrently with microtubule-modulating chemotherapeutic agents, such as paclitaxel. Further, cDNA microarray analysis was used to identify gene expression changes in LNCaP prostate carcinoma cells exposed to PC-SPES and estrogenic agents including diethylstilbestrol. Transcripts encoding cell cycle-regulatory proteins, α- and β-tubulins, and the androgen receptor were down-regulated by PC-SPES. A comparison of gene expression profiles resulting from these treatments indicates that PC-SPES exhibits activities distinct from those attributable to diethylstilbestrol and suggests that alterations in specific genes involved in modulating the cell cycle, cell structure, and androgen response may be responsible for PC-SPES-mediated cytotoxicity (Bonham *et al.*, 2002; Chavan *et al.*, 2006).

PREDICTON OF DRUG RESPONSE AND ADVERSE EFFECTS

Individual responses to the same drug may vary considerably. For example, cure rates with combined surgical and drug treatment of advanced colorectal carcinoma range from 20% to 40%, while the remainder of the patients experience little gain or even severe toxicity from chemotherapy. It is thus important to determine the SNP responsible for the response (or the lack of response) the drug having optimal benefits for that patient may be selected. In modern medicine, several attempts have been made to develop microarray genotyping system for the analysis of a panel of SNPs in genes encoding proteins involved in the prediction of response to different therapeutic agents. One such study was carried out for blood pressure regulation, and this system was applied to a pilot study demonstrating its feasibility in the pharmacogenetics of anti-hypertensive drug response (Schwartz & Turner, 2004). DNA microarrays may provide a suitable high-throughput platform for research and development of drugs from natural products. In natural products a broad repertoire of chemical entities act together on multiple targets that makes it necessary to study the changes in expression of multiple genes simultaneously. Novel technologies such as SAGE and DNA microarrays allow rapid and detailed analysis of thousands of transcripts,

providing a revolutionary approach to the investigation of gene expression. Thus, DNA microarray techniques might prove to be a reliable basis for predicting response (or lack of response) of individuals to herbal drugs.

Adverse Drug Reactions

All medicinal agents have potentially unexpected effects including toxicity, and herbals are no exception. As with other drugs, the risk of unexpected effects may be influenced by a user's age, gender, genetics, nutrition status, and concurrent disease states and treatments. As medicinal agents, herbal products should be regarded as having the same potential for unwanted effects as patented drugs. Unfortunately, a recent review of randomized, controlled trials evaluating herbal medicine revealed that only 15% of studies provided information on safety or side effects (Boullata & Nace, 2000). Adverse effects or adverse drug reactions (ADRs) are generally acknowledged to be one among the leading causes of death worldwide (Table 1). A study suggested that ADRs caused over 2.2 million serious conditions and were responsible for more than 100 000 deaths in the United States in 1994 (Lazarou, 1998). Thus the management of these adverse effects remains an important clinical problem. A drug might show adverse effects in some patients, but not in others. Populations and enzyme polymorphisms are known. For example, CYP2D6 (a variant of the enzyme cytochrome P450), an enzyme that metabolizes several commonly used drugs, shows great variability in individuals: some individuals are poor metabolizers, while others are rapid metabolizers. While 5–10% Blacks and Caucasians are poor metabolizers, few Asians are poor metabolizers. Ethiopians and Saudi Arabian are ultrarapid metabolizers (Patwardhan *et al.*, 2004). Recent studies have indicated that gender, too, may affect the activity of certain enzymes of the cytochrome P450 superfamily (Scandyln *et al.*, 2008). Genomic technologies may help to predict an individual's susceptibility towards a particular herbal drug and can help to prevent potential adverse effects. Of the limited number of dietary supplement adverse event reports to the FDA in 1998, 7% involved St. John's wort, 8% *Ginkgo biloba*, and at least 17% ephedra-containing products.

The use of some herbal supplements has been reported to be associated with oral manifestations, including aphthous ulcers, lip and tongue irritation, and swelling with feverfew; gingival bleeding with feverfew and ginkgo; tongue numbness with echinacea; xerostomia with St. John's wort; oral and lingual dyskinesia with kava; and salivation with yohimbe (Abebe, 2003).

