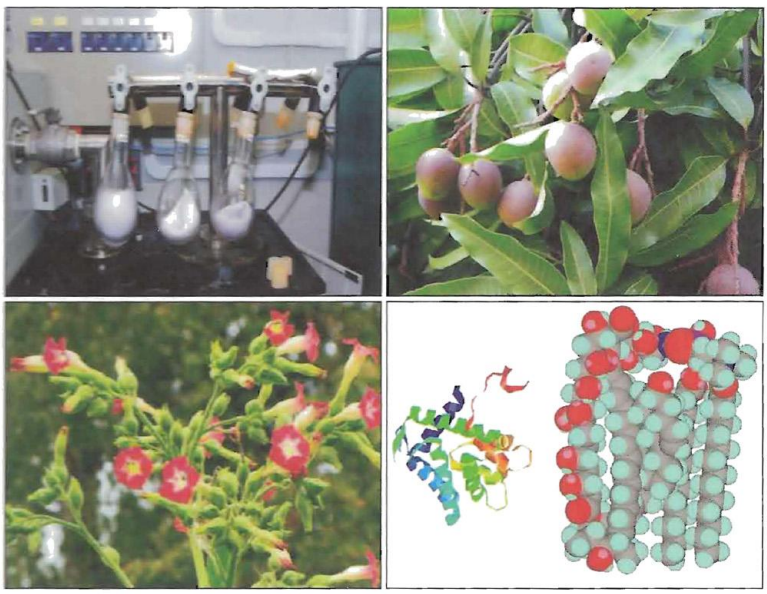
Comprehensive

Bioactive Natural Products

Vol 6 Extraction, Isolation & Characterization



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S C Taneja
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Studium Press LLC

Comprehensive
Bioactive Natural Products

Volume 6
Extraction, Isolation & Characterization

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Comprehensive Bioactive Natural Products

Vol. 6: Extraction, Isolation & Characterization

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

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Vol. 4* : *Antioxidants & Nutraceuticals
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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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M.Sc. M.Phil. Ph.D., FBRS FES. FNRS, FNSE, FNESA, FNAASc, FNASc.,
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(Former Director, NBRI, Lucknow)

08-06-2009

Foreword to the Series

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

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

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

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

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

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

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

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

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

(P. Pushpangadan)

About the Editors

Dr. Vijay Kumar Gupta

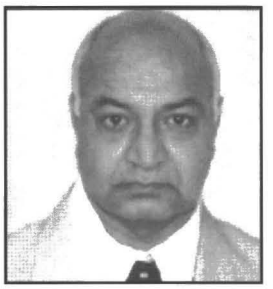
Dr. Vijay Kumar Gupta, Ph.D., FLS, London (born 1953-) Deputy Director & Head, Animal House, Indian Institute of Integrative Medicine (CSIR), Jammu, India. He did his M.Sc. (1975) and Ph.D. (1979) in Zoology both from University of Jammu, Jammu-India. His research capabilities are substantiated by his 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.

Dr. Subhash Chandra Taneja

Dr. Subhash Chandra Taneja (born 1950-) obtained his masters degree in Organic Chemistry in 1971 and thereafter completed Ph.D. in Birla Institute of Technology & Science, Pilani, India in 1975 under the guidance of Prof.

H.P. Tiwari. He is scientist of eminence and presently working as Scientist 'G', Indian Institute of Integrative Medicine (CSIR), Jammu. Dr. Taneja has over 100 publications in international journals, two book chapters and two review articles, 38 patents of which 14 are USP. He has also visited Institute of Organic Chemistry, Warsaw, Poland (1989-1990) under CSIR Scientist Exchange Programme. His areas of interest are chemistry and bioactivities of medicinal plants and generation of semi-synthetic libraries based on natural product scaffolds; design and synthesis of bioactive lead molecules in the area of inflammation, oncology, infectious diseases, immunomodulation; exploitation of microbial biodiversity for the identification newer sources of biocatalysts *e.g.* lipases, esterases, glucosidases, nitrilases, oxido-reductases etc. and development of new throughput screening techniques; synthesis and kinetic resolution of important dugs/intermediates using chemo-enzymatic methods; development of green methodologies for the synthesis of mono terpenes and perfumery molecules; development of new glycosylation methodologies; *C*- and *O*-glycosylation techniques; new synthetic protection-deprotection methodologies for carbohydrates, and their conversion to bioactive molecules and organic synthesis of natural products and bioactive molecules.

Dr. Bishan Datt Gupta



Dr. Bishan Datt Gupta (born 1951-) obtained his M.Sc. in chemistry in 1973 from Jammu University and then did his Ph.D. at Regional Research Laboratory, Jammu (now IIIM, Jammu) under the guidance of Dr. C. K. Atal. He is a well known scientist in the area of natural product chemistry and is at present working as Scientist 'G' at Indian Institute of Integrative Medicine (CSIR), Jammu. Dr. Gupta has more than sixty publications in international journals, one book chapter and eighteen patents. He has visited France under CSIR-CNRS Scientist Exchange Programme and has also participated in two international conferences at Kuala Lumpur and Hanoi. His areas of interest are natural product chemistry, especially drug development based on natural products which includes activity guided fractionation for isolation of pure biomolecules, their structure elucidation,

semi-synthesis as well as structure modifications. His work areas also include standardization of herbal drugs/formulations using modern analytical techniques (GC-MS, HPTLC, HPLC, LC-MS) on the basis of marker compounds.

Preface

For decades, natural products have been the sources of drugs and drug leads. A survey of the new chemical entities introduced as drugs worldwide during the last few decades reveals that many of these are natural products or are inspired by natural products. Current drug discovery from plants has mainly relied on bioactivity-guided isolation methods and has led to discoveries of several new drugs like paclitaxel and camptothecin. Spectacular progress in extraction and isolation techniques coupled with newer spectroscopic methods of identification, have contributed to the phenomenal success of natural product chemistry over the past few decades. A sound isolation strategy has helped in the isolation and characterization of many bioactive molecules. Nowadays, bioassay-guided fractionation of medicinal plants for isolation of bioactive molecules is a feature of routine in the attempt to isolate bioactive components from natural sources. These techniques are not only being restricted to plant sources but they are also being applied to microbial , marine and even fungal sources.

During the last two decades there has been remarkable progress in natural product research. With the outstanding developments in the areas of chromatography, spectroscopic techniques, and microplate-based ultrasensitive *in vitro* assays, natural product research is enjoying renewed attention for providing new and interesting chemical entities. The availability of hyphenated techniques, e.g. GC-MS, LC-PDA, LC-MS, LC-FTIR, LC-NMR, LC-NMR-MS, CE-MS, X-Ray analysis have made the analyses of the crude extracts or fractions from different natural sources, isolation and on-line detection of natural products, chemotaxonomic studies, chemical finger printing, quality control of herbal products and structure determination of isolates very interesting.

The present volume ***"Extraction, Isolation & Characterization"*** in the series **"Comprehensive Bioactive Natural Products"** is devoted to specific aspects of natural product isolation protocols, structure determination and a general overview of the techniques involved in natural product research, starting from extraction to structure determination of purified products. The topics have been contributed by the experts in the fields with relevant and up to date information and includes the studies on: Recent insights on the chemistry and pharmacology of withasteroids; Phenolic compounds from *Plumbago zeylanica* and their cytotoxicity; Extraction, characterization and biological properties of 4-*O*-methyl glucuronoxylan from hard wood - a review; Biologically active naphthaquinones from nature; Biological function of glycoproteins; Chemical constituents and pharmacology of the neotropical burseraceae; Are well-studied snake venoms well investigated? Strategy for isolation of new polypeptides from snake venom ; Extraction, isolation and characterization

of solanesol from *Nicotiana tobacum* L.; Cinnamon: molecular evidence for the health benefits through its insulin-like and anti-inflammatory effects; Flax cyanogenic glycosides; Isolation and preliminary characterization of antimicrobial proteins and peptides from *Ctenophores* and *Cnidaria*; Plants of the genus: *Commiphora*-their chemistry; Secondary metabolites and biological activities of some gentianaceae species from Serbia and Montenegro; Chitin and chitosan: extraction and characterization; Chemical composition of the mango stem bark extract (*Mangifera indica*).

We believe that the present volume will be a useful compendium of knowledge for natural product chemists, ethnobotanists, pharmacologists, pharmaceutical scientists as well as other researchers in traditional medicines.

Jammu, India

V.K. Gupta
S.C. Taneja
B.D. Gupta

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Table of Contents

About the Series *vii*
Foreword to the Series *ix*
Preface *xv*
Table of Contents of Volume 6 *xvii*

1. Recent Insights on the Chemistry and Pharmacology of Withasteroids 1-55
   MARIA LEOPOLDINA VERAS, OTILIA DEUSDÊNIA LOIOLA PESSOA, EDILBERTO ROCHA SILVEIRA, ANA ISABEL VITORINO MAIA, RAQUEL CARVALHO MONTENEGRO, DANILO DAMASCENO ROCHA, CLÁUDIA PESSOA, MANOEL ODORICO DE MORAES AND LETICIA VERAS COSTA-LOTUFO (BRAZIL)
2. Phenolic Compounds from *Plumbago zeylanica* and their Cytotoxicity 57-69
   NGUYEN A.-T., MALONNE H., FONTAINE J., BLANCO L., VANHAELEN M., FIGYS J., ZIZI M. AND DUEZ P. (BELGIUM, FRANCE)
3. Extraction, Characterization and Biological Properties of 4-*O*-Methyl Glucuronoxylan from Hard Wood – A Review. 71-95
   ALINE BARBAT, CHARLOTTE MOINE, PIERRE KRAUSZ AND VINCENT GLOAGUEN (FRANCE)
4. Biologically Active Naphthaquinones from Nature 97-118
   VINOTHKUMAR S.P. AND GUPTA JAYANTA KUMAR (INDIA)
5. Biological Function of Glycoproteins
   KWANG LIM AND KYE-TAEK LIM (SOUTH KOREA, CANADA) 119-142
6. Chemical Constituents and Pharmacology of the Neotropical Burseraceae 143-165
   JUNIOR VEIGA F. VALDIR AND RUDIGER L. ANDRÉ (BRAZIL)

7. Are Well-Studied Snake Venoms Well Investigated? Strategy for Isolation of New Polypeptides from Snake Venom 167-184
OSIPOV A.V., TSETLIN V.I. AND UTKIN YU.N. (RUSSIAN FEDERATION)

8. Extraction, Isolation and Characterization of Solanesol from *Nicotiana tabacum* L. 185-201
RAO NAGESWARA R. AND NARENDRA KUMAR TALLURI M.V. (INDIA)

9. Cinnamon: Molecular Evidence for the Health Benefits Through its Insulin-Like and Anti-Inflammatory Effects 203-227
CAO HEPING, URBAN F. JOSEPH JR. AND ANDERSON A. RICHARD (USA)

10. Flax Cyanogenic Glycosides 229-264
BARTHET VÉRONIQUE J. AND BACALA RAY (CANADA)

11. Isolation and Preliminary Characterization of Antimicrobial Proteins and Peptides from *Ctenophores* and *Cnidaria* 265-286
GRANT SUZANNE, GISONDI AMY, HORTANO WILLIAM, DEFILIPPO JOHN, AND BECK GREGORY (USA)

12. Plants of the Genus: *Commiphora*-their Chemistry 287-322
SELVAMANI P., GUPTA JAYANTA KUMAR AND SEN DHRUBOJYOTI (INDIA)

13. Secondary Metabolites and Biological Activities of some Gentianaceae Species from Serbia and Montenegro 323-340
ŠAVIKIN K., JANKOVIĆ T., KRSTIĆ MILOŠEVIĆ D., MENKOVIĆ N. AND MILOSAVLJEVIĆ S. (SERBIA)

14. Chitin and Chitosan: Extraction and Characterization 341-381
ELSABEE MAHER Z AND ALSAGHEER FAKHRIEA (EGYPT, KUWAIT)

15. Chemical Composition of the Mango Stem Bark Extract (*Mangifera indica* L) 383-412
NÚÑEZ SELLÉS A.J. AND RASTRELLI LUCA (ITALY, CUBA)

Appendix

Table of Contents of Other Volumes of the Series- Vols. 1 to 5 & 7 to 8 413-426

Index 427-437

1

Recent Insights on the Chemistry and Pharmacology of Withasteroids

MARIA LEOPOLDINA VERAS[1], OTILIA DEUSDÊNIA LOIOLA PESSOA[1,*], EDILBERTO ROCHA SILVEIRA[1], ANA ISABEL VITORINO MAIA[1], RAQUEL CARVALHO MONTENEGRO[2], DANILO DAMASCENO ROCHA[2], CLÁUDIA PESSOA[2], MANOEL ODORICO DE MORAES[2] AND LETICIA VERAS COSTA-LOTUFO[2]

ABSTRACT

The withasteroids constitute a remarkable group of steroidal lactones characteristic of some genera of the Solanaceae family. They present a wide spectrum of biological activity such as antibacterial, antileishmanial, antitrypanosomal, anti-inflammatory, antitumor, antistress, cytotoxic and immunostimulating effects. The great structural diversity allied to the interesting pharmacological properties has encouraged scientists throughout the world to devote a huge effort in the scrutiny of new sources, new structural entities and their pharmacological activities. The present review, from 1990, was based on a previous work by Ray and Gupta (1956-1994), to which 4 years of overlapping was taken to assure a higher reliability. More than 400 structures, separated by structural groups (withanolides, withaphysalins, physalins, ixocarpalactones, perulactones, and acnistins) with their botanical sources are provided. In addition, the pharmacological potential of these molecules is also discussed taking withaferin A as an example, since this is the most studied among the withasteroids. Until now several biological properties have been ascribed to this compound, including cytotoxic and anti-invasive activity in tumor cells; inhibition of cyclooxygenase, cholinesterase and angiogenesis; induction of reactive oxygen species and apoptosis. These data highlight its therapeutical potential as an anticancer agent.

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Key words : Withasteroids, steroidal lactones, withanolides, Solanaceae, pharmacological activities, anticancer agents

INTRODUCTION

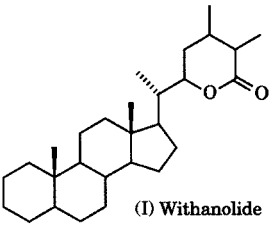
The Withasteroids are steroidal lactones (C-28) with an ergostane skeleton type, rich in oxygenated functions which are responsible for several natural modifications of the carbocyclic skeleton, as well as the side chain, resulting in compounds structurally diverse and complexes. The name withasteroids, proposed by Russian (Tursunova *et al.,* 1981), involves all C-28 ergostane steroids including those with modified skeletons. These types of secondary metabolites have been isolated especially from 21 genera of the Solanaceae family: *Acnistus*, *Brachistus, Datura*, *Deprea*, *Discopodium*, *Dunalia*, *Exodeconus*, *Hyoscyamus*, *Iochroma*, *Jaborosa*, *Lycium*, *Nicandra*, *Physalis*, *Salpichroa*, *Saracha*, *Solanum*, *Trechonaetes*, *Tubocapsicum*, *Vassobia*, *Withania* and *Witheringia*. Rarely, those compounds were found in the genera *Ajuga* (Labiatae), *Tacca* (Taccaceae) and *Cassia* (Leguminosae) and also from some marine organisms such as *Minabea* sp. Thus, withasteroids does not characterize a class of compounds exclusively of the Solanaceae family. The genera *Withania* and *Physalis* are the producers of withasteroids with the greatest structural variety.

In this review is presented a total of 618 withasteroids, including the source and plant part from where they have been isolated and some physical properties, contributing to the research in the natural products area, particularly with the chemistry of the Solanaceae.

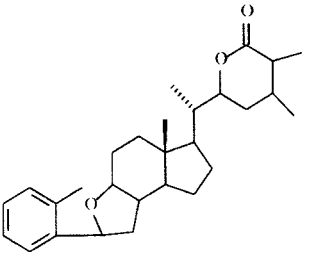
The withasteroids are compounds rich in oxygenated functions due to an enzymatic procedure elaborated by the plants, able to oxidize all the carbon atoms of the steroidal nucleus and also of the side chain, except the C-10. The presence of these functions allow the formation of new bonds and sometimes ring aromatization, resulting in a large variety of compounds of complex structures having a side chain constituted by nine carbon atoms presenting a γ or δ lactone or lactol moieties, characteristic of the withaesteroids. The withasteroids are classified in the following groups: withanolides (I), withanolides with an aromatic ring A (II), withanolides with an aromatic ring D (III), withaphysalins (IV), physalins (V), acnistins (VI), ixocarpalactones (VII) and perulactones (VIII) (Table 1). Until 1994, Ray and Gupta described the isolation and characterization of 218 withaesteroids. According to this survey, 400 new compounds have been

isolated, thus, the number of isolated and registered withasteroids ranks to 618, representing an increase of ca. 180% (Table 2).

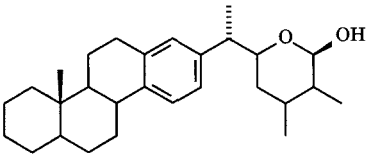
Table 1. Withasteroid types according to the structural modification



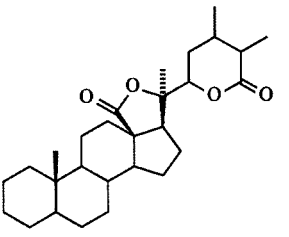
(I) Withanolide



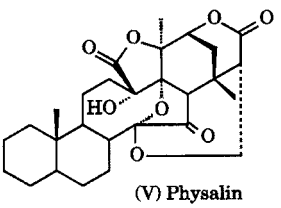
(II) Withanolide with aromatic ring A



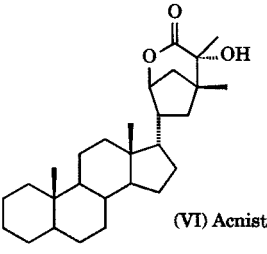
(III) Withanolide with aromatic ring D



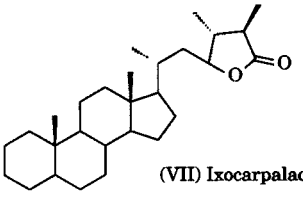
(IV) Withaphysalin



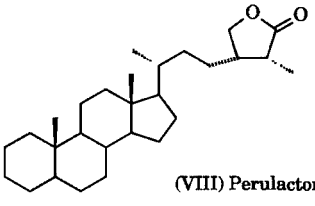
(V) Physalin



(VI) Acnistin



(VII) Ixocarpalactone



(VIII) Perulactone

Table 2. Withasteroids isolated after 1990 from Solanaceae, Labiatae, Taccaceae and Leguminosae

Plant name	Plant part	Compounds	References
Acnistus arborescens	Leaves	**1 – 3**	Minguzzi *et al.*, 2002
		4 – 6	Veras *et al.*, 2004b
		7, 8	Veras *et al.*, 2004a
		9 – 14	Usubillaga *et al.*, 2005
Acnistus ramiflorum	Leaves	**9, 10**	Usubillaga *et al.*, 1992
Ajuga bracteosa	Whole plant	**15 – 17**	Riaz *et al.*, 2004
Ajuga parviflora	Whole plant	**18, 19**	Khan *et al.*, 1999a
		20, 21	Nawaz *et al.*, 1999
		22, 23	Kahn *et al.*, 1999b
		24	Kahn *et al.*, 1999c
	*	**25**	Nawaz *et al.*, 2000
	*	**26, 27**	Nawaz *et al.*, 2000
Brachistus stramonifolius	Roots	**28 – 30**	Fang *et al.*, 2003
Cassia siamea	Barks	**31**	Srivastava *et al.*, 1992
Datura fastuosa	Leaves	**32**	Goel *et al.*, 1997
		33	Manickam *et al.*, 1994a
		34	Manickam *et al.*, 1994b
		35 – 37	Manickam *et al.*, 1993
	Flowers	**32, 38, 39**	Manickam *et al.*, 1998
Datura ferox	Leaves	**40, 41, 41A**	Veleiro *et al.*, 1999
		42 – 46	Cirigliano *et al.*, 1995
Datura innoxia	Aerial parts	**47, 48**	Siddiqui *et al.*, 2005a
		49, 50	Siddiqui *et al.*, 1999
		51, 52	Siddiqui *et al.*, 2002
		53	Siddiqui *et al.*, 2005b
Datura metel	Leaves	**45, 54 – 56**	Gupta *et al.*, 1992
	Aerial parts	**35, 57, 58**	Gupta *et al.*, 1991
		59	Manickam *et al.*, 1994b
	Flowers	**60**	Shingu *et al.*, 1990b
		61 – 63	Ma *et al.*, 2006
		64, 65	Jahromi *et al.*, 1993
		66 – 68	Yang *et al.*, 2007
		69 – 78	Pan *et al.*, 2007
Datura quercifolia	Leaves	**79**	Bandhoria *et al.*, 2006
Datura tatula	Aeaves	**35, 80**	Manickam *et al.*, 1996a
	Aerial parts	**81**	Manickam & Ray, 1996b
	Flowers	**82 – 84**	Srivastava *et al.*, 1996
Datura tatura	Aerial parts	**85, 86**	Shingu *et al.*, 1990c
Deprea orinocensis	Leaves	**87 – 90**	Luis *et al.*, 1994
		91	Echeverri *et al.*, 1995
Deprea subtriflora	*	**92 – 94**	Su *et al.*, 2003a
		95 – 104	Su *et al.*, 2003b