Table 1. Adverse effects of commonly used herbal drugs

Common Herbs	Adverse Effects
Chast tree fruit	Diarrhoea
Ephedrine (*ma huang*)	Hypertension, cardiac arryt ·mias,anxiety, restlessness, tremors, myocardial infarction, ·erebrovascular event, renal stones, seizures

Table 1. *Contd.*

Ginkgo biloba	Spontaneous bleeding
Kava	Hives
Paprika	Headache
Senna leaf	Hives
St. John's wort	Gastrointestinal disturbances, allergic reactions, dry mouth, confusion, fatigue, photosensitivity, serotonin syndrome

HERB-DRUG INTERACTIONS

Interactions of herbal products with conventional drugs have been described. These may be due to pharmacodynamic, pharmacokinetic drug inhibition or drug enhancement. Recent studies have highlighted that concurrent use of herbs may mimic, magnify, or oppose the effect of drugs. Certain commonly used herbal constituents (kava-kava) have been shown to directly inhibit CYP and p-glycoprotein (P-gp) activity *in vitro* (Weiss *et al.*, 2005), and some of them (like piperine and silymarin) were shown to act as P-gp inhibitors at dietary concentrations (Marchetti *et al.*, 2007). For St. John's wort, which is a commonly used herbal product obtained from *Hypericum perforatum* does show some adverse effects as well. Recent reports suggest important drug interactions with OCs, warfarin, cyclosporin and theophylline. Hypericum-containing products appear to induce hepatic enzymes of the cytochrome P450 group. In each of the reported cases, the patient has been female, though more women than men take hypericum extracts. Levels of the coadministered agent were reduced in each case report, and all such agents are metabolised by the P450 enzymes. *In vitro* studies indicate hypericum extracts are capable of inducing and approximately doubling cytochrome P450 activity. In one study involving human volunteers, area under the curve for phenprocoumon was markedly reduced when patients were pretreated with hypericum extract compared with placebo (Hu *et al.*, 2005). Genomic technologies, especially DNA microarrays, can be used for studying herb–drug interactions, and the mechanisms underlying these interactions.

TOXICOLOGICAL STUDIES

Genomic technologies also find widespread application in the field of toxicology. Toxicogenomics is the sub-discipline that merges genomics with toxicology. In toxicology research, gene expression profiling facilitates mechanism-based research on toxicant action by comparing results for an experimental compound with a database. Recently several studies have demonstrated the utility of microarray analysis for studying genome-wide effects of xenobiotics and the rapid identification of toxic hazards for novel drug candidates.

An example of such a platform is ToxBlot II, a custom microarray containing cDNAs representing 12564 human genes chosen on the basis of

their potential relevance to a broad range of toxicities (Pennie, 2002). ToxBlot II allows the simultaneous expression profiling of genes representing entire cellular pathways, making it possible to study the mechanisms of toxicity in great detail. cDNA microarray analysis was used to study the expression level of genes in oral fibroblast cell lines in response to exposure to ripe areca nut extract. The results showed up-regulation of IL-6 expression and down-regulation of PDGFR, APP-1 and KGF-1 expressions in multiple cell lines assayed. The down-regulation of KGF-1 expression in oral fibroblast cell lines potentially impairs the proliferation of overlying keratinocytes, which could partially explain the frequent epithelial atrophy observed in chronic areca chewers *in vivo*. This study helped to establish a novel toxicogenomic database for areca nut extract (Ko *et al.*, 2003). It would be extremely valuable if similar databases could be developed for other botanical products as well.

CONCLUSIONS

The Ayurveda is a systematic ancient system of medicine which offers a plethora of drugs to treat myriad ailments. A study of the 25 best-selling pharmaceutical drugs in 1997 found that 11 of them (42%) were biologicals, natural products or entities derived from natural products, with a total value of US$ 17.5 billion. The total sales' value of drugs (such as Taxol) derived from just one plant species (*Taxus baccata*) was US$ 2.3 billion in 2000 (Patwardhan, 2008). Despite the recent revolutions in biotechnology and genome research, an estimated 80% of the world population still has no access to modern medicine and obtain benefits from the time-tested alternative systems of medicine (Hegde, 2003). Scientists working in the fields of genetic engineering, toxigenomics and combinatorial chemistry should apply metagenomic approaches to identify novel natural product lead molecules. Recently, Kawamura *et al.* carried out genomic screening of a non-toxic, active component in Japanese herbal medicine formulation Keishi-bukuryo-gan (KGB) that regulates transcription of hemeoxygenase-1 (Kawamura *et al.*, 2006). With the advent of personalized medicine, it is not only important to screen herbal drugs but also to determine the gene expression profiles of patients currently being treated with herbal drugs.

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Antifungal Activity of Marine Bacterial Associates of Deep-Sea Origin Against Plant Pathogens

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ABSTRACT

Eighty three bacterial associates isolated from ten sponges, one gorgonoid, one antipatherians and from sediments were screened for their antifungal activity against five plant pathogens viz. Rhizoctonia solani, Fusarium oxysporum, Helminthosporium oryzae, Trichochonis padwickii and Curvularia lunata. Three bacterial associates have shown antifungal activity against all the plant pathogens studied. The isolates were halo-tolerant and thermo-tolerant in nature. The active components are water soluble and their production is nutrition dependent. The MIC values of the extra cellular product (ECP) of the three isolates ranged from 2.8–38.7 μg/ml.

Key words : Antifungal activity, marine bacteria, plant pathogens

INTRODUCTION

Since the discovery of penicillin microbial products have been proven to be a rich source of novel compounds with diverse biological activities (Davies, 1999; Imada & Hota, 1992; Berner *et al.*, 1997). A majority of antimicrobials in clinical use today are microbial products or their synthetic/semisynthetic analogs. Sponge symbionts are of biotechnological interest since bioactive compounds of potential medical importance isolated from sponges may be microbial in origin (Bewley & Faulkner, 1998; Bewley *et al.*, 1996; Stierle *et*

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al., 1988). Marine microorganisms have developed unique metabolic and physiological capabilities that not only ensure survival in extreme environments but also offer the potential for production of novel secondary metabolites (Williams, 1993) which are chemically interesting and biologically active against a number of human pathogens (Kobayashi, 1989).

Atafual *et al.* (1989) reported that a bacterium *Pseudomonas antimicrobica* showed antagonism against a wide range of fungi and bacteria including a number of plant pathogens. Antifungal activity of marine bacteria against plant pathogens has been reported by Mohapatra *et al.* (2002). We have reported antibacterial activity from marine bacteria against certain fish pathogens (Vimala *et al.*, 2001) However, very little work has been carried out for screening the antimicrobial compounds of marine origin against plant pathogens. The present investigation describes screening of 83 bacterial associates isolated from organisms of marine origin against five common rice pathogens for their antifungal activity.

MATERIALS AND METHODS

Media

Nutrient broth (NB), Nutrient agar (NA), Potato dextrose agar (PDA), Mycological peptone agar (MPA), Sabraud's dextrose agar (SDA), Czapeckdox agar (CA) were procured from Hi-media Ltd., Mumbai and prepared as per manufacturer's instruction. All the media were prepared with 50% pre-filtered sea-water unless mentioned otherwise.

Collection of Host Organism

Sponges, gorgonians and sediment samples were collected from a depth of 30 m by SCUBA divers off Gopalpur coast and brought to the laboratory aseptically as mentioned earlier (Bapuji *et al.*, 1999).

Isolation of Bacteria

Isolation of bacteria from sponges and gorgonians was carried out following the procedure reported earlier (Vimala *et al.*, 2000).

Identification of Isolates

Standard microbiological methods were used for identification of the isolates (Collins & Lyne, 1970; Moris & Swain, 1971; Bergey's manual of determinative bacteriology, 1993; Vimala, 2004; Vimala *et al.*, 2006). Thermal Death Point (TDP), NaCl tolerance, and pH tolerance of the isolates have been studied following the method of Rath and Subramanyam (1998).

Test Pathogens

The pathogenic fungi used as test organisms were obtained from culture collections maintained in Microbiology Department, Orissa University of Agriculture and Technology (O.U.A.T), Bhubaneswar, India. The pathogens are *Rhizoctonia solani, Trichochonis padwickii, Fusarium oxysporum, Helminthosporium oryzae* and *Curvularia lunata.* The fungal cultures were maintained on PDA slants and used in the study.

Inhibition Assay

In vitro inhibition assay was performed on PDA medium, following aerobic plate method of Fiddman and Rosall (1993).

Inhibition Assay through Fungal Weight Measurement

1 mL of freshly grown bacterial culture (containing 10^7 CFU/mL) and 0.1 mL of 72 h grown fungal pathogen culture (fungal spores were washed with 1 ml sterile distilled water) was inoculated onto 10 mL of PDB medium. Cultures were incubated on a rotary shaker at 30°C for 5 days for control experiment, fungus alone was inoculated. Briefly the 5-day-old dual culture was passed through pre-weighted Whatman No.-1 filter paper, and was dried for 3 h at 70°C and the weights were taken.