Table 2. *Contd.*

Plant name	Plant part	Compounds	Reference(s)
Discopodium penninervium	Leaves	**105 – 108**	Habtemariam *et al.*, 1993
		109	Habtemariam *et al.*, 2000
	Roots Barks	**110 – 112**	Habtemariam & Gray, 1998
		113	Wube *et al.*, 2008
Dunalia australis	Roots	**114 – 120**	Lischevski *et al.*, 1991
		121 – 124	Lischevski *et al.*, 1992
Dunalia brachyacantha	Leaves and flowers	**125 – 129**	Silva *et al.*, 1999
	Leaves	**130 – 132**	Bravo *et al.*, 2001
	Roots	**133**	Bravo *et al.*, 2001
Dunalia solanacea	Leaves	**11, 134, 135**	Luis *et al.*, 1994b
		136, 137	Luis *et al.*, 1994c
		138	Luis *et al.*, 1994a
Exodeconus maritimus	Whole plant	**112, 139 – 141**	Gil *et al.*, 1997
Hyoscyamus niger	Seeds	**142 – 144**	Ma *et al.*, 1999
Iochroma australe	Aerial parts	**1, 145, 146**	Vaccarini & Bonetto, 2000b
Iochroma coccineum	Aerial parts	**147 – 149**	Alfonso & Kapetanidis, 1991
		150 – 155	Alfonso *et al.*, 1993
Iochroma fuchsioides	Leaves and stems	**130, 156 – 160**	Raffauf *et al.*, 1991
Iochroma gesnerioides	Aerial parts	**161 – 169**	Alfonso & Kapetanidis, 1994
Jaborosa araucana	Aerial parts	**170**	Cirigliano *et al.*, 1996
Jaborosa bergii	Aerial parts	**171 – 176**	Nicotra *et al.*, 2003
Jaborosa caulescens var. *bipinnatifida*	Aerial parts	**177, 178**	Nicotra *et al.*, 2000
		179 – 183	Nicotra *et al.*, 2007
Jaborosa caulescens var. *caulescens*	Aerial parts	**184, 185**	Nicotra *et al.*, 2000
		186, 187	Nicotra *et al.*, 2007
Jaborosa integrifolia	Roots	**188 – 190**	Vaccarini & Bonetto, 2000a
Jaborosa kurtzii	Aerial parts	**191, 192**	Ramacciotti *et al.*, 2007
Jaborosa laciniata	Aerial parts	**193 – 198**	Cirigliano *et al.*, 2007
Jaborosa leucotricha	Aerial parts	**199 – 201**	Misico *et al.*, 1997
	Whole plant	**202 – 204**	Misico & Oberti, 1996
	Leaves	**205**	Veleiro *et al.*, 1992b
Jaborosa magellanica	Whole plant	**206 – 211**	Fajardo *et al.*, 1991
		212	Parvez *et al.*, 1991
		213	Cárcamo & Fajardo, 1993
	Seeds	**214**	Parvez *et al.*, 1990
Jaborosa odonelliana	Whole plant	**215 – 219**	Cirigliano *et al.*, 2000
	Leaves	**220, 221**	Cirigliano *et al.*, 2002
Jaborosa rotacea	Aerial parts	**222 – 233**	Nicotra *et al.*, 2006
Jaborosa runcinata	Aerial parts	**170, 234 – 238**	Cirigliano *et al.*, 1996
Jaborosa sativa	Aerial parts	**239 – 242**	Bonetto *et al.*, 1995
Nicandra physaloides	Whole plant	**243 – 247**	Shingu *et al.*, 1994

Table 2. *Contd.*

Plant name	Plant part	Compounds	Reference(s)
Physalis alkekengi var. *francheti*	Roots	**248 – 250**	Sunayama *et al.*, 1993
	Aerial parts	**251, 252**	Kawai *et al.*, 1992
		253	Kawai *et al.*, 1993
		254, 255	Chen *et al.*, 2007
	Leaves and stems	**256, 257**	Makino *et al.*, 1995b
	Leaves	**258**	Kawai *et al.*, 2001
		259, 260	Makino *et al.*, 1995a
Physalis angulata	Seeds	**261 – 263**	Shingu *et al.*, 1992b
	Leaves and stems	**264 – 266**	Shingu *et al.*, 1992a
		267	Shingu *et al.*, 1991
	Aerial parts	**268 – 271**	Nagafuji *et al.*, 2004
		272 – 274	Kuo *et al.*, 2006
		275 – 277	Abe *et al.*, 2006
	Whole plant	**278**	Chen *et al.*, 1990
		279 – 286	Damu *et al.*, 2007
Physalis chenopodifolia	Aerial parts	**287 – 291**	Maldonado *et al.*, 2004
Physalis cinerascens	Aerial parts	**292, 293**	Maldonado *et al.*, 2005
	Leaves	**294**	Anjaneyulyu & Rao, 1997
Physalis coztomatl	Aerial parts	**295 – 299**	Pérez-Castorena *et al.*, 2006
Physalis divericata	Aerial parts	**300**	Ma *et al.*, 2006
Physalis minima	Whole plant	**301**	Sen & Pathak, 1995
		29, 30, 302 – 306	Choudhary *et al.*, 2005
		307	Kawai *et al.*, 1996
Physalis minima	*	**308 – 314**	Ma *et al.*, 2007
Physalis mínima var. *indica*	Whole plant	**315, 316**	Choudhary *et al.*, 2007
Physalis peruviana	Calyces	**317, 318**	Dinan *et al.*, 1997
	Whole plant	**319, 320**	Ahmad *et al.*, 1999b
		321	Ahmad *et al.*, 1999a
		322 – 325	Ahmad *et al.*, 1999c
		326	Ahmad *et al.*, 1998
Physalis philadelphica	Fruits	**327**	Kennelly *et al.*, 1997
	Leaves and stems	**112, 328 – 333**	Su *et al.*, 2002
		334 – 337	Gu *et al.*, 2003
Physalis pruinosa	Aerial parts	**338, 339**	Shingu *et al.*, 1993
Physalis solanaceus	Leaves	**28, 29, 340, 341**	Pérez-Castorena *et al.*, 2004
Physalis viscosa	Roots and leaves	**317**	Silva *et al.*, 1993
	Leaves	**342**	Silva *et al.*, 1993
Salpichroa origanifolia	Leaves	**343 – 345**	Tettamanzi *et al.*, 1998
		346, 347	Tettamanzi *et al.*, 1996
	Leaves and stems	**348 – 352**	Tettamanzi *et al.*, 2001 Tettamanzi *et al.*, 2000
	Whole plant	**353**	Veleiro *et al.*, 1992a
		354 – 356	Veleiro *et al.*, 1994

Table 2. *Contd.*

Plant name	Plant part	Compounds	Reference(s)
Saracha viscosa	Roots	**29, 30, 340**	Ripperger & Kamperdick, 1998
Solanum cilistum	Leaves	**357 – 360**	Zhu *et al.*, 2001b
		361 – 366	Zhu *et al.*, 2001a
		367 – 372	Zhu *et al.*, 2001c
Solanum sisymbiifolium	Leaves	**373, 374**	Niero *et al.*, 2006
Tacca chantrieri	Rhizomes	**375, 376**	Yokosuka *et al.*, 2003
Tacca plantaginea	Whole plant	**377 – 381**	Liu *et al.*, 2006
Tubocapsicum anomalum	Aerial parts	**382, 383**	Shingu *et al.*, 1990a
	Leaves and stems	**384 – 394**	Hsieh *et al.*, 2007
	Roots	**395 – 398**	Hsieh *et al.*, 2007
Vassobia lorentzii	Aerial parts	**8, 333, 399 – 405**	Misico *et al.*, 2000
Withania adpressa	Leaves	**406**	Abdeljebbar *et al.*, 2007
Withania coagulance	Aerial parts	**407 – 410**	Atta-ur-Rahman *et al.*, 1998c
		411	Atta-ur-Rahman *et al.*, 1997
Withania coagulans	Whole plant	**412 – 414**	Atta-ur-Rahman *et al.*, 2003
		415	Atta-ur-Rahman *et al.*, 1993
		416 – 418	Atta-ur-Rahman *et al.*, 1998
		419	Nur-e-Alam *et al.*, 2003
		420 – 424	Atta-ur-Rahman *et al.*, 1998
Withania hunzikeri	Whole plant	**28**	Karikas *et al.*, 1998
Withania somnifera	Leaves	**294**	Anjaneyulyu & Rao, 1997a
		425 – 429	Jayaprakasam & Nair, 2003
		430 – 435	Misra *et al.*, 2005
		436	Bandhoria *et al.*, 2006
	Roots	**437 – 439**	Anjaneyulyu & Rao, 1997b
		440 – 446	Matsuda *et al.*, 2001
		447 – 451	Zhao *et al.*, 2002
		452, 453	Misra *et al.*, 2008
		454,	Subbaraju *et al.*, 2006
	Stem bark	**455 – 459**	Ali *et al.*, 1997
	Whole plant	**24, 460, 461**	Atta-ur-Rahman *et al.*, 1999
		435	Atta-ur-Rahman *et al.*, 1993
		462 – 464	Choudhary *et al.*, 1996
		465 – 466	Atta-ur-Rahman *et al.*, 1992

Table 2. *Contd.*

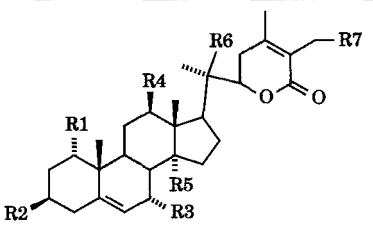
Plant name	Plant part	Compounds	Reference(s)
		467	Jamal *et al.*, 1995
		468, 469	Choudhary *et al.*, 2004
	Aerial parts	**470, 471**	Atta-ur-Rahman *et al.*, 1991
	Fruits	**472-474**	Kuroyanagi *et al.*, 1999
		475, 476	Abou-Douh, 2002
		477	Kumar *et al.*, 2004
Withania somnifera chemotype III	Leaves	**478, 479**	Bessalle & Lavie, 1992
Witheringia hunzikeri	Whole plant	**28**	Karikas *et al.*, 1998
Witheringia solanacea	Leaves	**28, 29, 340**	Jacobo-Herrera *et al.*, 2006

WITHANOLIDES

The withanolides constitute the most abundant group within the withasteroids. They frequently present an oxygenated function in C-1, C-22 and C-26, and are regarded as the probable precursors of the withanolides with aromatic ring A (II), with aromatic ring D (III), withaphysalins (IV), physalins (V) and acnistins (VI) (Ray & Gupta, 1994). They are of great phytochemical, chemotaxonomical and biological interest and are subdivided into two groups based on their side chain orientation: those with β-orientation (17β) and those considered rare, with α-orientation (17α) (Glotter *et al.*, 1991). These secondary metabolites have been isolated from the genera *Acnistus*, *Datura*, *Deprea*, *Discopodium*, *Dunalia*, *Exodeconus*, *Hyoscyamus*, *Iochroma*, *Jaborosa*, *Lycium*, *Minabea*, *Nicandra*, *Petunia*, *Physalis*, *Salpichroa*, *Saracha*, *Solanum*, *Trechonaetes*, *Tubocapsicum*, *Vassobia*, *Withania* and *Witheringia* (Solanaceae), *Ajuga* (Labiatae) *Cassia* (Caesalpiniaceae-Leguminosae) and *Tacca* (Taccaceae).

By the year 1994, Ray and Gupta registered the isolation of withanolides from 17 genera, 14 of which belonging to the Solanaceae family. Currently, reports about withanolides isolation from 25 genera, 21 of which belonging to the Solanaceae family were found in the literature. This represents an increase of 32% on the number of genera as a whole and 50% on the number of genera of the Solanaceae.

Withanolides Bearing a 5-ene System



(**86**) $R_1 = R_3 = R_7 = OH$, R_2 = O-β-D-Glc $[\alpha]^{15}_D$ -17.7 (*c* 0.52, C_5D_5N)

(**114**) R_1 = OAc, $R_2 = OH$, $[\alpha]^{24}_D$ +58.8 (*c* 0.17, $CHCl_3$)

(**115**) $R_1 = R_4 = R_6 = OH$, R_2 = OAc m.p. 135-137°C, $[\alpha]^{24}_D$ -16.7 (*c* 0.48, $CHCl_3$)

Contd.

Withanolides Bearing a 5-ene System

(**116**) R_1 = R_2 = OH, m.p. 117-119°C $[\alpha]^{23}_D$ +36.1 (*c* = 0.18, $CHCl_3$)

(**117**) R_1 = R_2 = R_4 = R_6 = OH, m.p. 172-175°C $[\alpha]^{24}_D$ +26.3 (*c* 0.76, $CHCl_3$)

(**118**) R_1 = R_6 = OH, R_2 = OAc, m.p. 152-158°C $[\alpha]^{23}_D$ +6.9 (*c* 0.44, $CHCl_3$)

(**119**) R_1 = OAc, R_2 = R_6 = OH, m.p. 235-240°C $[\alpha]^{24}_D$ +28.4 (*c* 0.31, $CHCl_3$)

(**120**) R_1 = R_2 = R_6 = OH, m.p. 260-265°C $[\alpha]^{25}_D$ +20.4 (*c* 0.28, $CHCl_3$)

(**121**) R_1 = OAc, R_2 = β-D-xyl(1→3), β-D-xyl(1→4), β-D-Glc, R_6 = OH, m.p. 123-126°C $[\alpha]^{22}_D$ -21.5 (*c* 0.2, $CHCl_3$)

(**122**) R_1 = OAc, R_2 = β-D-xyl(1→3), β-D-Glc(1→4), β-D-Glc, R_6 = OH, m.p. 118-120°C $[\alpha]^{22}_D$ -9.5 (*c* 0.3, $CHCl_3$)

(**123**) R_1 = OAc, R_2 = β-D-Glc(1→3), β-D-Glc(1→4), β-D-Glc, R_6 = OH, m.p. 117-123°C $[\alpha]^{22}_D$ -2.1 (*c* 0.2, $CHCl_3$)

(**124**) R_1 = OAc, R_2 = β-D-Glc(1→3), β-D-Glc(1→4), β-D-Glc, R_4 = R_6 = OH m.p. 120-124°C, $[\alpha]^{26}_D$ -23.4 (*c* 0.2, $CHCl_3$)

(**132**) R_1 = R_4 = OAc, R_2 = O-α-L-ram(1→4)-β-D-Glc), R_6 = OH, m.p. 135-137°C $[\alpha]^{25}_D$ -2.5 (*c* 1.0, MeOH)

(**133**) R_1 = OAc, R_4 = R_6 = OH, R_2 = O-β-D-xyl(1→3)-β-D-xyl(1→4)-β-D-Glc m.p. 119-121°C, $[\alpha]^{25}_D$ -5.3 (*c* 1.0, MeOH)

(**266**) R_1 = R_7 = OH, R_2 = O-β-D-Glc

(**322**) R_1 = OAc, R_2 = O-β-D-Glc, R_5 = R_6 = OH $[\alpha]^{25}_D$ +16.5° (*c* 0.49, MeOH)

(**323**) R_1 = OAc, R_2 = O-β-D-Glc, R_7 = OH $[\alpha]^{25}_D$ +29.3 (*c* 0.89, MeOH)

(**425**) R_1 = R_7 = OH, R_2 = O-Glc(1→6)-Glc-(1→4)-Glc

(**426**) R_1 = OH, R_2 = R_7 = O-β-D-Glc

(**443**) R_1 = R_7 = OH, R_2 = O-β-D-Glc(1→6)-β-D-Glc; $[\alpha]^{28}_D$ +5.2 (*c* 0.2, MeOH)

(**444**) R_1 = OH, R_2 = O-β-D-Glc(1→6)-β-D-Glc $[\alpha]^{28}_D$ +7.8 (*c* 0.3, MeOH)

(**445**) R_1 = R_6 = OH, R_2 = O-β-D-Glc(1→6)-β-D-Glc; $[\alpha]^{27}_D$ -11.6 (*c* 0.5, MeOH)

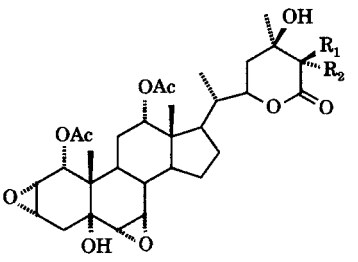
(**446**) R_1 = R_3 = OH, R_2 = O-β-D-Glc(1→6)-β-D-Glc; $[\alpha]^{29}_D$ +5.0 (*c* 0.1, MeOH)

(**448**) R_1 = OH, R_2 = O-β-D-Glc(1→6)-β-D-Glc, R_7 = O-β-D-Glc, $[\alpha]^{23}_D$ +10.4 (*c* 0.264, MeOH)

(**449**) R_1 = OH, R_2 = R_7 = O-β-D-Glc(1→6)-β-D-Glc

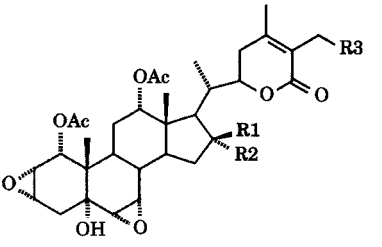
(**450**) R_1 = OH, R_2 = R_7 = O-β-D-Glc $[\alpha]^{23}_D$ +21.1 (*c* 0.11, MeOH)

(**474**) R_1 = OH, R_2 = O-β-D-Glc(1→6)-β-D-Glc



(**380**) R_1 = CH_3, m.p. 237-239°C $[\alpha]^{26}_D$ +66.1 (*c* 13.6, $CHCl_3$)

(**381**) R_1 = OH, m.p. 328-330°C $[\alpha]^{26}_D$ +198.5 (*c* 2.7, C_5D_5N)



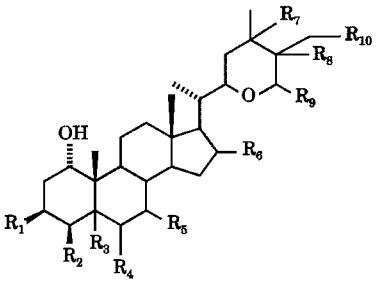
(**377**) R_1,R_2 = O, R_3 = OH, m.p. 246-247°C $[\alpha]^{26}_D$ +11.1 (*c* 3.3, $CHCl_3$)

(**378**) R_1 = OH, m.p. 253-255°C $[\alpha]^{26}_D$ +65.2 (*c* 2.6, $CHCl_3$)

(**379**) m.p. 208-210°C $[\alpha]^{26}_D$ +93.5 (*c* 4.2, $CHCl_3$)

1. All not specified R groups correspond to hydrogen atoms

Withanolides Bearing a 1-ol System



(**362**) R_1 = OH, R_7,R_8 = α-epoxide R_9 = O-β-D-Glc, $[\alpha]^{23}_D$ -54.4 (*c* 0.62, MeOH)

(**363**) R_1 = OH, R_7 = β-OMe, R_8 = α-OH R_9 = O-β-D-Glc, $[\alpha]^{23}_D$ -56.5 (*c* 0.46, MeOH)

(**364**) R_1 = OH, R_7 = R_8 = OH R_9 = O-β-D-Glc, $[\alpha]^{23}_D$ -63.7 (*c* 0.19, MeOH)

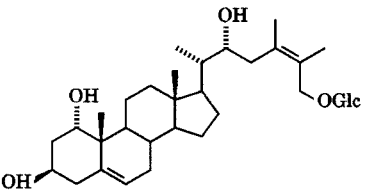
(**428**) R_1 = O-β-D-Glc, R_2 = R_6 = R_{10} = OH R_3 = R_4 = β-epoxide, Δ^{24}, R_9 = O

(**440**) R_1 = O-β-D-Glc, R_3 = α-OH R_4,R_5 = α-epoxide, R_9 = O, Δ^{24} $[\alpha]^{28}_D$ +48.6 (*c* 0.01, MeOH)

(**441**) R_1 = O-β-D-Glc, R_3 = R_{10} = OH, R_4,R_5 = α-epoxide, R_9 = O, Δ^{24} $[\alpha]^{28}_D$ -24.0 (*c* 0.01, MeOH)

(**442**) R_1 = O-β-Glc(1→6)-β-D-Glc, R_3 = α-OH R_4,R_5 = α-epoxide, R_9 = O, Δ^{24} $[\alpha]^{28}_D$ -9.6 (*c* 0.6, MeOH)

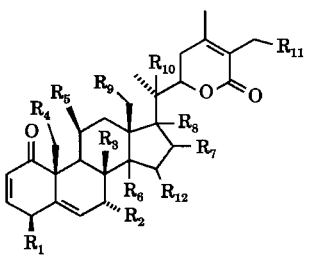
(**465**) R_1 = R_{10} = OH, R_9 = O, Δ^{24} $[\alpha]^{18}_D$ -4.72 (*c* 0.21, $CHCl_3$)



(**361**) $[\alpha]^{23}_D$ -24.3 (*c* 0.23, MeOH)

*All not specified R group correspond to hydrogen atoms

Withanolides Bearing a 2,5,24-trien-1-one System



(**22**) R_1 = R_6 = R_{10} = R_{11} = OH

m.p. 203-204°C, $[\alpha]^{25}_D$ +123 (*c* 0.028, MeOH)

(**85**) R_2 = OH, R_{11} = O-β-D-Glc

$[\alpha]^{21}_D$ -38.1 (*c* 0.72, C_5D_5N)

(**131**) R_1 = R_{10} = OH, R_9 = OAc

$[\alpha]^{25}_D$ +54.0 (*c* 0.94, $CHCl_3$)

(**159**) R_9 = OAc, R_{10} = OH, m.p. 132-135°C

(**160**) R_1 = R_{10} = OH, R_9 = OAc

$[\alpha]_D$ +66.3 (*c* 4.39, $CHCl_3$)

(**163**) R_1 = R_{11} = OH, R_2 = OAc

m.p. 149-152°C

(**290**) R_6 = R_8 = R_{10} = OH, R_9 = OAc

m.p. 150-151°C, $[\alpha]^{25}_D$ +17.5 (*c* 2.05, $CHCl_3$)

(**292**) R_6 = R_8 = R_4 = OH, m.p. 238-242°C

$[\alpha]^{20}_D$ +87 (*c* 0.85, MeOH)

(**295**) R_6 = R_{10} = OH, R_9 = OAc

$[\alpha]^{25}_D$ +62 (*c* 0.21, MeOH)

(**338**) R_1 = R_6 = R_8 = R_{10} = OH, m.p. 207-209°C

$[\alpha]_D$ +159.1 (*c* 0.46, MeOH)

*All not specified R groups correspond to hydrogen atoms

Contd.

Withanolides Bearing a 2,5,24-trien-1-one System

(**342**) R_1 = R_6 = R_8 = R_{10} = OH, m.p. 203-204°C

(**406**) R_6 = R_8 = R_{10} = R_{12} = OH

(**412**) R_{10} = OH, $[\alpha]^{25}_D$ +34 (*c* 0.0053, $CHCl_3$)

(**413**) R_{10} = R_{11} = OH, Δ^{14}

$[\alpha]^{25}_D$ +37 (*c* 0.0081, $CHCl_3$)

(**456**) R_3 = R_5 = R_7 = OH, m.p. 225-226°C

(**457**) R_3 = R_5 = OH, R_7,R_8 = α-epoxide

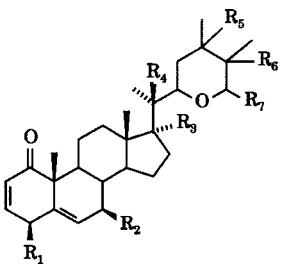
m.p. 230-231°C

(**458**) R_3 = R_9 = R_{10} = OH, m.p. 125-128°C

(**477**) R_{10} = O-β-D-Glc

*All not specified R group correspond to hydrogen atoms

Withanolides Bearing a 2,5-dien-1-one System



(**145**) R_1 = R_2 = R_4 = OH, R_7 = O, Δ^{24}

(**337**) R_1 = R_2 = R_4 = OH, R_7 = O

$[\alpha]^{20}_D$ -26.0 (*c* 0.082, CH_3CN)

(**367**) R_3 = R_7 = OH, R_5,R_6 = α-epoxide

m.p. 235-236°C, $[\alpha]^{25}_D$ -17.0 (*c* 0.22, MeOH)

(**368**) R_3 = OH, R_7 = β-OMe, R_5, R_6 = α-epoxide

m.p. 223-225°C, $[\alpha]^{25}_D$ -11.2 (*c* 0.22, MeOH)

(**369**) R_3 = OH, R_7 = α-OMe, R_5,R_6 = α-epoxide

m.p. 236-237°C, $[\alpha]^{25}_D$ -44.0 (*c* 0.13, MeOH)

(**370**) R_3 = OH, R_7 = O-β-D-Glc

R_5,R_6 = α-epoxide, $[\alpha]^{25}_D$ -42.1 (*c* 0.63, MeOH)

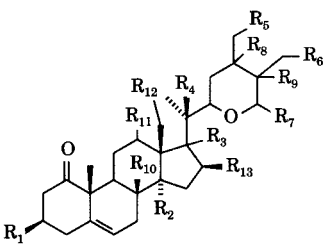
(**371**) R_3 = R_5 = R_6 = R_7 = OH, m.p. 223-224°C $[\alpha]^{25}_D$ +1.2 (*c* 0.44, MeOH)

(**372**) R_3 = R_6 = R_7 = OH, R_5 = OMe

m.p. 166-170°C, $[\alpha]^{25}_D$ -18.6 (*c* 0.40, MeOH)

*All not specified R group correspond to hydrogen atoms

Withanolides Bearing a 5-en-1-one System



(**18**) R_2 = R_4 = R_5 = OH, R_7 = O, $\Delta^{3,24}$

$[\alpha]^{25}_D$ +120.0 (*c* 0.25, MeOH)

(**19**) R_2 = R_4 = R_6 = OH, R_7 = O, Δ^{24}

$[\alpha]^{25}_D$ +64.0 (*c* 0.29, $CHCl_3$)

(**20**) R_2 = R_3 = R_4 = R_6 = OH, R_7 = O, $\Delta^{3,24}$

$[\alpha]^{21}_D$ +125.0 (*c* 0.058, MeOH)

*All not specified R groups correspond to hydrogen atoms

Contd.

Withanolides Bearing a 5-en-1-one System

(**21**) $R_2 = R_3 = R_6 = OH$, $R_7 = O$, Δ^{24}

$[\alpha]^{21}_D$ +57.0 (*c* 0.063, MeOH)

(**23**) $R_2 = R_3 = R_4 = R_6 = R_{10} = OH$, $R_7 = O$, Δ^{24} m.p. 273-275°C, $[\alpha]^{25}_D$ +74.0 (*c* 0.39, $CHCl_3$)

(**25**) $R_1 = R_2 = R_3 = R_4 = R_5 = OH$, $R_7 = O$, Δ^{24}

(**26**) $R_1 = R_3 = R_4 = OH$, $R_7 = O$, $\Delta^{14,24}$

(**94**) $R_4 = R_{12} = R_{13} = OH$, $R_7 = R_{11} = O$, Δ^3

$[\alpha]^{20}_D$ +54.3 (*c* 0.2, MeOH)

(**191**) $\Delta^{2,24}$, $R_7 = R_{11} = O$, $R_3 = OH$

$[\alpha]^{21}_D$ +18.6 (*c* 0.043, MeOH)

(**306**) $R_6 = OH$, $R_7 = O$, $\Delta^{14,24}$

$[\alpha]^{25}_D$ +113.0 (*c* 0.8, MeOH)

(**324**) R_1 = O-β-D-Glc, $R_4 = R_6 = OH$, $R_7 = O$ Δ^{24} $[\alpha]^{25}_D$ +73.4 (*c* 0.43, MeOH)

(**325**) R_1 = O-β-D-Glc, $R_2 = R_4 = R_6 = OH$ $R_7 = O$, Δ^{24}, $[\alpha]^{25}_D$ +86.3 (*c* 0.49, MeOH)

(**365**) $R_1 = HO_3SO$, $R_3 = R_7$ = α-OH R_8,R_9 = α-epoxide, $[\alpha]^{23}_D$ +8.6 (*c* 0.21, MeOH)

(**366**) $R_1 = HO_3SO$, $R_3 = R_9 = R_7 = OH$ R_8 = β-OMe, $[\alpha]^{23}_D$ +12.6 (*c* 0.71, MeOH)

(**411**) $R_2 = R_3 = R_4 = OH$, $R_7 = O$, $\Delta^{3,24}$

(**416**) R_1 = O-β-D-Glc, $R_2 = R_4 = OH$, $R_7 = O$ Δ^{24} $[\alpha]_D$ +35.0 (*c* 0.31, MeOH)

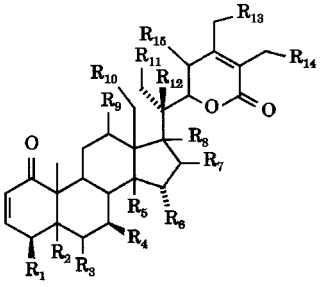
(**424**) R_1 = O-β-D-Glc, $R_2 = R_3 = R_4 = OH$, $R_7 = O$, Δ^{24}, $[\alpha]_D$ +30.0 (*c* 0.3, MeOH)

(**460**) R_1 = *O*-β-D-Glc, $R_4 = R_6 = OH$,

$R_7 = O$ $\Delta^{14,24}$, $[\alpha]_D$ +45.0 (*c* 0.31, MeOH)

*All not specified R group correspond to hydrogen atoms

Withanolides Bearing a 2-en-1-one System



(**32**) $R_2 = R_3 = R_{11} = R_{14} = OH$, m.p. 285-288°C

$[\alpha]_D$ +52.5 (*c* 0.4, Dioxane)

(**38**) $R_2 = R_3 = R_9 = R_{11} = R_{14} = OH$

m.p. 246-247°C

(**40**) $R_2 = R_3 = R_4 = R_9 = OH$, m.p. 277-278°C

$[\alpha]^{25}_D$ -31.5 (*c* 0.2, MeOH)

(**55**) $R_2 = R_3 = R_{11} = OH$

(**59**) $R_2 = R_3 = R_{11} = OH$, $R_{14} = OMe$

m.p. 275-278°C

(**61**) R_{14} = O-β-D-Glc, $\Delta^{4,6}$

$[\alpha]^{20}_D$ -36.0 (*c* 0.20, MeOH)

(**62**) $R_3 = R_4 = OH$, R_{14} = O-β-D-Glc, Δ^4

$[\alpha]^{20}_D$ -30.0 (*c* 0.20, MeOH)

*All not specified R groups correspond to hydrogen atoms

Contd.

Withanolides Bearing a 2-en-1-one System

(**66**) $R_2 = R_3 = R_9 = R_{11} = OH$, $R_{14} = OMe$

(**69**) $R_2 = R_3 = R_{11} = OH$, $R_{14} = \beta$-D-Glc

$[\alpha]^{20}_D$ +14.0 (*c* 0.10, MeOH)

(**83**) $R_3 = R_9 = R_{11} = OH$, Δ^4

(**84**) $R_2 = R_3 = R_9 = R_{11} = OH$

(**200**) $R_2 = R_3 = R_5 = R_8 = OH$, m.p. 181-182°C

(**261**) $R_2 = R_3 = R_5 = OH$, $R_6 = OAc$

R_{13} = O-D-Glc, Δ^{16}, $[\alpha]_D$ +56.7 (*c* 0.66, MeOH)

(**262**) $R_2 = R_3 = R_5 = OH$, $R_6 = OAc$

R_7,R_8 = β-epoxide, $[\alpha]_D$ +70.7 (*c* 1.1, MeOH)

(**263**) $R_2 = R_3 = R_5 = OH$, $R_6 = OAc$ R_7,R_8 = β-epoxide, R_{13} = O-β-D-Glc

$[\alpha]_D$ +31.3 (*c* 0.76, MeOH)

(**265**) R_2 = α-Cl, $R_3 = R_5 = OH$, $R_6 = OAc$, Δ^{16}

(**269**) R_2 = Cl, $R_3 = R_5 = OH$, $R_6 = OAc$

$[\alpha]^{27}_D$ +66.0 (*c* 0.85, MeOH)

(**270**) $R_2 = R_3 = R_5 = OH$, $R_6 = OAc$

$[\alpha]^{27}_D$ +30.9 (*c* 1.15, MeOH)

(**271**) $R_2 = R_3 = R_5 = R_8 = OH$, $R_6 = OAc$

m.p. 190-193°C, $[\alpha]^{27}_D$ +92.8 (*c* 2.0, MeOH)

(**275**) $R_2 = R_3 = R_5 = R_8 = R_{14} = OH$, R_6 = OAc

(**276**) $R_2 = R_3 = R_5 = R_{15} = OH$, $R_6 = OAc$, Δ^{16}

(**279**) $R_1 = R_2 = R_3 = R_5 = R_8 = R_{12} = OH$

$[\alpha]^{25}_D$ +8.6 (*c* 0.02, MeOH)

(**287**) $R_2 = R_3 = R_5 = R_8 = R_{12} = OH$, R_{10} = OAc m.p. 204-205°C, $[\alpha]^{22}_D$ +17.5 (*c* 1.7, MeOH)

(**288**) $R_2 = R_3 = R_5 = R_8 = R_{12} = R_{13} = OH$, R_{10} = OAc, m.p. 209-210 °C

$[\alpha]^{22}_D$ +62.09 (*c* 1.53, MeOH)

(**291**) $R_2 = R_3 = R_8 = R_{12} = OH$, $R_{10} = OAc$, Δ^{14} m.p. 185-187°C,

$[\alpha]^{25}_D$ +7.77 (*c* 0.9, $CHCl_3$)

(**296**) $R_2 = R_3 = R_{10} = R_{12} = OH$

$[\alpha]^{25}_D$ +53.0 (*c* 0.29, MeOH)

(**297**) $R_3 = R_5 = R_8 = R_{12} = OH$, $R_{10} = OAc$, Δ^4 $[\alpha]^{25}_D$ -48.0 (*c* 0.23, MeOH)

(**299**) $R_2 = R_3 = R_{12} = OH$, $R_{10} = OAc$

m.p. 303-305°C, $[\alpha]^{25}_D$ +65.0 (*c* 0.28, MeOH)

(**339**) R_2 = Butoxyl, $R_3 = OAc$

$R_5 = R_8 = R_{12} = OH$, $[\alpha]_D$ +47.0 (*c* 0.47, MeOH)

(**384**) $R_1 = R_2 = R_3 = R_8 = OH$

m.p. 178-180°C, $[\alpha]^{24\,4}_D$ +100.3 (*c* 0.1, MeOH)

(**385**) $R_1 = R_2 = R_3 = R_7 = OH$, $R_8 = CH_3$, Δ^{13} m.p. 214-216°C, $[\alpha]^{24\,4}_D$ +178.9 (*c* 0.1, MeOH)

(**386**) $R_1 = R_2 = R_7 = OH$, $R_3 = Cl$, $R_8 = CH_3$, Δ^{13} m.p. 264-266°C, $[\alpha]^{24\,4}_D$ +32.5 (*c* 0.1, MeOH)

(**420**) $R_2 = R_3 = R_5 = R_6 = R_8 = R_{12} = OH$

$[\alpha]_D$ 98.0 (*c* 0.45, MeOH)

(**439**) $R_2 = R_5 = R_{12} = OH$, Δ^7

m.p. 294-295°C, $[\alpha]_D$ +67.77 (*c* 0.09, $CHCl_3$)

(**459**) $R_4 = R_{10} = R_{12} = R_{14} = OH$, Δ^4

m.p. 144-146°C

(**464**) $R_1 = R_2 = R_3 = R_{14} = OH$

$[\alpha]^{20}_D$ 272 (*c* 0.01, $CHCl_3$)

(**466**) $R_1 = R_{14} = OH$, R_5,R_6 = α-epoxide

$[\alpha]^{18}_D$ -2.66 (*c* 0.6, $CHCl_3$)

(**473**) $R_1 = R_2 = R_3 = R_{14} = OH$

(**475**) $R_2 = R_3 = R_5 = R_8 = R_{12} = OH$

m.p. 278-280°C

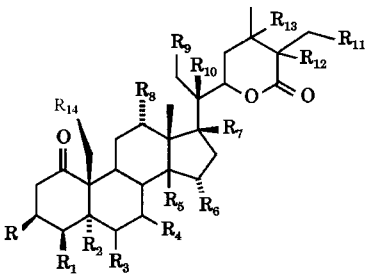
(**478**) R_2 = Cl, $R_3 = R_5 = R_8 = R_{12} = OH$

m.p. 180-182°C

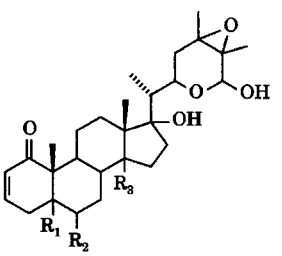
(**479**) $R_2 = R_5 = R_8 = R_{12} = OH$, R_3 = Cl

*All not specified R groups correspond to hydrogen atoms

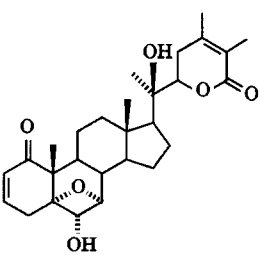
Withanolides Bearing a 1-one System



(**17**) R = OMe, $R_1 = R_3 = R_{11}$ = OH, Δ^{24}
$[\alpha]^{31}_D$ +29.0 (*c* 0.04, $CHCl_3$)
(**41**) $R_2 = R_3 = R_4 = R_8$ = OH, Δ^2 R_{13},R_{12} = α-epoxide,
$[\alpha]^{25}_D$ -50.5 (*c* 0.2, MeOH)
(**54**) $R_2 = R_3 = R_9$ = OH, m.p. 290-292°C
(**63**) R_4 = OH, R_{11} = O-β-D-Glc, $\Delta^{3,5,24}$
$[\alpha]^{20}_D$ -21.0 (*c* 0.20, MeOH)
(**67**) $R_2 = R_3 = R_6 = R_9$ = OH, Δ^{24}
(**189**) $R_3 = R_{11}$ = OH, $\Delta^{4,24}$
(**190**) $R_2 = R_3 = R_{11}$ = OH, Δ^{24}
(**204**) $R_1 = R_2 = R_3 = R_{11} = R_{14}$ = OH, Δ^{24}
m.p. 204-205°C
(**211**) $R_2 = R_3 = R_7$ = OH, Δ^{24}
m.p. 289°C, $[\alpha]_D$ +109.0 (*c* 0.85, MeOH)
(**286**) $R_1 = R_2 = R_3 = R_5 = R_7 = R_{10}$ = OH
R_6 = OMe, Δ^{24}, $[\alpha]^{25}_D$ -12.8 (*c* 0.1, MeOH)
(**293**) $R_2 = R_3 = R_5 = R_7 = R_{10}$ = OH, Δ^2 m.p. 179-182°C, $[\alpha]^{20}_D$ +58.2 (*c* 0.158, MeOH)
(**319**) $R_2 = R_3 = R_5 = R_{10} = R_{11}$ = OH, Δ^{24}
$[\alpha]_D$ +72.5 (*c* 0.23, MeOH)
(**419**) $R_2 = R_3 = R_5 = R_6 = R_7 = R_{10} = R_{11}$ = OH
(**463**) $R_1 = R_2 = R_3 = R_{11}$ = OH
$[\alpha]^{20}_D$ 162.0 (*c* 0.024, $CHCl_3$)



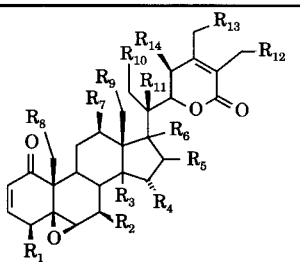
(**176**) R_1 = Cl, $R_2 = R_3$ = OH
m.p. 173°C, $[\alpha]^{21}_D$ +13.2 (*c* 0.004, $CHCl_3$)
(**373**) R_1,R_2 = epoxide
(**374**) $R_1 = R_2$ = OH



(**452**) m.p. 242°C
$[\alpha]^{30}_D$ +12.73 (*c* 0.14, MeOH)

*All not specified R groups correspond to hydrogen atoms

Withanolides Bearing a 5β,6β-epoxy-2,24-diene-1-one System



(**1**) $R_1 = R_{11}$ = OH, R_2 = OAc, m.p. 151-153°C
(**2**) $R_1 = R_{11}$ = OH, $R_2 = R_5$ = OAc
m.p. 163-166°C
(**3**) $R_2 = R_5$ = OAc, m.p. 132-137°C

Contd.