The difference in dry weight between fungal culture grown with the bacteria or the control culture with out any bacterium was recorded according to Broekaert *et al.* (1990).

Microscopic Observation of Fungal Mycelium for Morphological Changes

Fungal culture was taken from the nearest edge of inhibition zone onto a clean glass slide and stained with lacto phenol cotton blue for the control experiment, culture grown without any bacterium was taken, the slides were observed under a Nikon inverted microscope.

Effect of Different Media on Antifungal Activity of the Isolates

Study of the nutrient effects on production of antifungal compounds using different media *viz.* potato dextrose agar (PDA), Sabouraud's dextrose agar (SDA), Mycological peptone agar (MPA), Nutrient agar (NA), Czapeckdox agar (CA) was carried out following the method described above. The production of antifungal compounds was measured in terms of zones of inhibition against the fungi.

Inhibition Assay through Bacterial ECP

Preparation of Bacterial ECP

Bacterial ECP was prepared following the method of Chythanya *et al.* (2002). Marine bacteria isolates were cultured in 5 × 1-L batch culture

with ZM broth (Hi-media). Fermentation was carried out at 28–30°C in an orbital shaker for 48 h. The bacterial cultures were harvested by centrifugation at 4°C (12,000 RPM, 30 min) and then filtered through membrane filter paper 0.22 um (Sartorius). The filtered supernatant was taken as crude ECP. The supernatant was concentrated by Lyophilisation (Heto FD-3 Freeze dryer).

Determination of Minimum Inhibitory Concentration (MIC) by Tube Dilution Method

The MIC of ECP of three bacterial isolates against the five plant pathogens was determined by the tube dilution method described by Janssen *et al.* (1986).

RESULTS

83 bacteria were isolated and identified from 10 sponges, one gorgonian, one antipatherian and sediment. They were found to belong to nine genera, including *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Citrobacter*, *Klebsiella*, *Vibrio*, and *Micrococcus* (Vimala, 2004; Vimala *et al.*, 2006)

Four sedentary organisms out of a total of twelve have harboured these active bacteria. *Psammaplysilla purpureae* yielded the greatest number of antagonistic bacteria and *Acanthella ramosa* the lowest. Out of the 83 bacterial isolates screened, seven (8.4%) have shown antagonism against the test fungi.

The pH, salinity tolerance and thermal death point of the active isolates were characterized. Thermal death point of *Bacillus subtilis* (RRL-36) and *Acinetobacter* sp. (RRL-74) was found to be 100°C while the TDP for *Bacillus stearothermophilus* (RRL-15) was found to be 90°C. *Acinetobacter* sp. (RRL-74) showed maximum NaCl tolerance of 35%. This shows that the isolates are thermo-tolerant and halo-tolerant in nature. All the active isolates showed optimum growth at a pH of 7.

The zones of inhibition of the bacterial isolates against the fungal pathogens on PDA are presented in Table 1.

Table 1. Antifungal activity of bacteria on potato dextrose agar plates by spot inoculation method

Bacteria	**Plant Pathogens**				
	Curvularia lunata	**Fusarium oxysporum**	**Helmintho sporium oryzae**	**Rhizocto-nia solani**	**Trichocho-nis padwicki**
Acinetobacter sp.	+	++	+	++	+
Acinetobacter sp.	Trace	-	-	-	-

Table 1. *Contd.*

B. stearothermophilus	++	++	++	++	+
B. subtilis	++	++	++	+	++
Klebsiella sp.	Trace	+	+	+	-
P. aeruginosa	Trace	-	+	+	-
Pseudomonas sp.	Trace	-	-	-	-

++ = 10 mm, + = 5 mm, trace < 5 mm, = no zone of inhibition

Acinetobacter sp. (RRL-74), *Bacillus subtilis* (RRL-36) and *B. sterothermophilus* (RRL-15) inhibited the growth of all the five fungal pathogens tested. *Klebsiella* sp. (RRL-83) has shown antagonism against *R. solani*, *F. oxysporum* and *H. oryzae* but no zones of inhibition was recorded against *T. padwickii.*

The percentage dry weight reduction is shown in Table 2.