Withanolides Bearing a 5β,6β-epoxy-2,24-diene-1-one System

(**33**) $R_{10} = R_{12} = OH$, m.p. 245-247°C
$[\alpha]_D$ +30.2 (*c* 0.53, $CHCl_3$)

(**39**) $R_7 = R_{10} = OH$

(**81**) $R_{10} = OH$, m.p. 275-277°C

(**82**) $R_7 = R_{10} = OH$

(**105**) $R_5 = OAc$, $R_6 = OH$
$[\alpha]_D$ = + 22 (c 0.1, $CHCl_3$)

(**106**) $R_5 = R_6 = OH$
$[\alpha]_D$ = +48 ° (*c* 0.1, $CHCl_3$)

(**107**) $R_5 = OAc$, $[\alpha]_D$ = +30° (*c* 0.1, $CHCl_3$)

(**108**) $R_6 = OH$

(**127**) $R_1 = R_5 = OH$, m.p. 250-251°C

(**128**) $R_1 = R_9 = OH$, m.p. 150°C

(**129**) $R_1 = R_5 = OH$, $R_9 = O$, m.p. 238-240°C

(**130**) $R_1 = R_{11} = OH$, $R_9 = OAc$, m.p. 148-153°C $[\alpha]_D$ +71.9° (*c* 5.0, $CHCl_3$)

(**146**) $R_1 = R_2 = R_{11} = OH$

(**147**) $R_1 = R_{12} = OH$

(**148**) $R_1 = OH$, $R_9 = OAc$

(**149**) $R_1 = OH$, $R_5 = OAc$

(**152**) $R_1 = R_{12} = OH$, $R_9 = OAc$, m.p. 134-138°C

(**153**) $R_1 = OH$, $R_5 = OAc$, $R_{12} = OH$ m.p. 242-244°C

(**154**) $R_1 = R_5 = OH$, $R_9 = OAc$, m.p. 153-157°C

(**156**) $R_1 = R_{11} = OH$, m.p. 253-255°C

(**161**) $R_1 = OH$, $R_5 = R_9 = OAc$, m.p. 131-134°C

(**162**) $R_1 = OH$, $R_7 = R_5 = OAc$, m.p. 115-118°C

(**188**) $R_{12} = OH$

(**202**) $R_8 = R_{12} = OH$, m.p. 287-288°C

(**264**) $R_3 = OH$, $R_4 = OAc$, Δ^{16}
$[\alpha]_D$ +31.3 (*c* 0.76, MeOH)

(**267**) $R_1 = R_3 = OH$, $R_4 = OAc$
R_5,R_6 = β-epoxide, m.p. 242,5-243°C
$[\alpha]^{31}_D$ +105.8 (*c* 0.52, MeOH)

(**268**) $R_3 = OH$, $R_4 = OAc$, R_5,R_6 = β-epoxide
$[\alpha]^{27}_D$ +61.4 (*c* 0.87, MeOH)

(**278**) $R_1 = R_3 = OH$, $R_4 = OAc$
m.p. 152-153°C, $[\alpha]^{20}_D$ +23.9 (*c* 0.018, $CHCl_3$)

(**280**) $R_1 = R_3 = R_4 = R_6 = R_{11} = OH$
$[\alpha]^{25}_D$ +197.2 (*c* 0.1, MeOH)

(**289**) $R_3 = R_6 = R_{11} = OH$, $R_9 = OAc$
m.p. 156-157°C

(**298**) $R_9 = OAc$, $R_{11} = OH$
$[\alpha]^{25}_D$ +61 (*c* 0.21, MeOH)

(**317**) $R_1 = R_3 = R_6 = R_{11} = OH$

(**318**) $R_3 = R_6 = R_{11} = R_{13} = OH$

(**320**) $R_1 = R_3 = R_4 = OH$, m.p. 142-144°C
$[\alpha]_D$ +68 (*c* 0.2, MeOH)

(**333**) $R_1 = R_9 = R_{11} = OH$, m.p. 171-173°C
$[\alpha]^{20}_D$ +52.0 (*c* 0.15, MeOH)

(**387**) $R_1 = R_5 = OH$, $R_6 = Me$, Δ^{13}
m.p. 223-225°C, $[\alpha]^{24\,3}_D$ -0.57 (*c* 0.1, MeOH)

(**388**) $R_1 = OH$, R_5,R_6 = α-epoxide
m.p. 233-235°C, $[\alpha]^{24\,4}_D$ +22.3 (*c* 0.1, MeOH)

(**395**) $R_1 = R_6 = OH$, m.p. 200-202°C
$[\alpha]^{25}_D$ +75.7 (*c* 0.07, MeOH)

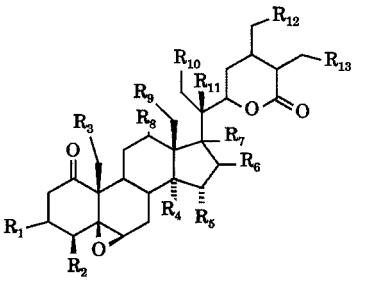
(**396**) $R_1 = R_{11} = OH$, R_5,R_6= α-epoxide
m.p. 245-247°C, $[\alpha]^{26\,5}_D$ +14.4 (*c* 0.12, MeOH)

(**397**) $R_1 = R_{14} = OH$, R_5,R_6= α-epoxide
m.p. 223-225°C, $[\alpha]^{25\,2}_D$ -34 (*c* 0.1, MeOH)

(**430**) $R_1 = OH$, Δ^{16}, m.p. 268°C
$[\alpha]^{30}_D$ +92.6 (*c* 0.25, $CHCl_3$)

*All not specified R groups correspond to hydrogen atoms

Withanolides Bearing a 5β,6β-epoxy-1-one System



(**15**) R_1 = OMe, R_2 = R_{12} = OH, Δ^{24}

(**16**) R_1 = OMe, R_2 = R_{12} = OH, R_3 = COOH, Δ^{24}

(**80**) R_{10} = OH, Δ^{24}, m.p. 288-290°C

(**150**) R_2 = OH, R_9 = OAc, Δ^2, m.p. 106-112°C

(**151**) R_2 = OH, R_6 = OAc, Δ^2, m.p. 191-196°C

(**155**) R_1 = R_2 = OH, R_9 = OAc, Δ^{24} m.p. 148-154°C

(**157**) R_1 = OMe, R_2 = R_{11} = OH, Δ^{24} m.p. 222-228°C, $[\alpha]_D$ -119.3 (*c* 2.8, $CHCl_3$)

(**158**) R_1 = OMe, R_2 = R_{11} = OH, R_9 = OAc, Δ^{24}, m.p. 242-245°C,

$[\alpha]_D$ +13.2 (*c* 7.87, $CHCl_3$)

(**164**) R_1 = OMe, R_2 = R_{13} = OH, Δ^{24}

(**165**) R_1 = OMe, R_2 = OH, R_9 = OAc, Δ^{24}

(**166**) R_1 = OMe, R_2 = OH, R_6 = OAc, Δ^{24}, m.p. 127-131°C

(**213**) R_8 = O, R_7 = OH, Δ^{24}, m.p. 216°C

$[\alpha]_D$ -21.2 (*c* 4.1, MeOH)

(**214**) R_8 = O, R_7 = OH, Δ^{24}

(**277**) R_1 = OMe, R_4 = OH, R_5 = OAc, $\Delta^{16,24}$

(**167**) R_1 = OMe, R_2 = R_{13} = OH, R_9 = OAc, Δ^{24}, m.p. 130-134°C

(**168**) R_1 = OMe, R_2 = R_{13} = OH, R_6 = OAc, Δ^{24}, m.p. 139-143°C

(**169**) R_1 = OMe, R_2 = R_6 = OH, R_9 = OAc, Δ^{24}

(**203**) R_3 = R_7 = R_{13} = OH, Δ^{24}, m.p. 220-222°C

(**281**) R_2 = R_4 = R_7 = R_{11} = OH, R_5 = OCH_3, Δ^{24} $[\alpha]^{25}_D$ -69.2 (*c* 0.1, MeOH)

(**282**) R_4 = R_7 = R_{11} = OH, R_2 = OCH_3, Δ^{24} $[\alpha]^{25}_D$ +127.5 (*c* 0.1, MeOH)

(**283**) R_4 = R_{11} = OH, $\Delta^{16,24}$

$[\alpha]^{25}_D$ +18.5 (*c* 0.02, MeOH)

(**284**) R_1 = OCH_3, R_4 = R_6 = R_7 = R_{11} = OH, Δ^{24} $[\alpha]^{25}_D$ -4.1 (*c* 0.03, MeOH)

(**294**) R_1 = OMe, R_2 = OH, Δ^{24}, m.p. 256-257°C, $[\alpha]_D$ -40.47 (*c* 0.43, $CHCl_3$)

(**327**) R_1 = OMe, R_2 = R_6 = R_{11} = OH

$[\alpha]^{22}_D$ +47.4 (*c* 0.1, $CHCl_3$)

(**427**) R_1 = R_2 = OH, R_{13} = O-β-D-Glc, Δ^{24}

(**429**) R_{13} = OH

R_2 = 2,2-dimethylcyclopropanone, Δ^{24}

(**462**) R_1 = R_2 = R_{13} = OH, R_4,R_5 = α-epoxide, Δ^{24} $[\alpha]^{20}_D$ -26.0 (*c* 0.6, $CHCl_3$)

(**467**) R_1 = OMe, R_2 = R_{13} = OH, Δ^{24}

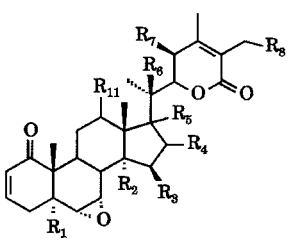
(**468**) R_2 = R_7 = R_{13} = OH, Δ^{24}

$[\alpha]^{25}_D$ +12.0 (*c* 0.11, $CHCl_3$)

(**472**) R_1 = OMe, R_2 = R_{11} = OH

*All not specified R groups correspond to hydrogen atoms

Withanolides Bearing a 6,7-epoxy-2-en-1-one System



(**42**) R_1 = R_{11} = OH, m.p. 271-272°C

(**43**) R_1 = R_3 = R_{11} = OH, m.p. 266-267°C

(**44**) R_1 = R_8 = R_{11} = OH, m.p. 271-273°C

(**45**) R_1 = R_8 = R_{11} = OH,

m.p. 260-262°C, $[\alpha]_D$ +76.67

Contd.

Withanolides Bearing a 6,7-epoxy-2-en-1-one System

(**46**) R_1 = OH, R_{11} = O, m.p. 264-266°C

(**56**) R_1 = OH

(**58**) R_1 = R_8 = OH, m.p. 292-294°C

$[\alpha]_D$ +92.3 (*c* 1.47, $CHCl_3$)

(**68**) R_1 = R_3 = R_{11} = OH

(**110**) R_1 = R_6 = OH

(**111**) R_1 = OH, R_5 = β-OH

(**112**) R_1 = OH, R_5 = α-OH, m.p. 264-267°C

$[\alpha]^{20}_D$ +64.5 (*c* 0.08, MeOH)

(**113**) R_1 = R_5 = R_{11} = OH

(**139**) R_1 = OH, R_4 = β-OH, Δ^{17}, m.p. 260-263°C

(**140**) R_1 = OH, R_4 = α-OH, Δ^{17}, m.p. 273-274°C

(**141**) R_1 = R_4 = R_5 = OH, m.p. 278-279°C

(**436**) R_1 = R_5 = OH

(**437**) R_1 = R_6 = R_8 = OH, m.p. 271-273°C

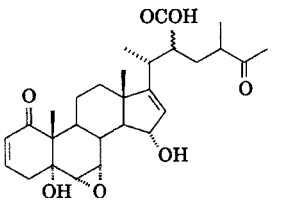
$[\alpha]_D$ +74.88 (*c* 0.44, MeOH)

(**453**) R_1 = OH, R_4 = OAc, Δ^{17}, m.p. 238-240°C

$[\alpha]^{30}_D$ +0.97 (*c* 0.24, MeOH)

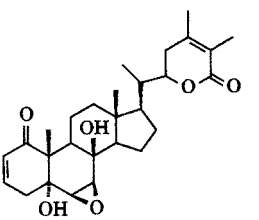
(**470**) R_7 = OH

(**476**) R_1 = R_2 = R_5 = R_7 = OH, m.p. 260-262°C



(**246**) m.p. 173-175 °C

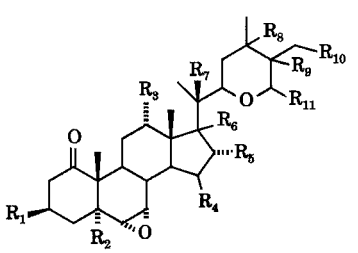
$[\alpha]^{25}_D$ + 57.8 (*c* 0.25, $CHCl_3$)



(**455**) m.p. 251-252°C.

*All not specified R groups correspond to hydrogen atoms

Withanolides Bearing a 6α,7α-epoxy-1-one System



(**79**) R_2 = R_3 = OH, R_8, R_9 = α-epoxide R_{11} = O, Δ^2

(**142**) R_2 = OH, R_8,R_9 =α-epoxide, R_{11} = O, Δ^2 m.p. 288-290°C, $[\alpha]_D$ 132.8 (*c* 0.013, MeOH)

(**143**) R_2 = R_{11} = OH, R_8, R_9 = α-epoxide, Δ^2 m.p. 241-243°C, $[\alpha]_D$ 98.4 (*c* 0.012, MeOH)

(**144**) R_2 = R_{11} = OH, R_5 = OAc

R_8,R_9 = α-epoxide, Δ^2, m.p. 262-264°C

(**243**) R_2 = R_3 = R_{11} = OH, R_8, R_9 = α-epoxide, Δ^2 m.p. 228-231°C, $[\alpha]^{24}_D$ +44.6 (*c* 1.03, $CHCl_3$)

Contd.

Withanolides Bearing a 6α,7α-epoxy-1-one System

(**244**) $R_2 = R_4 = R_{11} = OH$

R_8, R_9 = α-epoxide, $\Delta^{2,16}$

m.p. 224-226°C, $[\alpha]^{24}_D$ +75.8 (*c* 0.46, $CHCl_3$)

(**245**) $R_2 = R_4 = R_{11} = OH$, R_8, R_9 = α-epoxide, $\Delta^{2,16}$ $[\alpha]^{24}_D$ +22.6 (*c* 0.61, $CHCl_3$)

(**431**) $R_2 = R_7 = OH$, $R_{11} = O$, Δ^2

(**432**) $R_2 = R_6 = R_{10} = OH$, $R_{11} = O$, Δ^2

(**433**) $R_1 = R_2 = R_6 = OH$, $R_{11} = O$, Δ^{24}

m.p. 258°C, $[\alpha]^{30}_D$ +66.0 (*c* 0.25, MeOH)

(**434**) $R_1 = OSO_3H$, $R_2 = R_6 = OH$, $R_{11} = O$, Δ^{24}

m.p. 158°C, $[\alpha]^{30}_D$ +59.4 (*c* 0.25, MeOH)

(**438**) $R_2 = R_7 = OH$, $R_{11} = O$, Δ^2, m.p. 281-283 °C $[\alpha]_D$ -123.99 (*c* 0.53, $CHCl_3$)

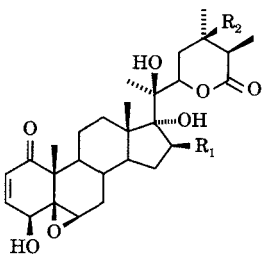
(**469**) $R_1 = R_2 = R_7 = OH$, $R_{11} = O$, Δ^{24}

$[\alpha]^{25}_D$ -196 (*c* 0.006, MeOH)

(**471**) $R_6 = OH$, $R_{11} = O$, $\Delta^{4,24}$

*All not specified R groups correspond to hydrogen atoms

Withanolides Bearing a 5,6-epoxy-2-en-1-one System



(**328**) m.p. 274-275°C

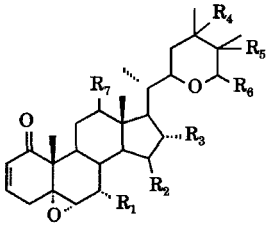
$[\alpha]^{20}_D$ -6.2 (*c* 0.08, MeOH)

(**329**) R_2 = OH, m.p. 260-261°C

$[\alpha]^{20}_D$ +74.3 (*c* 0.16, MeOH)

(**330**) R_1 = OH, m.p. 276-280°C

$[\alpha]^{20}_D$ +23.5 (*c* 0.08, MeOH)



(**41A**) $R_1 = OH$, $R_6 = O$, Δ^{24}

$[\alpha]^{25}_D$ 43.5 (*c* 0.2, MeOH)

(**348**) $R_3 = R_6 = OH$, R_4, R_5 = α-epoxide, Δ^{13},

m.p. 157-159°C, $[\alpha]^{25}_D$ -6.0 (*c* 0.05, MeOH)

(**349**) $R_2 = R_6 = OH$, R_4,R_5 = α-epoxide, Δ^{16}

m.p. 159-161°C, $[\alpha]^{25}_D$ +36.0 (*c* 0.05, MeOH)

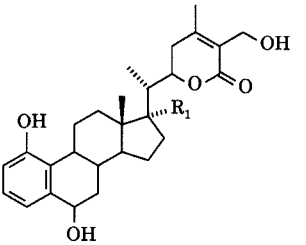
(**356**) $R_2 = O$, $R_6 = OH$, R_4,R_5 = α-epoxide, Δ^{16}, m.p. 154-155°C

Withanolides Bearing an Aromatic Ring A System

These withanolides are also denominated jaborols because they were isolated only from plants of the genus *Jaborosa*, from where the name comes from. The first to be isolated was the jaborol, from *Jaborosa magellanica* by

*All not specified R groups correspond to hydrogen atoms

Fajardo and co-workers (1987), followed by jaborosalactone 7 (**199**) and jaborosalactone Q (**205**), both isolated from *Jaborosa leucotricha* (Veleiro *et al.,* 1992b; Misico *et al.,* 1997). They are unique in their structure.

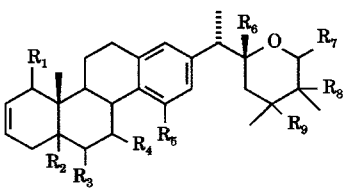


(**199**) R_1= OH, m.p. 185-186°C

(**205**) R_1 = H, m.p. 176-177°C

Withanolides Bearing an Aromatic Ring D System

These withanolides differ from the last ones due to the presence of an aromatic ring D instead of ring A. They are denominated nicandrenones, because nicandrenone (**31**) and nicandrenolactone were the first ones to be isolated for the first time from *Nicandra physaloids* and, salpichrolide A (**353**) from *Salpichroa origanifolia* (Ray & Gupta, 1994). From that date on, 10 of these secondary metabolites were isolated: salpichrolides B (**354**), C (**355**), E (**346**), F (**347**), G (**343**), H (**344**), I (**345**), J (**351**), K (**352**) and M (**350**), all isolated from *Salpichroa origanifolia* (Veleiro *et al.,* 1994; Tettamanzi *et al.,* 1996, 1998, 2001). The withanolide nicadrenone (**31**) was also isolated from *Cassia siamea* (Srivastava *et al.,* 1992), the only report of this type of compound from the Caesalpinaceae-Leguminosae family.



(**31**) R_1 = O, R_2 = R_7 = OH, R_3, R_4 = α-epoxide R_8,R_9 = β-epoxide, m.p. 117°C

(**343**) R_1 = O, R_2, R_3 = α-epoxide, R_5 = R_7 = OH, R_8,R_9 = β-epoxide, m.p. 155-156°C

(**344**) R_1 = O, R_2, R_3 = α-epoxide R_7 = R_8 = R_9 = OH

(**345**) R_1 = O, R_2, R_3 = α-epoxide, R_7 = R_8 = OH, Δ^{23}

(**350**) R_1 = O, R_2, R_3 = α-epoxide R_7 = R_8 = R_9 = OH, $[\alpha]^{25}_D$ -50.8 (*c* 0.05, MeOH)

(**351**) R_1 = O, R_2, R_3 = α-epoxide, R_9 = OH, R_6,R_8 = β-epoxide, m.p. 172-173°C

$[\alpha]^{25}_D$ -25.0 (*c* 0.04, MeOH)

(**352**) R_1 = O, R_2, R_3 = α-epoxide R_6 = R_8 = R_9 = OH, $[\alpha]^{25}_D$ -28.0 (*c* 0.03, MeOH)

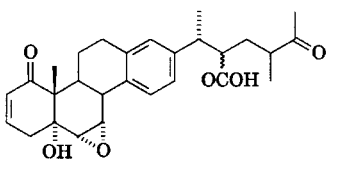
(**353**) R_1 = O, R_2, R_3 = α-epoxide R_8,R_9 = β-epoxide, R_7 = OH, m.p. 179-180°C

(**354**) R_1 = R_7 = OH, R_2, R_3 = α-epoxide

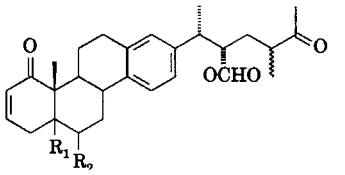
R_8,R_9 = β-epoxide, m.p. 164-165°C

(**355**) R_1 = O, R_2 = R_3 = R_7 = OH

R_8,R_9 = β-epoxide, m.p. 179-180°C



(**247**) $[\alpha]^{26}_{D}$ +15.9 (*c* 1.72, $CHCl_3$)



(**346**) R_1, R_2 = α-epoxide, m.p. 180-181°C

(**347**) R_1 = α-OH, R_2 = β-OH

Modified Withanolides

The modified withanolides are designated in this way because they are different from most withanolides commonly found. Compounds like cilistol p (**357**), pm (**358**), pl (**359**) and u (**360**), isolated from the leaves of *Solanum cilistum* (Zhu *et al.*, 2001a), show a ketal group at C-26 and a 3,5-cyclopropane ring. The compounds denominated TH-6 (**382**) and TH-12 (**383**) were obtained from *Tubocapsicum anomalum* (Shingu *et al.*, 1990). The subtrifloralactones D (**98**) and E (**99**), isolated from *Deprea subtriflora* by Su *et al.* (2003b), are withasteroids with just 27 carbon atoms (*nor*-withasteroids) missing the methyl group in C-18, and presenting a carbonyl group in C-12.