Table 2. Antifungal activity of the isolates through percentage dry weight reduction*

Pathogen	% Dry matter reduction		
	***Acinetobacter* sp.**	***B. subtilis***	***B. stearothermophilus***
Trichochonis padwickii	43.3	35.3	32.5
Rhizoctonia solani	33.7	48.7	30.3
Helminthosporium oryzae	39.4	36.8	30.7
Fusarium oxysporum	19.4	33	25
Curvularia lunata	44.2	40.1	30.8

* Reduction in dry weight presented in comparison to controls

A percentage dry weight reduction of 48.7% was seen in dual culture of *Rhizoctonia solani* with *Bacillus subtilis.* The percentage dry weight reduction of *Curvularia lunata* grown along with *Bacillus subtilis* is 40.1%. Basha and Ulaganathan (2002), who showed a 60% reduction of in the dry weight of *Curvularia lunata* grown with Bacillus strain BC121. *Trichochonis padwickii* when grown along with *Acinetobacter* sp. showed a 43.3% reduction in dry weight.

Microscopic Observation

From the microscopic observation it was observed that, RRL-74, inhibited *Trichochonis padwickii* due to the formation of abnormal hyphae with condensation and swelling of mycelial tips. However, no such distortion on the fungal mycelia was reported in control sets. Similar results were also found with the fungus when tested against RRL-36 and RRL-15 shrinkage of fungal cell cytoplasm was observed in both the cases. But the three isolates resulted in abnormal swelling of fungal mycelia in case of *Fusarium*

oxysporum, on the other hand the controlled filaments were observed to be thin and straight.

Also a microscopic change was being observed, in case of *Rhizoctonia solani* when tested against the three active isolates. Both RRL-74 and RRL-15 resulted in shrinkage of cell cytoplasm, in comparison to control sets. In addition to this an interesting phenomenon was observed in case of *R. solani* when tested against RRL-36. The fungal cells were approximately ten times longer than the normal cells, indicating a probable activity through cell wall inhibition.

Similar bulging of fungal mycelia was also observed against *Curvularia lunata* and *Helminthosporium oryzae* by the three bacterial isolates RRL-74, RRL-36 and RRL-15. The bulging of the mycelia was more prominent in mycelia present in inhibition zone against RRL-36. In addition to bulging of fungal hyphae, cytoplasmic shrinkage was also being reported against *H. oryzae* by the active isolate RRL-15.

The antifungal activity in different media is shown in Table 3. Our studies showed highest antifungal activity on nutrient agar and mycological peptone agar followed by potato dextrose agar with 7, 5 and 4 instances of maximum zones respectively. Sabouraud's dextrose agar showed only one instance of maximum zone whereas, Czapeckdox did not exhibit impressive zones against all the test pathogens.

Table 3. Antifungal activity (Zone of inhibition in mm) of the isolates in different media

Media	Active bacterial isolates														
	***Acinetobacter* sp.**					***Bacillus subtilis***					***Bacillus stearothermophilus***				
	Pathogens					**Pathogens**					**Pathogens**				
	RS	TP	FO	HO	CL	RS	TP	FO	HO	CL	RS	TP	FO	HO	CL
PDA	12	4	10	4	5	3	11	6	10	12	8	5	8	8	14
SDA	10	6	6	8	4	6	8	8	12	13	6	20	14	4	9
MPA	8	20	16	16	12	16	9	10	14	12	8	7	10	16	8
NA	8	20	22	28	20	14	11	14	12	17	-	5	-	15	6
CDA	-	8	4	-	-	-	-	-	-	-	-	10	-	-	-

However in our study poor activity of isolate RRL-15 on nutrient agar could be attributed to its inhibited growth on the above media, as growth and metabolic activities are responsible for the production of secondary metabolites.

Solvent extraction of the intra cellular product obtained by sonication yielded negative results. The lyophilised ECP showed activity. The MIC values of ECP ranged from 4.8–38.7 μg/ml (Table 4).

Table 4. MIC values of ECP (μg/ml) of the three isolates

Plant pathogen	MIC value (mg/mL)		
	RRL-74	**RRL-36**	**RRL-15**
C. lunata	9.6	6.6	4.7
F. oxysporum	9.6	3.3	2.8
H. oryzae	4.8	13.2	4.7
R. solani	9.6	6.6	4.7
T. padwickii	38.7	13.2	9.4

Values repesent antifungal activity in terms of zone sizes in mm RS – *Rhizoctonia solani*, TP – *Trichochonis padwickii*, FO – *Fusarium oxysporum*, HO – *Helminthosporium oryzae*, CL – *Curvularia lunata*. (–) no zone of inhibition.