(**357**) $R_1 = R_2 = R_3$ = OH, R_4 = O-β-D-Glc

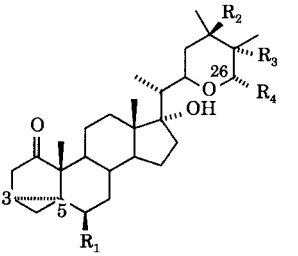
$[\alpha]^{23}_{D}$ -77.8 (*c* 0.23, MeOH)

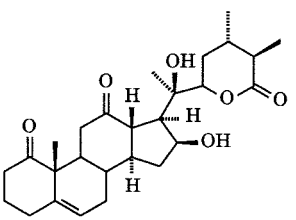
(**358**) R_1 = OMe, $R_2 = R_3$ = OH, R_4 = O-β-D-Glc $[\alpha]^{23}_{D}$ -65.2 (*c* 0.36, MeOH)

(**359**) $R_1 = R_3$ = OH, R_2 = OMe, R_4 = O-β-D-Glc $[\alpha]^{23}_{D}$ -125.0 (*c* 0.36, MeOH)

(**360**) R_1 = OH, R_2,R_3 = α-epoxide

R_4 = O-β-D-Glc, $[\alpha]^{23}_{D}$ -102.0 (*c* 0.15, MeOH)

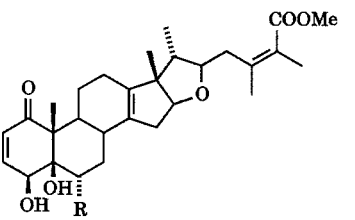




(**98**) Δ^2, m.p. 196-197°C

$[\alpha]^{23}_{D}$ +2.0 (*c* 0.15, MeOH)

(**99**) Δ^3, $[\alpha]^{23}_{D}$ +12.9 (*c* 0.05, MeOH)

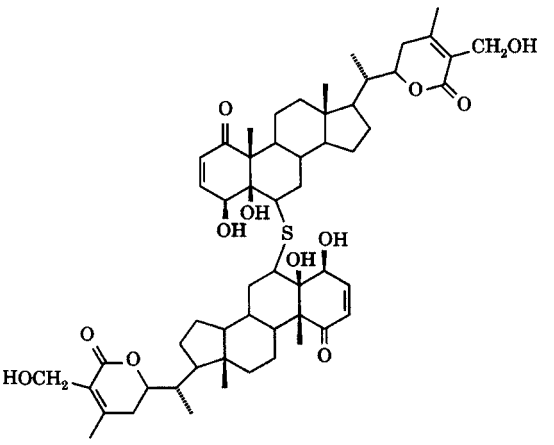


(**382**) R = Cl, m.p. 190-192°C

$[\alpha]_{D}$ -120.4 ($CHCl_3$)

(**383**) R = OH, $[\alpha]_{D}$ -65.8 ($CHCl_3$)

*All not specified R groups correspond to hydrogen atoms



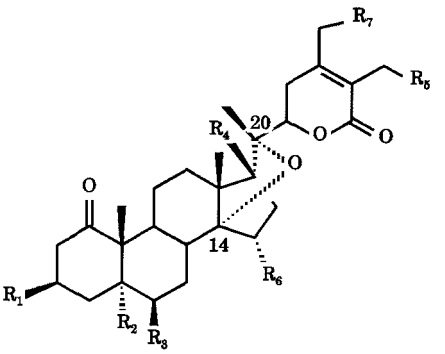
(**454**) m.p. 180-182°C, $[\alpha]_D$ +161.0 (*c* 0.09, MeOH)

$[\alpha]_D$ + 240.1 (*c* 0.385, DMSO)

A group of modified withanolides presenting a heterocycle ring through the carbon atoms C-14/C-20 with configuration α (17α) was isolated mainly from plants of the genus *Withania*. The first report of this type of compound dates from 1993, with the isolation of coagulin (**415**) from the same source (Atta-Ur-Rahman *et al.,* 1993). Lately, another group of compounds, which were named coagulin B (**407**), C (**408**), D (**409**) and E (**410**) as isolated from *W. coagulance*, while the coagulins I (**421**), J (**422**), K (**426**), M (**418**), N (**417**) and **414** were obtained from *W. coagulans*.

Compounds **24** and **27** were isolated from *Ajuga parviflora* (Khan *et al.,* 1999; Nawaz *et al.,* 2000). Khan and co-workers denominated compound **24** of ajugin, which constitute the first report of withanolides from the Labiatae family. In the same year, Atta-Ur-Rahman and co-workers isolated this compound from *W. coagulans* and denominated it coagulin R.

In 1998 and 1999 the withanolides **321** and **326**, respectively, were isolated from *Physalis peruviana* by Ahmad's group, while the jaborosalactones 18 (**171**), 19 (**173**), 20 (**174**), 21 (**175**) and **172,** with an uncommon structure and having hydroxyl groups in C-14 and C-17, both with 17β configuration, were isolated from *Jaborosa bergii* by Nicotra *et al.* (2003). A new C-27 *nor*-withanolide, designated withaphysanolide A (**300**), with a pyran ring, was isolated from aerial parts of *Physalis divericata* together with the known withaphysalins A, C, D and E, and the physalins A, B, D, F and H (Ma *et al.,* 2007).



(**24**) $R_1 = R_4 = OH$, Δ^5

$[\alpha]_D$ +35.0 (*c* 0.31, MeOH)

(**27**) $R_7 = OH$, $\Delta^{2,5}$

(**321**) R_1 = O-β-D-Glc, $R_4 = OH$, Δ^5 m.p. 210-211°C $[\alpha]^{25}_D$ +78.0 (*c* 0.0077, MeOH)

(**326**) $R_4 = OH$, $\Delta^{3,5}$, m.p. 192-193°C

$[\alpha]^{25}_D$ -11.0 (*c* 0.0062, $CHCl_3$-MeOH)

(**407**) $R_5 = OH$, $\Delta^{2,5}$

(**408**) $R_4 = OH$, $\Delta^{2,5}$

(**409**) $\Delta^{2,5}$

(**410**) $\Delta^{3,5}$

(**414**) $R_4 = OH$, $\Delta^{3,5}$

$[\alpha]^{25}_D$ = -11.0 (*c* 0.0062, $CHCl_3$-MeOH)

(**415**) $R_4 = R_5 = OH$, $\Delta^{3,5}$

$[\alpha]^{25}_D$ -11.0 (*c* 0.0062, $CHCl_3$-MeOH)

(**417**) R_1 = O-β-D-Glc, $R_4 = R_6 = OH$, Δ^5

$[\alpha]_D$ +106.0 (*c* 0.46, MeOH)

(**418**) $R_2 = R_3 = R_5 = OH$

$[\alpha]_D$ +64.0 (*c* 0.46, $CHCl_3$)

(**421**) $R_4 = R_5 = R_6 = OH$, Δ^2

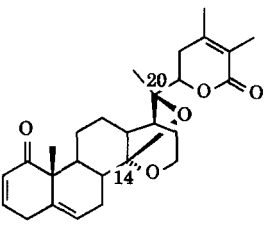
$[\alpha]_D$ +14.0 (*c* 0.3, MeOH)

(**422**) $R_1 = R_5 = OH$, Δ^5

$[\alpha]_D$ +45.0 (*c* 0.35, MeOH)

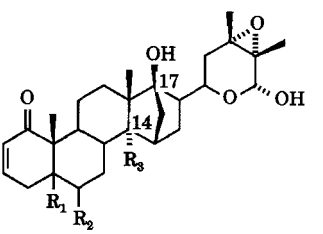
(**423**) R_1 = O-β-D-Glc, Δ^5

$[\alpha]_D$ +106.0 (*c* 0.46, MeOH)



(**300**) m.p. 221-222°C

$[\alpha]^{20}_D$ +103.0 (*c* 0.30, $CHCl_3$)



(**171**) R_1,R_2 = β-epoxide, $R_3 = OH$, m.p. 214-216°C, $[\alpha]^{21}_D$ +30.6 (*c* 0.001, $CHCl_3$)

(**172**) R_1 = α-Cl, R_2 = β-OH, $R_3 = OH$

(**173**) R_1,R_2 = β-epoxide, $\Delta^{8,14}$,

m.p. 148°C, $[\alpha]^{21}_D$ +89.2 (*c* 0.0045, $CHCl_3$)

(**174**) R_1 = α-OH, R_2 = β-OH, $\Delta^{8,14}$,

m.p. 220-222°C, $[\alpha]^{21}_D$ +90.7 (*c* 0.0035, $CHCl_3$)

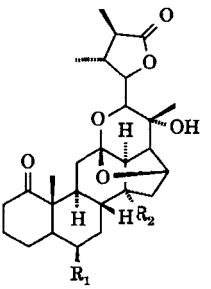
(**175**) R_1 = α-Cl, R_2 = β-OH, $\Delta^{8,14}$,

m.p. 183-185°C

The withanolides jaborosalactone R (**239**), S (**240**) and T (**241**) were isolated from *Jaborosa sativa* by Bonetto and co-workers (1995). In 2000, Nicotra and his group, isolated compounds **184** and **185** from the *Jaborosa caulescens* var. *caulescens* and, in 2006, the jaborosalactona 37 (**233**) was obtained from *Jaborosa orelacea*. More recently, Nicotra and co-workers (2007), isolated seven new withanolides, being five from *J. caulescens* var. bipinatifida

denominated jaborosalactones 39 (**183**), 40 (**181**), 42 (**179**) and, 12-O-ethyljaborosalactone 42 (**180**), besides two compounds from *J. caulescens* var. caulescens, jaborosalactone 38 (**186**) and 18-O-methyljaborosalactone 38 (**187**). These metabolites have in their structure a hemiketal ring formed between a hydroxyl group in C-21 and a carbonyl in C-12, but they keep a δ-lactone in the side chain, characteristic of the withanolides. Seven new trechonolides were isolated from *J. laciniata* (Cirigliano *et al.*, 2007), which were designated jaborosalactones 45 (**193**), 46 (**195**), 47 (**196**), 48 (**197**) 49 (**198**) and 12-O-methyljaborosalactone 45 (**194**).

The subtrifloralactones A (**95**), B (**97**), C (**96**), K (**92**) and L (**93**) were isolated from *Deprea subtriflora* (Su *et al.*, 2003). These *nor*-withanolides have as characteristic a skeleton containing 27 carbon atoms and a hemiketal or ketal moiety. The main differences among them are in the rings A and B, as it can be observed for compounds **92**, **93**, **95-97**.



(**93**) R_1 = OH, $\Delta^{2,4}$

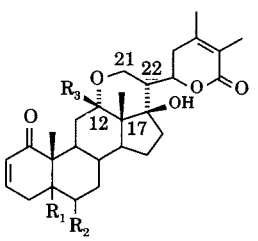
(**95**) $\Delta^{2,5}$, m.p. 221-222°C

$[\alpha]^{23}_D$ -13.3 (*c* 0.15, MeOH)

(**96**) R_2 = OH, $\Delta^{2,5}$

$[\alpha]^{23}_D$ -30.5 (*c* 0.21, MeOH)

(**97**) $\Delta^{3,5}$, $[\alpha]^{23}_D$ +14.8 (*c* 0.13, MeOH)



(**184**) R_1,R_2 = β-epoxide, R_3 = OH

(**185**) R_1,R_2 = β-epoxide, R_3 = OMe

(**233**) R_1 = α-Cl, R_2 = R_3 = OH

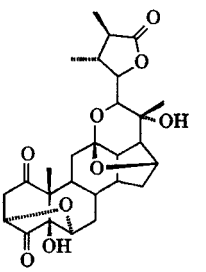
m.p. 205°C, $[\alpha]^{21}_D$ +62.85 (*c* 0.0047, $CHCl_3$)

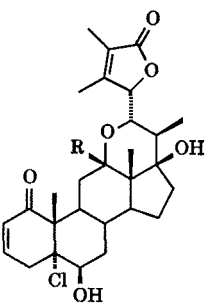
(**239**) R_2 = R_3 = OH, Δ^4

(**240**) R_1 = R_2 = R_3 = OH, m.p. 265-266°C

(**241**) R_1 = β-OH, R_2 = α-Cl, R_3 = OMe,

m.p. 234-235°C





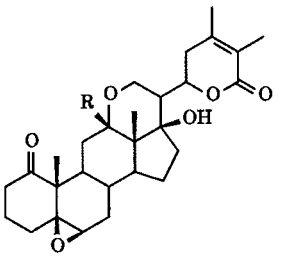
(**179**) R = OH, 23R, m.p. 203-204°C

$[\alpha]^{21}_D$ +17.2 (*c* 0.0099, $CHCl_3$)

(**180**) R = OEt, 23R, m.p. 200-202°C

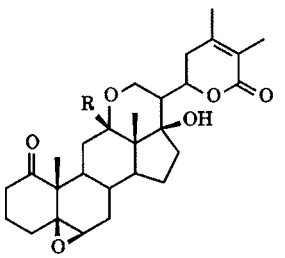
$[\alpha]^{21}_{D}$ +26.6 (*c* 0.0016, $CHCl_3$)

(**198**) R = OH, $[\alpha]^{20}_{D}$ -73.5 (*c* 0.17, MeOH)



(**181**) 23R, $[\alpha]^{21}_{270}$ +58.0 (*c* 0.0002, MeOH)

(**182**) 23S, $[\alpha]^{21}_{270}$ +24.0 (*c* 0.0002, MeOH)



(**183**) R = OH, m.p. 156-158°C

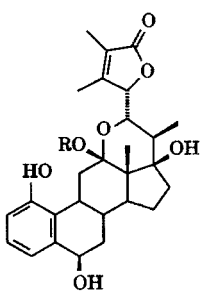
$[\alpha]^{21}_{270}$ +16.3 (*c* 0.00003, MeOH)

(**186**) R = OH, Δ^2, m.p. 237-239°C

$[\alpha]^{21}_{D}$ +56.5 (*c* 0.034, MeOH)

(**187**) R = OMe, Δ^2

$[\alpha]^{21}_{D}$ +71.7 (*c* 0.019, MeOH)

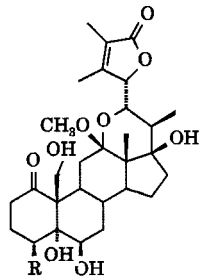


(**193**) R = H, m.p. 202-203°C

$[\alpha]^{20}_{D}$ -33.5 (*c* 0.16, MeOH)

(**194**) R = Me, m.p. 195-196°C

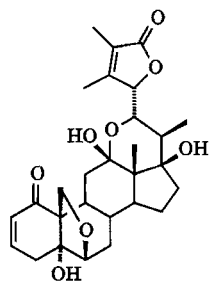
$[\alpha]^{20}_{D}$ -56.5 (*c* 0.17, MeOH)



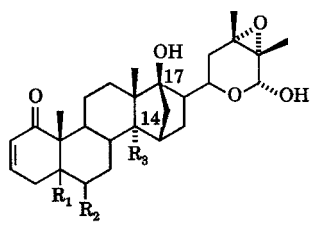
(**195**) R = H, Δ^2, $[\alpha]^{20}_{D}$ -25.4 (*c* 0.16, MeOH)

(**196**) R = OH, m.p. 220-222°C

$[\alpha]^{20}_{D}$ -32.3 (*c* 0.17, MeOH)



(**197**) $[\alpha]^{20}_{D}$ -42.8 (*c* 0.18, MeOH)



(**171**) R_1,R_2 = β-epoxide, R_3 = OH, m.p. 214-216°C, $[\alpha]^{21}_{D}$ +30.6 (*c* 0.001, $CHCl_3$)

(**172**) R_1 = α-Cl, R_2 = β-OH, R_3 = OH

(**173**) R_1,R_2 = β-epoxide, $\Delta^{8,14}$

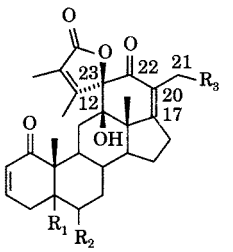
m.p. 148°C, $[\alpha]^{21}_{D}$ +89.2 (*c* 0.0045, $CHCl_3$)

(**174**) R_1 = α-OH, R_2 = β-OH, $\Delta^{8,14}$ m.p. 220-222°C, $[\alpha]^{21}_{D}$ +90.7 (*c* 0.0035, $CHCl_3$)

(**175**) R_1 = α-Cl, R_2 = β-OH, $\Delta^{8,14}$ m.p. 183-185°C

*All not specified R groups correspond to hydrogen atoms

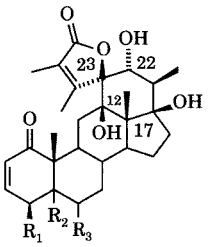
The jaborosalactones 1 (**234**), 2 (**170**), 3 (**236**), 4 (**235**), 5 (**238**) and 6 (**237**), were isolated from the aerial part of *Jaborosa runcinata*, by Cirigliano and coworkers (1996). All of these metabolites have in their side chain a carbon-carbon double bond located in C-17 of the intact steroidal nucleus, an spiranoid γ-lactona in C-23 and an additional ring junction through C-12 and C-17, forming a 17(20)-en-22-keto system. The jaborosalactones 4-6 also contain an additional hydroxyl in C-21.



(**170**) $R_1 = R_2 = OH$, m.p. 250-251°C
(**234**) R_1,R_2 = β-epoxide, m.p. 269-270°C
(**235**) R_1,R_2 = β-epoxide, $R_3 = OH$
m.p. 273-274°C
(**236**) $R_1 = Cl$, $R_2 = OH$, m.p. 262-264°C
(**237**) $R_1 = Cl$, $R_2 = R_3 = OH$
(**238**) $R_3 = OH$, Δ^5, m.p. 234-235°C

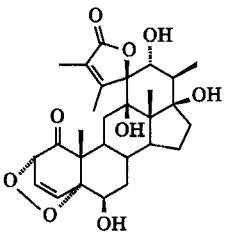
Jaborosalactone P (**215**) isolated from *Jaborosa odonelliana* (Monteagudo *et al.*, 1990) was the first withanolide containing a spiranoid γ-lactona in the side chain. Lately, Cirigliano and co-workers (2002), reported the isolation of six new withanolides, from the same species, which were denominated jaborosalactones 10-15 (**218**), (**216**), (**217**), (**219**), (**220**) and (**221**). These compounds only differ in the substitution pattern of rings A and B. To jaborolactone 15 (**221**), the difference resides in the presence of an endoperoxy system.

Su and co-workers (2003) isolated three withanolides from *Deprea subtriflora,* which present in their structure a ketal moiety at C-12 and an ester at C-16, they are known as subtrifloralactone H (**102**), I (**103**) and J (**104**).

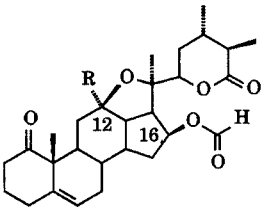


(**215**) Δ^5, m.p. 262-264°C
(**216**) R_2, R_3 = β-epoxide
$[\alpha]^{25}_D$ -14.0 (*c* 0.06, MeOH)
(**217**) $R_1 = OH$, R_2,R_3 = β-epoxide,
$[\alpha]^{25}_D$ -10.8 (*c* 0.04, MeOH)
(**218**) $R_2 = Cl$, $R_3 = OH$, m.p. 264-265°C
$[\alpha]^{25}_D$ -7.37 (*c* 0.05, MeOH)
(**219**) $R_2 = R_3 = OH$
$[\alpha]^{25}_D$ -10.1 (*c* 0.07, MeOH)
(**220**) $R_2 = OMe$, $R_3 = OH$, m.p. 269-270°C
$[\alpha]^{25}_D$ -6.5 (*c* 0.06, MeOH)

*All not specified R groups correspond to hydrogen atoms

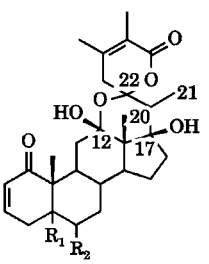


(**221**) $[\alpha]^{25}_{D}$ -6.3 (*c* 0.05, MeOH)

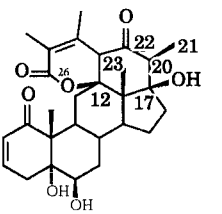


(**102**) Δ^3, R = OEt, $[\alpha]^{23}_{D}$ +49.5 (*c* 0.07, MeOH)
(**103**) Δ^2, R = OEt, $[\alpha]^{23}_{D}$ -17.3 (*c* 0.12, $CHCl_3$)
(**104**) Δ^2, R = OMe, $[\alpha]^{23}_{D}$ +10.8 (*c* 0.15, MeOH)

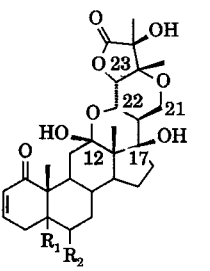
The withanolides designated jaborosalactones 26-30 (**222 - 226**), were isolated from *Jaborosa rotacea* (Nicotra *et al.*, 2006), and jaborosalactone 43 (**192**) from *J. kurtzii* (Ramcciotti & Nicotra, 2007). They present in the steroidal skeleton two ketal carbons located at C-12 and C-22, that may have been originated from ketone functions in those carbons, resulting in a new six members ring with a hydroxyl group having β orientation at C-12 and a spiranoid center at C-22, leading up to the formation of a δ-lactone. Differing from the withanolides previously mentioned, the withasteroids jaborosalactone 31 (**227**) has a bond between C-12 and C-23, forming a six members ring resulting in a δ-lactone bridge at C-26 and C-12, while the jaborosalactones 35 (**231**) and 36 (**232**) comprise two six member rings, one of them formed through the epoxy bridge at C-12 and C-22 and the other at C-22-C-23, with the formation of a γ-lactone.



(**192**) Δ^5, m.p. 167°C
$[\alpha]^{21}_{D}$ +12.9 (*c* 0.044, MeOH)
(**222**) R_1,R_2 = β-epoxide, m.p. 218-220°C
$[\alpha]^{21}_{D}$ +3.3 (*c* 0.0036, $CHCl_3$)
(**223**) $R_1 = R_2$ = OH, m.p. 194°C
$[\alpha]^{21}_{D}$ +11.8 (*c* 0.0029, $CHCl_3$)
(**224**) R_1 = Cl, R_2 = OH, m.p. 185-187°C
$[\alpha]^{21}_{D}$ -0.6 (*c* 0.0045, $CHCl_3$)
(**225**) R_1 = OH, R_2 = Cl
$[\alpha]_{D}$ +4.2 (*c* 0.002, $CHCl_3$)
(**226**) R_2 = OH, Δ^4, $[\alpha]_{D}$ -32.0 (*c* 0.0016, MeOH)

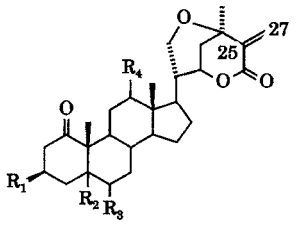


(**227**) $[\alpha]_{D}$ -25.9 (*c* 0.0005, $CHCl_3$)



(**231**) $R_1 = R_2$ = OH
(**232**) R_1,R_2 = β-epoxide, m.p. 210-212°C
$[\alpha]^{21}_{D}$ -13.10 (*c* 0.048, $CHCl_3$)

Withametelin (**35**), obtained from *Datura metel*, was the first isolated hexacyclic withanolide with a bicycle system in the side chain. This metabolite was later isolated from other species like *D. tatula*, *D. fastuosa* and *D. ferox* (Gupta *et al.*, 1991; Manickam, 1996; Manickam *et al.*, 1993; Cirigliano *et al.*, 1995). Besides the withametelin, the withametelins B (**57**), F (**64**) and G (**65**) (Gupta *et al.*, 1991; Jahromi *et al.*, 1993), were also isolated from *D. metel* while withafastuosin C (**34**) was obtained from *D. fastuosa* (Manickam *et al.*, 1994). These metabolites present in their structure an exocyclic double bond located in C-25. Withafastuosin A (**36**) and B (**37**), isolated from *D. fastuosa* and, daturametelin D (**60**) from *D. metel* differ from the other compounds due the lack of the exocyclic double bond. Recently, were isolated from the flowers of *D. metel* the withametelins I to O (**70–76**), 1,10-*seco*-withametelin B (**77**) and 12β-hydroxy-1,10-*seco*-withametelin B (**78**) (Pan *et al.*, 2007). Compounds **77** and **78** possess a seven members β, γ unsaturated lactone located in ring A.



(**34**) R_1 = OMe, R_2,R_3 = β-epoxide m.p. 235-237°C

(**35**) $\Delta^{2,5}$, m.p. 210°C, $[\alpha]_D$ -64.4 ($CHCl_3$)

(**57**) R_3 = OH, $\Delta^{2,4}$, m.p. 283-285°C $[\alpha]_D$ -153.3 ($CHCl_3$)

(**64**) R_2,R_3 = β-epoxide, Δ^2

(**65**) R_2 = R_3 = OH, Δ^2

(**70**) R_2,R_3 = β-epoxide, R_4 = OH, Δ^2 m.p. 185-187°C $[\alpha]^{20}_D$ -59.0 (*c* 0.20, $CHCl_3$)

(**71**) R_2 = R_3 = R_4 = OH, Δ^2 m.p. 203-205°C, $[\alpha]^{20}_D$ -35.0 (*c* 0.09, $CHCl_3$)

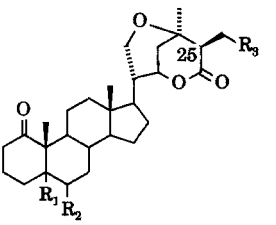
(**72**) R_3 = R_4 = OH, $\Delta^{2,4}$ m.p. 244-247°C, $[\alpha]^{20}_D$ -100.0 (*c* 0.10, $CHCl_3$)

(**73**) R_4 = OH, $\Delta^{2,5}$, m.p. 219-222°C $[\alpha]^{20}_D$ -122.0 (*c* 0.20, $CHCl_3$)

(**74**) R_4 = OH, $\Delta^{3,5}$, m.p. 145-148°C $[\alpha]^{20}_D$ -70.0 (*c* 0.10, $CHCl_3$)

(**75**) R_1 = OH, R_2, R_3 = β-epoxide m.p. 236-239°C, $[\alpha]^{20}_D$ -130.0 (*c* 0.09, $CHCl_3$)

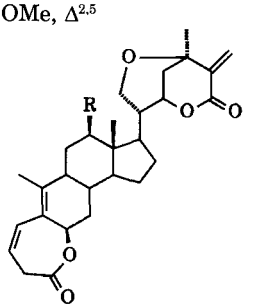
(**76**) R_2 = R_3 = OH, Δ^2, m.p. 161-164°C $[\alpha]^{20}_D$ -50.0 (*c* 0.12, $CHCl_3$)



(**36**) R_1,R_2 = β-epoxide, R_3 = OH

(**37**) R_1,R_2 = β-epoxide, R_3 = OH, Δ^2 m.p. 255°C, $[\alpha]_D$ -5.87 (*c* 1.5, $CHCl_3$)

(**60**) R_3 = OMe, $\Delta^{2,5}$

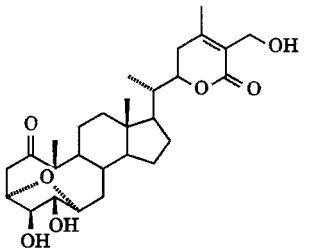


(**77**) R = H, m.p. 232-234°C $[\alpha]^{20}_D$ -39.0 (*c* 0.10, $CHCl_3$)

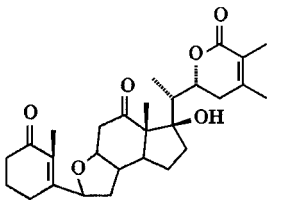
(**78**) R = OH, m.p. 157-159°C $[\alpha]^{20}_D$ -60.0 (*c* 0.20, $CHCl_3$)

*All not specified R groups correspond to hydrogen atoms

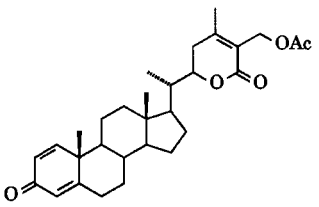
The withanolide **447** isolated from *Withania somnifera* by Zhao and co-workers (2002), is the first example of a group of withanolides containing an ether bridge between C-3 and C-6. Withasomidienone (**435**) also from *W. somnifera*, (Atta-Ur-Rahman *et al.*, 1993), the withanolide with a cyclohexadienone system, was the first report of this kind of metabolite from Solanaceae. Compounds with the same system had already been isolated from corals (*Minabea* sp) (Ksebati & Schmitz, 1988). The metabolite (+)-projaborol (**210**) was obtained from *Jaborosa magellanica* by Fajardo *et al.* (1991). Chantriolides A (**375**) and B (**376**), isolated from *Tacca chantrieri* (Taccaceae), are glucosides with a high degree of oxygenation.



(**447**) $[\alpha]^{23}_{D}$ -17.4 (*c* 0.109, MeOH)

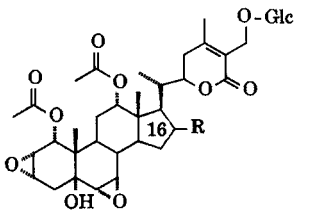


(**210**) m.p. 163°C, $[\alpha]_{D}$ +32.0 (*c* 1.24, MeOH)



(**435**) m.p. 213-215°C

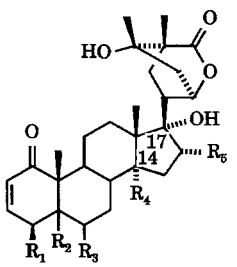
$[\alpha]^{30}_{D}$ +24.6 (*c* 0.25, MeOH)



(**375**) R = O, $[\alpha]^{25}_{D}$ -4.0 (*c* 0.1, MeOH)

(**376**) R = β-OH, $[\alpha]^{25}_{D}$ +54.0 (*c* 0.1, MeOH)

The withajardins are a group of withanolides characterized by the presence of aβ orientated side chain (17β) possessing a [2,2,2] bicycle system constituted through a δ-lactone. The withajardins A (**87**), B (**89**), C (**90**) and D (**88**) were isolated from *Deprea orinocensis* by Luis *et al.* (1994), while withajardin E (**91**), a 14-deoxi-withajardin, was isolated from the same species one year later by Echeverri and co-workers (1995). Lately, from *Tubocapsicum anomalum* a C-6 chlorinated withanolide was isolated, which was named tubonolide A (**394**) by Hsieh *et al.* (2007).



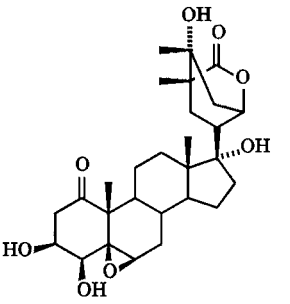
(**87**) $R_1 = R_4$ =OH, Δ^5, m.p. 225°C

(**88**) R_1 = OAc, R_4 = OH, Δ^5, m.p. 182°C

(**89**) $R_1 = R_4$ = OH, R_2,R_3 = β-epoxide m.p. 228°C

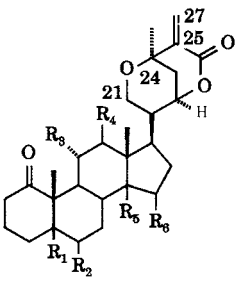
(**90**) R_1 = OAc, R_2,R_3 = β-epoxide, R_4 = OH, m.p. 268°C

(**394**) $R_1 = R_2 = R_5$ = OH, R_3 = Cl, m.p. 200-202°C $[\alpha]^{26\,6}_{D}$ +10.2 (*c* 0.1, MeOH)



(91)

The withanolides denominated witharifeen (**47**), daturalicin (**48**), withametelinol (**49**), withametelinone (**50**), daturacine (**53**), withametelinol A (**51**) and B (**52**) were isolated from *Datura innoxia* (Siddiqui *et al.*, 1999, 2002, 2005a, 2005b). These metabolites present a 21,24-epoxy-1-oxo-25(27)-ene system. The metabolite denominated physanolide A (**272**) was recently isolated from *Physalis angulata* by Kuo *et al.* (2006).



(**47**) $R_3 = R_4 =$ OH, $\Delta^{2,5}$, m.p. 166-167°C

$[\alpha]^{25}_D$ -109.0 (*c* 0.09, $CHCl_3$)

(**48**) $R_1,R_2 =$ β-epoxide, $R_5,R_6 =$ α-epoxide, Δ^2

m.p. 140-141°C, $[\alpha]^{25}_D$ -26.7 (*c* 0.14, $CHCl_3$)

(**49**) $R_4 =$ OH, $\Delta^{2,5}$, m.p. 148-149°C

$[\alpha]^{27}_D$ -105.0 (*c* 0.04, $CHCl_3$)

(**50**) $R_4 =$ O, $\Delta^{2,5}$, m.p. 152-153°C

$[\alpha]^{27}_D$ +15.6 (*c* 0.128, $CHCl_3$)

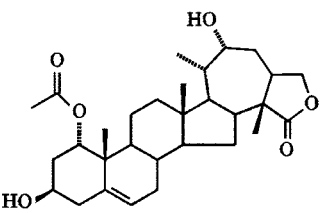
(**51**) $R_1 =$ OH, $\Delta^{2,9}$, m.p. 212-214°C

$[\alpha]^{28}_D$ +32.4 (*c* 0.074, MeOH)

(**52**) $R_4 =$ OH, $\Delta^{3,5}$, m.p. 197-198°C

$[\alpha]^{28}_D$ +0.7 (*c* 0.28, MeOH)

(**53**) $R_2 =$ O, $R_5 =$ OMe, Δ^2



(272)

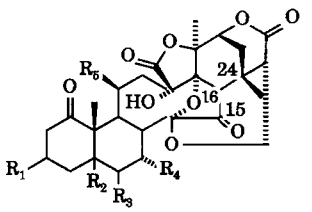
Physalins

Physalins, 13,14-*seco*-16,24-cyclo-withanolides with a γ-lactone in ring D are among the withasteroid, with the most complex structures. They are

*All not specified R groups correspond to hydrogen atoms

classified as types A and B according to the absence or presence of the ether linkage C_{14}-O-C_{27} (Makino *et al.,* 2002; Kawai *et al.,* 2002). They comprise a carbonyl at C-15, and a ring at the carbon atoms C-16 and C-24, that seems to be formed by an intramolecular Michael reaction.

The physalins are the most frequently constituents occurring for the genus *Physalis*. The isolation of the first physalin dates back to 1969 (physalin A), however, until 1992 only 16 of these metabolites (A-Q) had been isolated (Ray & Gupta, 1994) *i.e.* physalins N (**251**), O (**252**), R (**256**), S (**257**), and isophysalin G (**248**). The physalin **307** and the neophysalin **253** were isolated from *P. alkekengi*. Physalin S (**257**) isolated from the genus *Physalis* is the first example of a withasteroid containing a cyclopropane ring. Recently, from *P. alkekengi* were isolated the new neophysalins W (**254**) and X (**255**) (Chen *et al.,* 2007), which differ only in the configuration at C-3. Damu and co-workers (2007), isolated from *P. angulata* a new physalin, designated as physalin W (**285**), but structurally different of **254**, also known by the same denomination. Lately, *P. solanaceus* was investigated for the first time, resulting in the isolation of several physalins known as: physalin A (**341**), B (**28**), D (**340**) and F (**29**). Choudary and co-workers (2005) isolated from *P. minima* three new physalins **302**, **303** and **304**, together with the known physalin H (**30**) and isophysalin B (**305**). Recently, from the same species, two new physalins, **315** and **316** were isolated. This reinforces the importance of these constituents as chemotaxonomic markers for the genus (Pérez-Castorena *et al.,* 2004).



(**28**) $\Delta^{2,5}$, m.p. 247-250°C

(**29**) R_2,R_3 = β-epoxide, Δ^2, m.p. 295-300°C

(**30**) R_2 = Cl, R_3 = OH, Δ^2

(**248**) R_1 = OH, $\Delta^{4,6}$, $[\alpha]^{20}_D$ -32.0 (*c* 0.19, $CHCl_3$)

(**251**) R_4 = OH, $\Delta^{2,5}$, m.p. 252-254°C

$[\alpha]^{24}_D$ -124.0 (*c* 0.14, Me_2CO)

(**258**) R_2 = R_3 = OH

(**273**) R_1 = OMe, R_2,R_3 = epoxide

(**285**) R_1 = OMe, $[\alpha]^{25}_D$ +6.8 (*c* 0.02, MeOH)

(**301**) R_2 = OH, R_3 = OEt, Δ^2, m.p. 265-266°C

$[\alpha]^{25}_D$ -43.0 (*c* 1.0, MeOH)

(**302**) R_2 = R_3 = R_5 = OH, Δ^2

$[\alpha]^{25}_D$ -211.0 (*c* 3.1, Me_2CO)

(**303**) R_2 = OH, Δ^2, $[\alpha]^{25}_D$ -20.0 (*c* 1.8, MeOH)

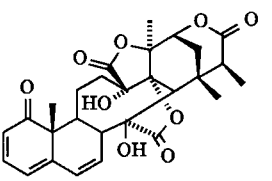
(**304**) R_1 = OMe, R_2,R_3 = β-epoxide

$[\alpha]^{25}_D$ -122.0 (*c* 1.1, CH_2Cl_2)

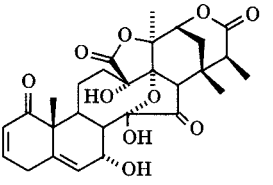
(**305**) $\Delta^{3,5}$, $[\alpha]^{25}_D$ -8.5 (*c* 3.5, CH_2Cl_2)

(**307**) R_2 = OEt, R_3 = OH, Δ^2, m.p. 224-226 °C
$[\alpha]^{25}_D$ -88.0 (*c* 0.15, Me_2CO)

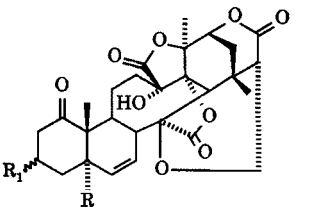
(**340**) R_2 = R_3 = OH, Δ^2, m.p. 302-305°C



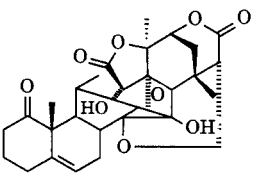
(**249**) $[\alpha]_D$ -67.0 (*c* 0.24, MeOH)



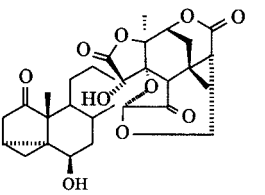
(**252**) m.p. 272-273°C
$[\alpha]^{24}_{D}$ -115.0 (*c* 0.1, Me_2CO)



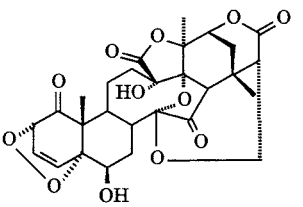
(**250**) $\Delta^{2,4}$, $[\alpha]_{D}$ +21.0 (*c* 0.26, MeOH)
(**253**) R = OH, Δ^{2}
(**254**) R_1 = β-OH, Δ^{4}
(**255**) R_1 = α-OH, Δ^{4}



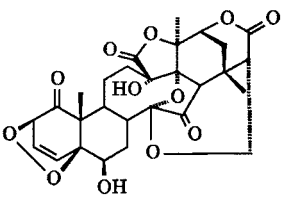
(**256**) m.p. >300°C
$[\alpha]^{15}_{D}$ -177.0 (*c* 0.13, Me_2CO)



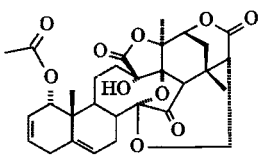
(**257**) m.p. 287-289°C
$[\alpha]^{15}_{D}$ -118.0 (*c* 0.08, Me_2CO)



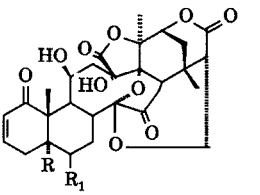
(**259**) m.p. >300°C, $[\alpha]^{20}_{D}$ -224.0 (*c* 0.04)



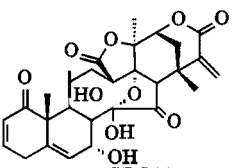
(**260**) m.p. >300°C, $[\alpha]^{20}_{D}$ +38.0 (*c* 0.06)



(**274**)



(**315**) R = OMe, R_1 = H
$[\alpha]^{25}_{D}$ -122.0 (*c* 1.1, CH_2Cl_2)
(**316**) R = OH, R_1 = O
$[\alpha]^{25}_{D}$ -211.0 (*c* 3.1, $(CH_3)_2CO$)

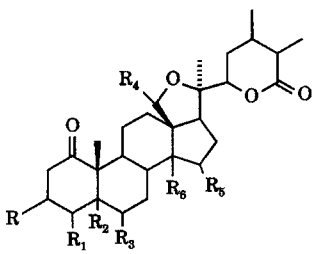


(**341**) m.p. 272-274°C

*All not specified R groups correspond to hydrogen atoms

Withaphysalins

These compounds are the intermediates formed during the biosynthesis of physalins from withanolides. Except withaphysalin C, all the others have a carbocycle skeleton intact from the ergostan precursor and seem to be structurally similar to the physalins, due to the presence of a γ-lactone or lactol fused to ring D of the steroidal nucleus. Until 1994, Ray and Gupta, registered the isolation of five withaphysalins designated as withaphysalins A, B, C, D and E, which were obtained from *Physalis minima* and *P. minima* var. *indica* (Glotter *et al.,* 1975; Kirson *et al.,* 1976; Sahai & Kirson, 1984; Sinha *et al.,* 1987). Recently withaphysalins F (**399**), G (**400**), H (**401**), I (**402**), J (**403**), K (**404**), L (**405**), M (**4**), N (**6**), O (**5**) and four others as C-18 epimeric mixtures (**7**, **8**, **125** and **126**) (Veras *et al.,* 2004a, 2004b; Misico *et al.,* 2000) were reported. From *Physalis minima* six new withaphysalins **308-311**, **313** and **314** (Ma *et al.,* 2007) were also isolated. The same researchers also isolated the withaphysalin P (**312**), with a carbonyl group at C-14 differing from reported withaphysalins (Ma *et al.,* 2007). These type of compounds were isolated from different genera of the Solanaceae family, such as, withaphysalins F-L from *Vassobia lorentii*, withaphysalins M-O and **7** from *Acnistus arborescens*, **308** - **314** from *P. minima*, **125** and **126** from *Dunalia brachyacantha*, while **8** was isolated from *V. lorenzii* and *A. arborescens*.