DISCUSSION

In vitro inhibition of growth of fungus on PDA on co-inoculation with bacteria was taken as evidence for the antifungal activity of the bacteria. The fungal hyphae failed to grow over the bacterial spot after five days of incubation. The degree of inhibition however varied among the seven isolates. This suggests that the antibiotic substance produced by them may not be identical compounds.

In our observation the active isolates belonged to the genera *Acinetobacter*, *Pseudomonas*, *Bacillus* and *Klebsiella*. Lemos *et al.* (1985) observed that 11–43% of the bacterial isolates belonging to the genera *Pseudomonas* and *Alteromonas* (isolated from the marine algae) showed antibiotic activity. We found seven active isolates from a total of 83 isolates. Gjessing and Ruskin (1977), reported that seven from a total of 16 isolates of Caribbean sponges produced antimicrobial activity.

The inhibition was also observed through fungal weight reduction. Highest percentage dry matter reduction was observed in case of *Bacillus subtilis* which is followed by *Acinetobacter* sp. and *Bacillus stearothermophilus* .This clearly shows that the reduction of the dry weight of fungus grown along with the bacteria is due to the anti fungal activity of the bacteria.

An experiment was designed to study the morphological deformities (if any) caused by the active isolates. We observed elongation and swelling of fungal mycelia, shrinkage of cell cytoplasm and elongation of fungal cells. Similar to this, Fidmman and Rosall (1993), observed clear differences in hyphal morphology when compared to the control sets. Hyphae were less prolific, and some were swollen although no elongation of hyphae or lysis was observed. Extensive vacuolation of hyphae, lysis and dissolution of fungal mycelia of *Aspergillus niger* by *B. subtilis* is reported by Podile and Prakash (1996).

In our results the isolates showed abnormal swelling of mycelia in case of *Fusarium oxysporum*. A similar result has been observed by Barrows–Broaddus and Kerr (1981), while studying the antagonistic activity of *Arthrobacter* (isolated from soil) against *Fusarium* sp. Hyphae growing at the farthest edge of the culture plate from *Arthrobacter* had an abnormal appearance and hyphae growing nearest to the *Arthrobacter* colony were distorted bulged, and bloated.

Basha and Ulaganathan (2002), where they reported the deformities in fungal mycelia of *C.lunata* when tested for antagonistic activity against *Bacillus* sp. (BC 121).

From the above discussion it is clearly evident that *Bacillus subtilis* resulted in greater mycelial deformities when compared to *Bacillus stearothermophilus* and *Acinetobacter* sp. It is important here to mention that the antifungal activity of *Bacillus stearothermophilus* of marine origin is unique and not reported in literature.

Further, when the activity was assayed on different media, it was observed that antifungal activity varies greatly depending upon the media on which the bacteria are grown.

The overall observation reveals that the peptone containing media showed better inhibitory results along with favourable growth of both isolates and pathogens. When *R. solani* is considered, the sugar containing media showed better inhibitory results with RRL-74. This fall in line with the observations of Fiddman and Rosall (1993), who reported that high sugar containing media Sabraud's glucose agar (SGA), PDA and Diagnostic sensitivity agar (DST) produced greater *in vitro* antifungal activity whereas, low sugar concentration NA and Tryptic soya agar (TSA) produced low activity. However, in our study the inhibition of *R. solani* was better in peptone media for RRL-74 and RRL-15. It is interesting to observe that the Czapeckdox medium which contains sucrose showed negative results in overall studies.

In the present study the antifungal compounds produced by these isolates are concluded to be water soluble as active inhibition was observed on agar medium.

We observed that the antifungal substance produced by the bacterial isolates is water soluble and are not cell bound. In their experiments Lemos *et al.* (1985) demonstrated that antibiotic activity of bacteria isolated from inter tidal seaweeds are cell associated.

Above observations reveal the mycolytic activity of the three bacterial isolates *i.e. Bacillus subtilis* (RRL-36), *Bacillus stearothermophilus* (RRL-15) and *Acinetobacter* sp. (RRL-74). Further, studies will help in developing the bacterial isolates as potential biological control agents against the plant pathogens.

Over all the present report has shown that the antifungal substances produced by the marine isolates are active against the class deuteromycetes

to which all the present pathogens belong. The production of antifungal compounds is dependent on the medium on which the bacteria are grown and the active components are water soluble. However, attempts are currently underway to study the nature of active components. Since these bacterial isolates are of deep sea origin, studies such as these could be a prerequisite for tapping the antifungal compounds for soil amendment against these pathogens.

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