(**7**) R_1 = β-OH, R_2,R_3 = β-epoxide, R_4 = OH (18*R/S*), $\Delta^{2,24}$, m.p. 165-168°C

$[\alpha]^{20}_D$ +102.7 (*c* 0.8, $CHCl_3$)

(**8**) R_1 = β-OH, R_2,R_3 = β-epoxide, R_4 = OH (18*R/S*), Δ^{24}, m.p. 212-216°C

$[\alpha]^{20}_D$ +50.0 (*c* 0.06, $CHCl_3$)

(**125**) R_1 = OAc, R_2,R_3 = β-epoxide, R_4 = OH (18*R/S*), $\Delta^{2,24}$

(**126**) R_1 = OAc, R_2,R_3 = β-epoxide, R_4 = OH (18*R/S*), Δ^{2}

(**308**) R = R_4 = OMe, R_2,R_3 = β-epoxide, Δ^{24}

(**309**) R_2 = R_3 = OH, R_4 = OMe, $\Delta^{2,24}$

(**310**) R_2 = R_4 = OMe, R_3 = OH, $\Delta^{2,24}$

(**311**) R_2 = R_3 = R_5 = OH, R_4 = OMe, $\Delta^{2,24}$

(**399**) R_1 = OH, R_4 = OH (18*R/S*), R_2,R_3 = β-epoxide, $\Delta^{2,24}$, m.p. 256-258°C $[\alpha]^{25}_D$ +46.9 (*c* 0.06, $CHCl_3$)

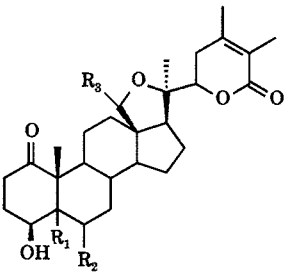
(**404**) R_1 = O, R_4 = OMe (18*R*), $\Delta^{2,5,24}$

$[\alpha]^{25}_D$ +41.1 (*c* 0.25, $CHCl_3$)

(**405**) R_1 = O, R_4 = OMe (18*S*), $\Delta^{2,5,24}$

$[\alpha]^{25}_D$ +38.9 (*c* 0.04, $CHCl_3$)

*All not specified R groups correspond to hydrogen atoms



(**4**) R_1,R_2 = β-epoxide, R_3 = O, D^2

m.p. 237-239°C, $[\alpha]^{20}_D$ +56.0 (*c* 0.33, C_5H_5N)

(**5**) R_1,R_2 = β-epoxide, R_3 = OEt, D^2

m.p. 263-266°C, $[\alpha]^{20}_D$ +96.0 (*c* 0.05, $CHCl_3$)

(**6**) R_1,R_2 = β-epoxide, R_3 = O

m.p. 295-299°C, $[\alpha]^{20}_D$ -25.0 (*c* 0.4, C_5H_5N)

(**400**) R_3 = OH (18*R/S*), $D^{2,5}$, m.p. 264-266°C

$[\alpha]^{25}_D$ +97.6 (*c* 0.13, MeOH)

(**401**) R_3 = OMe (18*R*), $D^{2,5}$

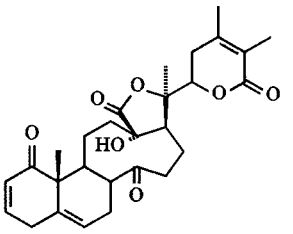
$[\alpha]^{25}_D$ +47.9 (*c* 0.20, $CHCl_3$)

(**402**) R_3 = OMe (18*S*), $D^{2,5}$

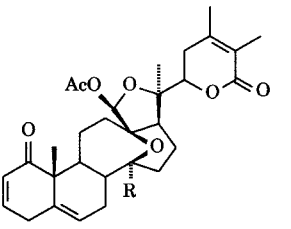
$[\alpha]^{25}_D$ +54.9 (*c* 0.18, $CHCl_3$)

(**403**) R_3 = O, $D^{2,5}$, m.p. 215-217°C

$[\alpha]^{25}_D$ +60.4 (*c* 0.15, $CHCl_3$)



(312)



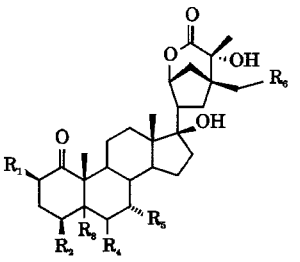
(313) R = OH

(314) R = OAc

Acnistins

They are withasteroids containing a [3,2,1] bicyclic system in place of a side chain, similar to the modified withanolides. In the review, Ray and Gupta (1994) registered the isolation of the acnistins A (**9**) and E (**10**) from *Acnistus ramiflorum* (Usubillaga *et al.*, 1992). Several other acnistine are known, including the acnistins B (**138**), C (**136**), D (**137**), F (**11**), G (**134**) and H (**135**) all isolated from *Dunalia solanaceae*. Usubilaga *et al.* (2005) isolated from *Acnistus arborescens* the known acnistins A (**9**), E (**10**) e F (**11**) and three new ones I (**12**), K (**13**) and L (**14**). Recently, 17-epi-acnistin-A (**109**) was isolated from *Discopodium penninervium*, which differs in the stereochemistry at C-17 due to β orientation of the side chain (Habtemariam *et al.*, 2000). From *Tubocapsicum anomalum* Hsieh *et al.* (2007) isolated six new secondary metabolites which were designated anomalides A (**389**), B (**391**), C (**390**), D (**393**), E (**392**) e F (**398**) respectively.

*All not specified R groups correspond to hydrogen atoms



(**9**) R_3,R_4 = β-epoxide, D^2

m.p. 258-260°C, $[\alpha]^{24}_{578}$ -22.0 (*c* 0.020, EtOH)

(**10**) R_2 = OH, R_3,R_4 = β-epoxide, D^2

m.p. 265-267°C, $[\alpha]^{24}_{578}$ -36.5 (*c* 0.020, EtOH)

(**11**) R_3 = R_4 = OH, D^2

(**12**) R_2 = R_6 = OH, D^2

(**13**) R_4 = R_6 = OH, D^4

(**14**) R_2 = R_6 = OH, R_3,R_4 = epoxide

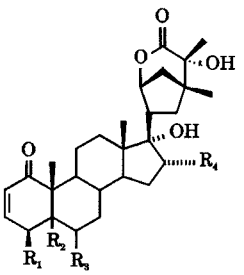
(**134**) R_3,R_4 = β-epoxide

(**135**) R_1 = R_3 = OH, $D^{3,6}$

(**136**) R_3,R_4 = β-epoxide, R_5 = OAc, D^2

(**137**) R_5 = OAc, $D^{2,5}$

(**138**) $D^{2,4,6}$, m.p. 212°C



(**389**) R_1 = OH, R_2,R_3 = β-epoxide

m.p. 170-172°C, $[\alpha]^{24}{}_{D}$ +12.9 (*c* 0.09, MeOH)

(**390**) R_1 = R_4 = OH, R_2,R_3 = β-epoxide

m.p. 280-282°C, $[\alpha]^{24}{}_{D}$ +3.4 (*c* 0.1, MeOH)

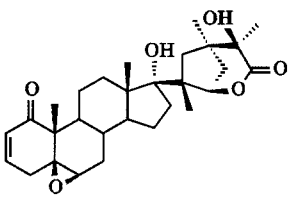
(**391**) R_1 = R_2 = R_3 = OH, m.p. 168-170°C $[\alpha]^{24}{}_{D}$ +26.5 (*c* 0.1, MeOH)

(**392**) R_2 = R_3 = R_4 = OH, m.p. 182-184°C $[\alpha]^{24}{}_{D}$ -2.5 (*c* 0.1, MeOH)

(**393**) R_1 = R_2 = R_4 = OH, R_3 = Cl

m.p. 196-198°C, $[\alpha]^{24}{}_{D}$ +5.6 (*c* 0.1, MeOH)

(**398**) R_1 = R_2 = R_4 = OH, m.p. 190-192°C $[\alpha]^{26}{}_{D}$ +4.76 (*c* 0.07, MeOH)



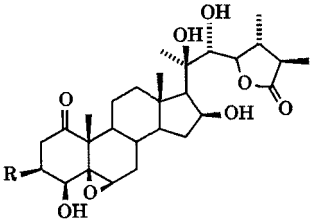
(**109**) m.p. 240-250°C

Ixocarpalactones

The compounds designated as ixocarpalactones have a γ-lactone in its side chain. Until the 80's, the isolation of just seven ixocarpalactones had been registered: ixocarpalactones A and B, taccalonolide A, B and D and trechonolide A and B (Lavie *et al.*, 1987; Chen *et al.*, 1987; Chen *et al.*, 1988; Kirson *et al.*, 1979). More recently, from *Physalis philadelphica* (Su *et al.*, 2002; Gu *et al.*, 2003), ixocarpalactones A (**332**), B (**331**), 2,3-dihydroxy-3β-methoxyixocarpalactone A (**334**), 2,3-dihydroxy-3β-methoxyixocarpalactone

*All not specified R groups correspond to hydrogen atoms

B (**335**) and **336** were isolated. The metabolites **100** and **101**, designated subtrifloralactones F and G respectively, were isolated from *Deprea subtriflora* (Su *et al.*, 2003).

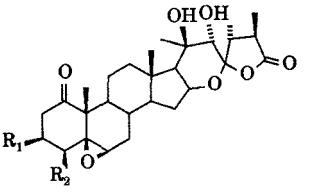


(**332**) Δ^2, m.p. 292-293°C

$[\alpha]^7_D$ +90.0 (*c* 0.20, MeOH)

(**334**) R = OMe, m.p. 240-243°C

$[\alpha]^{20}_D$ -39.0 (*c* 0.088, CH_3CN)



(**331**) R_1 = H, R_2 = OH, Δ^2, m.p. 145-148°C

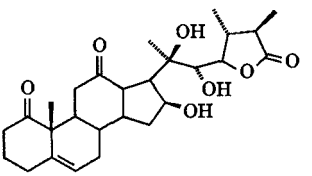
$[\alpha]^{20}_D$ -19.6 (*c* 0.25, $CHCl_3$)

(**335**) R_1 = OMe, R_2 = OH, m.p. 190-192°C

$[\alpha]^{20}_D$ -117.0 (*c* 0.076, CH_3CN)

(**336**) R_1 = H, R_2 = OH, m.p. 178-180°C

$[\alpha]^{20}_D$ -93.0 (*c* 0.089, CH_3CN)

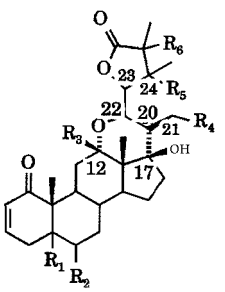


(**100**) D^2, $[\alpha]^{23}_D$ -3.8 (*c* 0.13, MeOH)

(**101**) D^3, $[\alpha]^{23}_D$ +28.5 (*c* 0.13, MeOH)

The ixocarpalactones also have in their side chain, a γ-lactone and a hemiketal C-12/C-22 moieties. Metabolites of this nature were isolated from *Jaborosa magellanica* by Fajardo *et al.* 1991 and Parvez *et al.* 1991, and designated as jaborosotetrol (**206**), (-)-jaborolone (**207**), (+)-jaborochlorotriol (**208**), (-)-jaborochlorodiol (**209**) respectively, besides **212**, **177** and **178** were also obtained from *J. caulescens* var. *bipinnatifida* (Nicotra *et al.*, 2000). 12-O-ethyljaborotetrol (**201**) and jaborosalactone U (**242**) were obtained from *J. leucotricha* (Misico *et al.*, 1997) and *J. sativa* (Bonetto *et al.*, 1995), respectively. Jaborosalactones 32 (**228**), 33 (**229**) and 34 (**230**) were isolated from *J. rotaceae* by Nicotra *et al.* (2006), the last one being an epimer of jaborochlorotriol (**177**) at C-23.

*All not specified R groups correspond to hydrogen atoms



(**177**) $R_3 = R_4 = OH$, $D^{5,24}$, 23*R*

178) $R_3 = R_4 = OH$, $D^{5,24}$, 23*S*

(**201**) $R_1 = R_2 = OH$, $R_3 = OMe$, D^{24}

m.p. 210-215°C

(**206**) $R_1 = R_2 = R_3 = OH$, D^{24}, m.p. 246-250°C

$[\alpha]_D$ -20 (*c* 1.07, MeOH)

(**207**) $R_1 = R_3 = OH$, $R_2 = O$, D^{24}, m.p. 264°C

$[\alpha]_D$ -33 (*c* 3.55, MeOH)

(**208**) $R_1 = R_3 = OH$, $R_2 = Cl$, D^{24}, m.p. 232°C

$[\alpha]_D$ +13.0 (*c* 0.24, MeOH)

(**209**) $R_2 = Cl$, $R_3 = OH$, $D^{4.24}$

m.p. 188-190°C $[\alpha]_D$ -10.0 (*c* 2.04, MeOH)

(**212**) $R_1 = OH$, $R_2 = Cl$, $R_3 = OMe$, D^{24}

(**228**) $R_1,R_2 = \beta$-epoxide, $R_3 = OH$, D^{24}

m.p. 180-182°C

$[\alpha]^{21}_D$ +25.3 (*c* 0.099, $CHCl_3$)

(**229**)$R_1 = R_2 = R_3 = OH$, D^{24}, m.p. 191-192°C

$[\alpha]_D$ +26.2 (*c* 0.0144, $CHCl_3$)

(**230**) $R_1 = R_3 = OH$, $R_2 = Cl$ D^{24}

$[\alpha]_D$ +7.15 (*c* 0.0112, $CHCl_3$)

(**242**) $R_1,R_2:R_5,R_6 = \beta$-epoxide, $R_3 = OH$

PHARMACOLOGY

Several plants mentioned in Table 2 are extensively used in traditional medicine, and accumulated knowledge has attributed their biological properties to the presences of withasteroids. *Withania somnifera*, for example, is widely used in many ayurvedic preparations and is popularly known as "Indian ginseng" (Zhao *et al.*, 2002; Senthil *et al.*, 2007). Its extracts and isolated compounds displayed antitumor, antiarthritic, antipyretic, anti-inflammatory, immunomodulatory, anticholinesterase and anti-stress effects, and effectiveness against various types of cardiovascular problems, hiperlipidemia and obesity (Sbohat *et al.*, 1967; Sheti *et al.*, 1970; Budhiraja & Sudhir, 1987; Devi *et al.*, 1992; Agrawal *et al.*, 1999; Archana & Namasivayam, 1999; Jayaprakasam *et al.*, 2003; Mary *et al.*, 2003; Senthil *et al.*, 2007). *Physalis* spp., on the other hand, have been considered of a great medicinal value all over the world, since compounds isolated from *P. angulata*, *P. peruviana* and *P. minima* display a wide spectrum of biological activities such as antimicrobial, antitumor, anti-inflammatory, immunomodulatory, cytotoxic, immunosuppressive, trypanocidal and molluscicidal (Chiang *et al.*, 1992a, 1992b; Kawai *et al.*, 1996, 2002; Tomassini *et al.*, 2000; Ribeiro *et al.*, 2002; Santos *et al.*, 2003; Soares *et al.*, 2003; Nagafuji *et al.*, 2004; Wu *et al.*, 2004a, 2004b; Lee & Houghton, 2005).

These are only a few examples of the diversity of biological properties attributed to Solanaceae species. This review focuses on the chemical diversity

associated with pharmacological properties of the isolated metabolites, despite the medicinal uses of the plant source. In the following sections, the activity of withasteroids against neurodegenerative disorders, parasite and microbial infections, as well as their anti-inflammatory, immunomodulatory and antitumor effects will be discussed.

Effects on Neurodegenerative Disorders

The interest on the effects of the withanolides against neurodegenerative disorders raised from the popular belief that *Withania somnifera* acts as "ginseng" with beneficial effects on nervous systems. In 2000, a group headed by Katsuko Komatsu started to study the effects of the methanolic extract from roots of *W. somnifera* against human neuroblastoma cell lines (SK-N-SH and SH-SY5Y), and they found that the extracts and some isolated withasteroids, withanolide A (**110**) and withanoside IV (**443**) and VI (**445**), possess significant neurite outgrowth activities (Tohda *et al.,* 2000; Zhao *et al.,* 2002). Another study, using rat cortical neurons, showed that withanolide A predominantly extended axons, while dendrites were extended by withanosides IV (**443**) and VI (**445**) (Kuboyama *et al.,* 2002).

Tohda *et al.* (2004) reported that rat cortical neurons were damaged by Aβ(25–35), which is an active partial fragment of amyloid β and causes both dendritic and axonal atrophy, synaptic loss (Grace *et al.,* 2002) and memory impairment (Maurice *et al.,* 1996). Withanolide A (**110**), withanosides IV (**443**) and VI (**445**) were then tested to see whether they could prevent, or not, these damages. In fact, both dendritic and axonal atrophy were prevented by the three compounds, and as mentioned earlier, the effects of withanolide A (**110**) were more specific to axons (98.6% of the control) while withanosides IV (**443**) and VI (**445**) were more specific to dendrites. In addition, both withanosides not only prevented the atrophy but also induced the growth of longer dendrites (106.3 and 117.4% of the control, respectively). These compounds did not only regenerate neurites, but were also able to reconstruct synapses. The results indicated that withanolide A (**110**) and withanosides IV (**443**) and VI (**445**) facilitated the reconstruction of both post-synaptic and pre-synaptic regions in neurons in which severe synaptic loss had already occurred. Again the effects were specific to each region, while withanosides IV (**443**) and VI (**445**) tended to increase post-synaptic structures (86.0 and 83.6% of the control, respectively); withanolide A (**110**) acted on pre-synaptic structures (108.1% of the control). Moreover neurites and synapses could be generated in the cerebral cortex and hippocampus and memory deficit be ameliorated in mice treated with withanolide A (**110**) (Kuboyama *et al.,* 2005).

Another approach to access the beneficial effects of withanolides on central nervous system was to measure their inhibiting properties against acetylcholinesterase (AChE) (Riaz *et al.,* 2004; Choudhary *et al.,* 2004, 2005). It is worthwhile mentioning that according to cholinergic hypothesis, the most promising therapeutic strategy to treat Alzheimer disease is to activate central cholinergic functions (Perry, 1986). Choudhary *et al.* (2004, 2005) assessed the potential of several withanolides from *W. somnifera* in inhibiting AChE enzyme. The withaferin A (**147**) was one of the compounds tested with IC_{50} value of 20.5 μm (concentration required for 50% inhibition of AChE). Galantamine was used as positive control and presented IC_{50} of 0.50 μm. Another type of withanolides, the bracteosins, were also assessed in order to measure their inhibitory activity against cholinesterase enzymes, with IC_{50} values ranging from 25.2 to 49.2 μm (Riaz *et al.,* 2004).

Anti-Parasitary Activity

In the last few years several medicinal plants have provided many secondary metabolites with leishmanicidal and trypanocidal activities, but due to their high cytotoxicity they have never made into clinical trials. Leishmania infection and Chagas' disease (American trypanosomiasis) are still some of the most serious public health in Latin America.

Since the 1940's, pentavalent antimonial drugs have been the main treatment for Leishmania infection. However, several problems underlying the treatment with these drugs like parasite resistance, severe side effects due to the drugs itself or the combination with other drugs, and costs are the most common ones. In fact, the research for new leishmanicidal compounds has been growing in recent years and withasteroids being the promising chemical group. Choudhary *et al.* (2005) studied the effects of physalins isolated from *Physalis minima*. All tested compound showed significant *in vitro* leishmanicidal activities against promastigotes of *Leishmania major* being the physalin H (**30**) the most potent with IC_{50} of 6.03 μm. Later on, the same group isolated two new physalins: 16,24-cyclo-13,14-*seco*-ergosta-2-ene-18,26-dioic acid-14 : 17,14 : 27-diepoxy-11β,13,20, 22-tetrahydroxy-5α-methoxy-1,15-dioxo-γ-lactone δ-lactone (**315**) and 16,24-cyclo-13,14-*seco*-ergosta-2-ene-18,26-dioic acid-14 : 17,14 : 27-diepoxy-5α,11β,13,20,22-pentahydroxy-1,6,15-trioxo-γ-lactone δ-lactone (**316**). Both the compounds showed potent leishmanicidal activity against the promastigotes of *Leishmania major* (Choudhary *et al.,* 2007).

Cardona *et al.* (2006) also demonstrated the activity of six types of acnistins and four types of withajardins against *Leishmania (V) panamensi* in amastigote forms. The acnistins and withajardins have two different bicyclic system attached at C-17, but in spite of these and other differences they have similar groups in rings A and B. The most potent withasteroids

were acnistin E (**10**) and withajardin B (**89**), with IC_{50} of 2.1 and 2.2 μm respectively, which have three common features: a 2-en-1-one system, a 5β,6β-epoxy and one hydroxyl group at C-4. Lack of any of these functionalities compromises the activity of these compounds, suggesting that they are important for the activity. Moreover, 4-deoxy-type compounds were active (acnistins A (**9**) and C (**136**)) but the acetylation of the C-4 hydroxy and epoxy groups without C2, C3-double bond (acnistin G (**134**)) caused reduction in the activity. Also, the α,β-unsaturated carbonyl group is less important than the epoxide ring in acnistins (Cardona *et al.,* 2006).

Nagafuji *et al.* (2004) and Abe *et al.* (2006) screened *in vitro* 15 withasteroids, isolated from the MeOH extract of the aerial parts of *Physalis angulata* L. (Solanaceae), against epimastigotes and trypomastigotes forms of *Trypanossoma cruzi*. The results showed a higher activity of all compounds against trypomastigotes, which is the infective form of the parasite, having the MC_{100} (minimum concentration at which all the trypomastigotes become immobilized after 24 h incubation) in the range of 2 to 9 μm for the most active compound. More recently, Vieira *et al.* (2008) screened 215 compounds, isolated from plants of the Northeast of Brazil, against epimastigotes forms of *T. cruzi*. Among these compounds tested only 8 were considered active and the withaphysalins O (**5**) and M (**4**) stood out with IC_{50} values of 0.6 and 1.4 μm, respectively.

Antimicrobial Activity

Plant extracts and fractions containing withasteroids are extensively studied against several types of bacteria, but only a few reports show the antimicrobial activity of the isolated compounds. Januario *et al.* (2002) demonstrated the effects of physalins against *Mycobacterium tuberculosis* H(37) Rv strains, where physalins B (**28**) and D (**340**) presented MIC (Minimal Inhibition Concentrations) values of > 128 μg/mL and 32 μg/mL, respectively. More recently, Silva *et al.* (2005) tested physalin B (**28**) against several gram positive and gram negative bacteria and great effects were observed against *N. gonorrhoeae* and *S. aureus* strains. Withaferin A was also screened for its antibacterial activity, showing significant results when compared to streptomycin sulfate (Shanazbanu *et al.,* 2006).

Anti-inflammatory and Immunomodulatory Effects

Withania somnifera leaves are popularly used as anti-inflammatory in India. In 2003, Jayaprakasam and Nair isolated from leaves of *W. somnifera* a series of withanolides, including withaferin A (**147**), and tested the ability

of these compounds to inhibit cycloxygenases 1 and 2 (COX-1 and COX-2), physiological and pathological enzymes, respectively, responsible for the formation of inflammatory mediators. All withanolides were able to inhibit COX-2 enzyme at 100 μg/mL withaferin A (**147**) being the most active one. Aspirin, ibuprofen, rofecoxib, naproxen, celecoxib and valdecoxib, all anti-inflamatory compounds used in clinics, were also tested as positive controls and the rates of COX-2 inhibition were 7, 59, 80, 95, 98 and 99%, respectively. It is worthy to mention that withaferin A (**147**) (100 μg/mL) was more active than aspirin (180 μg/mL), inhibiting COX-2 enzyme up to 39% and 7%, respectively. The withanolides were also tested at 250 μg/mL, but no improvement in the response was observed. None of the withanolides tested showed any activity against COX-1 enzyme, what makes it interesting, since COX-1 is responsible to maintain the physiological status of several organs, specially the stomach mucus. Wube *et al.* (2008) also reported the selective inhibition of COX-2 enzyme by a withanolide isolated from *Discopodium penninervium*. Besides, the effects directly on cycloxygenase enzymes, some studies have shown that physalins and withanolides also act inhibiting the nuclear factor-kappa B (NF-κB), which also regulates the expression of cytokines, chemokines, adhesion factors, and inducible pro-inflammatory receptors (Ichikawa *et al.*, 2006; Jacobo-Herrera *et al.*, 2006). In 2007, Kaileh *et al.* showed that withaferin A (**147**) potently inhibited NF-κB activation by preventing the tumor necrosis factor-induced activation by IκB kinase β (Kaileh *et al.*, 2007).

Immune system cells play an important role in host infection diseases. Soares *et al.* (2003) showed that physalins have a potent immunosuppressive effect in macrophages and in lipopolysaccharide-induced shock, inhibiting 90 to 100% on the Nitric Oxide (NO) production by macrophages, while dexamethasone (steroidal anti-inflamatory) only inhibited 30%. The production of pro-inflammatory cytokines, NO, TNF-α, interleukin-2, interleukin-6 and interleukin-12 as well as lymphocyte proliferation, were all reduced by physalins B (**28**), F (**29**) and G (Soares *et al.*, 2006), while an anti-inflammatory cytokine, IL-10 was enhanced (Vieira *et al.*, 2005). Overall, these effects are remarkably similar to the effects observed for dexamethasone. The physalins also display an interesting response on BALB/c mice transplanted with hearts (into the ear pinna) of newborn CBA mice. After 30 days of treatment with 1 mg/day/animal of physalins B (**28**), F (**29**) or G, all mice (6/6) presented viable grafts with vascularization, intact myofibers and a discrete inflammation. On the other hand, only 2 mice of the control group (2/6) presented viable grafts, while the others had their grafts rejected on the transplantation site, as inflammation and fibrotic areas associated with necrotic myofibers were observed. The mechanism by which physalins acts in the immune system remains unknown, but it

seems to be different from that of dexamethasone since their effects were not blocked by glucocorticoid receptor antagonist RU486, suggesting that the physalins might act activating the same receptor by another mechanism other than glucocorticoid or activating other receptors (Soares *et al.*, 2006).

Antitumoral

The antitumoral effects of withasteroids are probably the most studied of their pharmacological activities. These studies started in 1965 with the isolation and structural elucidation of withaferin A (**147**) (Kupchan *et al.*, 1965). Kupchan and collaborators (1969) tested withaferin A (**147**) *in vivo* against two tumor models: the Sarcoma 180 tumor in mice and the Walker intramuscular carcinosarcoma 256 in rats. At a dose of 20 mg/kg, withaferin A (**147**) showed an inhibitory effect of 62% in both tumor models (Kupchan *et al.*, 1969). Since then, withaferin A (**147**) and other withasteroids aroused the interest of several scientists in studying the antitumor effects of these compounds.

Minguzzi *et al.* (2002) isolated from the leaves of *Acnistus arborescens* three withanolides with cytotoxic activity against BC-1 (breast), Lu1 (lung), Col2 (colon), KB (oral epidermoid carcinoma), KB-V1 (vinblastine-resistant KB cell line) and LNCaP (hormone-dependent prostate cancer) cell lines.

Jayaprakasam *et al.* (2003) tested 12 withanolides, isolated from the leaves of *Withania somnifera*, against NCI-H460 (lung), HCT-116 (colon), SF-298 (central nervous system), and MCF-7 (breast) cancer cell lines, where withaferin A (**147**) was the most potent among the withanolides tested with IC_{50} of 0.24, 0.36, 0.28, and 0.27 μg/mL, respectively. Mohan *et al.* (2004) demonstrated that withaferin A (**147**) (7 μg/kg/day) is a potent inhibitor of angiogenesis in the FGF-2 Matrigel™ model in mice. More recently, Pan *et al.* (2007) showed that the withametelins I (**70**), K (**72**), L (**73**) and N (**75**), isolated from the flowers of *Datura metel*, also possess cytotoxic effects against A549 (lung), BGC-823 (gastric), and K562 (leukemia) cancer cell lines, with IC_{50} values ranging from 0.05 to 3.5 μm. Also in 2007, fifteen new withanolides, isolated from the stems, roots, and leaves of *Tubocapsicum anomalum*, displayed significant cytotoxic activity against five human cancer cell lines (hepatocellular carcinoma Hep G2 and Hep 3B, breast carcinoma MCF-7 and MDA-MB-231, lung carcinoma A-549), with IC_{50} values of the most potent compound ranging from 0.13 to 3.11 μg/mL (Hsieh *et al.*, 2007). Ma *et al.* (2007) also showed that physalins A (**341**), B (**28**) and H (**30**) have cytotoxic effects against human colorectal carcinoma HCT-116 cells and human non-small cell lung cancer NCI-H460, with IC_{50} values ranging from 0.3 to 1.9 μm. Lee *et al.* (2008) demonstrated the cytotoxic effect of withangulatin A (**264**) against gastric (AGS) and colorectal (COLO 205) carcinoma cell lines, with IC_{50} of 1.8 and 16.6 μm.

The withaphysalins, another group of withasteroids, also showed cytotoxic effects against SF268 (central nervous system), B16 (murine melanoma), MCF-7 (breast), HCT-8 (colon), CEM, HL-60 and K-562 (leukemia) cell lines. Withaphysalins F (**399**), M (**4**), N (**6**) and O (**5**) caused 32 to 99.5% inhibition of DNA synthesis in HL-60 and K-562 and also induced cells to apoptosis and necrosis (Veras *et al.,* 2004a; Rocha *et al.,* 2006). These findings corroborated the findings of Senthil *et al.* (2007) that showed that the apoptosis induction of withasteroids may be *via* mitochondrial depolarization, releasing the cytochrome c, leading to caspase activation. The study demonstrated a decrease in the Bcl-2 expression and an increase in the levels of Bax in HL-60 cells treated with a withanolide isolated from *Withania somnifera.* This increase of Bax expression is associated to loss of mithochondrial membrane potential and consequently to the release of cytochrome c, which in turn activates caspase 9 and then caspase 3 (Wei *et al.,* 2001; Thornberry, 1998).

Yang *et al.* (2006) showed both *in vitro* and *in vivo* that the primary target of withaferin A is the tumor cells proteasome. The proteasome is a proteinase complex responsible for the degradation of endogenous proteins like cyclins, transcription factors, tumor suppressors and others (Glotzer *et al.,* 1991; Goldberg, 1995).

Another mechanism proposed to withasteroids is the interference in the cytoskeleton of cells, which is important to the maintenance of cell shape, cellular locomotion, intracellular transport and cellular division (Grzanka *et al.,* 2003). Falsey *et al.* (2006) showed that withaferin A can covalently bind to annexin 2, part of the membrane-binding protein family, leading to F-actin bundling. Later on, Bagagna-Mohan *et al.* (2007) showed that withaferin A (**147**) also binds to the intermediate filament protein, vimentin. Any of these alterations in the cytoskeletal architecture may lead cells to induce apoptosis (Grzanka, 2001).

There are also reports that the double bond between carbons C-2 and C-3 are responsible for the cytotoxic effects of withasteroids (Fuska *et al.,* 1984; Su *et al.,* 2003; Magalhães *et al.,* 2006; Damu *et al.,* 2007). The SAR studies have been conducted mainly with physalins, and led to the conclusion that the presence of the conjugated cyclohexenone moiety is essential for cytotoxicity. Physalin D (**340**) that does not contain this functionality is inactive in tumor cells (Chiang *et al.,* 1992a). Furthermore, the hydrogenation of 5α-ethoxy-6β-hydroxy-5,6-dihydrophysalin B forming 5α-ethoxy-6β-hydroxy-2,3,5,6-tetrahydrophysalin B increased the IC_{50} value 23.4 times for tumor cells, and abolished the activity in sea urchin eggs (Magalhães *et al.,* 2006). However, other structural requirements seem to be necessary for cytotoxic activity, as the compounds having the double bound between C-2 and C-3

may be inactive as well (Magalhães *et al.*, 2006). Similarly, Antoun *et al.* (1981) demonstrated that the 5,6-epoxy ring of physalins is also responsible for antitumor activity, and Lee and Houngton (2005) recently revalidated the importance of the functional groups located at C-5 and C-6 as well. According to Magalhães *et al.* (2006), cytotoxic activity of physalins is probably determined by the combination of the conjugated cyclohexenone moiety and the presence of an oxygen located at both C-5 and C-6. However, it seems that the structure requirements for cytotoxic activity are far from being decisively elucidated. Nonetheless, withasteroids could be considered potential antineoplastic compounds, and may also explain the ethnobotanical use of Solanaceae species in the treatment of cancer.

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Phenolic Compounds from *Plumbago zeylanica* and their Cytotoxicity

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ABSTRACT

*Bio-guided fractionation of an active methanol extract of Plumbago zeylanica aerial parts led to the isolation of eight phenolic compounds, 4-hydroxy-3,5-dimethoxylbenzoic acid (**1**), 3-hydroxy-4-methoxybenzoic acid (**2**), N-E/N-feruloyl tyramine (**3a**, **3b**), (R,S)-plumbagic acid (**4**), (R,S)-methyl plumbagate (**5**), (3R,4R)-isoshinanolone (**6**), plumbagin (**7**) and 7-hydroxy-2,5-dimethyl-chromen-4-one (**8**). The structure and configuration of the compounds isolated were identified by 1-2D NMR, MS, chiral HPLC techniques and CD analysis. Compound **7** was the most cytotoxic with a marked activity against different human cell lines (leukemia K562, IC_{50}= 0.23 µg/mL), (breast cancer MCF7, IC_{50}= 0.24 µg/mL), (Bowes melanoma, IC_{50}= 0.26 µg/mL); it was found to induce apoptosis in K562 cells and to depolarize the mitochondrial membrane.*

Key words : *Plumbago zeylanica*, plumbagic acid, methyl plumbagate, plumbagin, cytotoxicity, apoptosis, bio-guided assay

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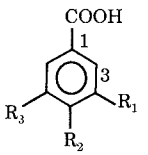
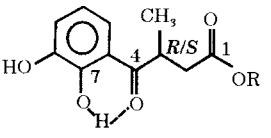
INTRODUCTION

The aerial parts of *Plumbago zeylanica* Linn. (Plumbaginaceae) are used in Vietnamese traditional medicine for the treatment of rheumatic pain, sprains, scabies, skin diseases, wounds, ulcers, inflammations and cancer (Vo, 1997; Do, 1999). Previous investigations of the plant composition revealed the presence of naphthoquinones, steroids, glucose, naphthalenones, alkanes, triterpenes, trace elements and amino acids (Veluri *et al.,* 1999; Gupta *et al.,* 1998). Recently, we reported the presence of non-polar cytotoxic compounds from *Plumbago zeylanica* aerial parts (Nguyen *et al.,* 2004). As the phenolic compounds from this plant have been scarcely investigated, we focused our study on these compounds and evaluated their cytotoxicity on several human cancer cell lines. This paper reports the isolation, the structure determination, the configuration identification and the cytotoxicity of eight phenolic compounds (**1–8**) (Fig 2.1) from the methanolic extract of *Plumbago zeylanica*. In addition, the mechanism of action of plumbagin (**7**), a cytotoxic principle from this plant, on the mitochondrial membrane potential of human cancer cells has been investigated.



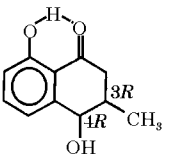
1 **2**

R_1	OCH_3	OH
R_2	OH	OCH_3
R_3	OCH_3	H



4 **5**

R	H	CH_3



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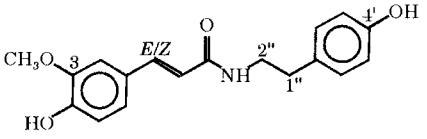
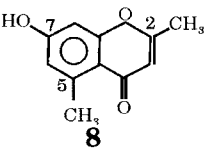


3a **3b**
E form *Z* form



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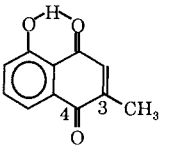

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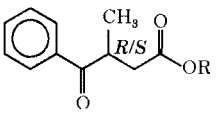

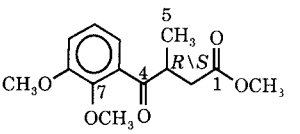

10 **11** **9**

R H CH_3

Fig 2.1. Phenolic compounds **(1–8)** isolated from *Plumbago zeylanica*, diazomethylated derivatives of **4** and **5** **(9)** and CD/HPLC chiral references **(10–11)**

MATERIALS AND METHODS

General Experimental Procedures

TLCs were run on silica gel (preparative: 0.25 mm, 20 × 20 cm, analytical: 0.03 mm, 10 × 20 cm; Merck, Darmstadt, Germany) using eluents, A (toluene-ethyl acetate-acetic acid, 40:10:5), B (ethyl acetate-acetic acid-formic acid-water 50:5.5:5.5:13) and C (petroleum ether (40–60°C)-diethylether-acetic acid 70:30:3). Detections of compounds were made under UV light (254 and 366 nm) before and after spraying with methanolic potassium hydroxide 10% or with ethanolic sulfuric acid 3% and heating at 150°C for 10 min; CC were run on silica gel C_{18} (20 × 2 cm *i.d.*, 20 g) and silica gel 60 (230–400 mesh, 20 × 2 cm *i.d.*, 30 g, Merck, Darmstadt, Germany).

Chiral HPLC separations were achieved on a Chiralcel OD-H (250 x 4.6 mm, *i.d.*) column (Daicel chemical industries, Ltd, Japan) eluting with different mixtures of n-hexane and ethanol (Merck, Darmstadt, Germany). On-line UV spectra were recorded with a diode-array detector (Hewlett Packard 1100, G1315A, Darmstadt, Germany). CD spectra were recorded on a CD spectrometer J-710 (JASCO, Japan) in the range 190 nm to 360 nm. Each spectrum was the accumulation of four scans at 50 nm/min with a 1 nm slit width and a time constant of 0.5 s. The CD spectra then were analyzed using the J-700 Standard Analysis (v.1.10.00) software. Samples were dissolved in MeOH (Merck, Darmstadt, Germany). The apparatus was purged with N_2 during the time of CD recording.

1D NMR including ^1H, ^{13}C-NMR, DEPT 90, DEPT 135 and 2D NMR including COSY, HMQC, HMBC spectra were recorded on a Bruker Avance 300 and a Varian Unity 600 spectrometers at 25°C, respectively. Chemical shifts (δ) are expressed in (ppm) using TMS as an internal standard. EI and EIHR mass spectra were obtained with an Autospec M apparatus (Micromas, UK) at temperature 200°C and an electron energy 70 eV. Optical rotation was recorded on a Perkin-Elmer 141 polarimeter at 25°C.

Chemicals

All cell culture material was obtained from Life Technologies (Paisley, Scotland). The Annexin V-FITC® kit was purchased from BD Bioscience (Bornem, Begium) and the DePsipher® kit was from Trevigen (Brussels, Belgium). Plumbagin standard (99.5%) was from Roth (Karlsrhure, Germany). All other chemicals were purchased from Sigma-Aldrich (Brussels, Belgium).

Plant Material

Aerial parts of *Plumbago zeylanica* Linn. (Plumbaginaceae) (identified by Prof. Dr. V.C. Vu) were collected in November, 2002 in Hanoi. A voucher specimen (N°501) is deposited in the herbarium of Hanoi University of Pharmacy, Vietnam.

Extraction, Isolation and Semi-Synthesis

The ground dried plant material (300 g) was extracted with 3 l MeOH at room temperature. Half part of the residue (12.8 g) obtained after evaporation under reduced pressure of the methanol extract was chromatographed on a C_{18} column using H_2O-MeOH (1–0, 1–1, 0–1) as eluent to yield three fractions M_1 (4.2 g), M_2 (1.2 g) and M_3 (1.0 g). Fraction M_3 inhibited the proliferation of several human cancer cell lines: leukemia K562 (IC_{50}= 2.21 µg/mL), breast cancer MCF7 (IC_{50}=2.34 µg/mL) and Bowes melanoma (IC_{50} = 2.11 µg/mL), and was selected for further fractionation. 0.5 g of M_3 was chromatographed on a column of silica gel C_{18} using H_2O-MeOH gradient to yield 6 fractions $M_{3.1}$, $M_{3.2}$, M_{33}, M_{34}, M_{35} and M_{36}. Purification of fraction M_{31} by *prep.* TLC using solvent A (3 developments) afforded **1** (1.4 mg) and **2** (6.2 mg). Using the same chromatographic conditions, fraction M_{33} afforded **4** (35.0 mg) and **5** (7.2 mg); fraction M_{34} (two developments) gave **6** (6.5 mg) and fraction M_{35} afforded **3a/b** (5.8 mg) and **8** (1.3 mg). *Prep.* TLC on silica gel of fraction $M_{3.6}$ (first development on 8 cm with solvent B, second development on 16 cm with solvent C) afforded **7** (2.0 mg). The purity of these isolated compounds was 99% by analytical HPLC and ^{1}H-NMR spectrum analysis.

Compound **9** was obtained by methylation of **4** and **5** using diazomethane. A solution of diazald (1 g) in ether (10 mL) was added to a solution potassium hydroxide (501 mg) in ethanol (1 mL) maintained at 65°C and an ethereal solution of diazomethane was simultaneously distilled off (Salmoun, 2002). The samples (10 mg of **4** or 7 mg of **5**) were dissolved in 10 mL of the ethereal solution of diazomethane and magnetically stirred at room temperature;

the products were checked daily by TLC' 1D and 2D NMR spectra. After 3 days, the reaction was complete and the methylation products were purified by *prep.* TLC using solvent A to yield compound **9**. Compounds **10** (*S*, $[\alpha]_D$ - 3.1°(*c* 1.6, $CHCl_3$), *ee* 80%), and **11** (*S*, $[\alpha]_D$ - 0.8° (*c* 1.6, $CHCl_3$), *ee* 80%) were prepared by enzymatic resolution of 3-substituted-4-oxaesters (Blanco *et al.*, 1993) and used as reference for chiral HPLC and CD analysis.

***4-Hydroxy-3,5-dimethoxybenzoic acid* (1):** ^{1}H-NMR (600 MHz, CD_3OD): δ 7.33 (2H, s, H-2, H-6), 3.88 (6H, s, 2 $OC\underline{H}_3$); ^{13}C-NMR (150 MHz, CD_3OD): δ 122.83 (C-1), 108.18 (C-2, C-6), 148.75 (C-3, C-5), 141.35 (C-4), 170.78 ($\underline{C}O_2H$), 56.61 and 56.60 (2 $O\underline{C}H_3$).

***3-Hydroxy-4-methoxybenzoic acid* (2):** ^{1}H-NMR (600 MHz, CD_3OD): δ 7.55 (1H, H-2), 7.54 (1H, d, J = 8.6 Hz, H-6), 6.83 (1H, d, J = 8.6 Hz, H-5), 3.89 (3H, s, $OC\underline{H}_3$); ^{13}C-NMR (150 MHz, CD_3OD): δ 123.9 (C-1), 114.0 (C-2), 148.7 (C-3), 152.6 (C-4), 115.9 (C-5), 125.3 (C-6), 171.0 ($\underline{C}O_2H$), 56.5 ($O\underline{C}H_3$).

***N-(E/Z)-feruloyl tyramine* (3a/b):** ^{1}H-NMR, ^{13}C-NMR and MS: Data in agreement with the literature values (Ma *et al.*, 2004).

***Plumbagic acid* (4):** $[\alpha]_D^{25}$: - 20.0° (*C* 0.5, $CHCl_3$); chiral HPLC: mobile phase 95% n-hexane and 5% EtOH, flow rate 1.4 mL/min, UV detection at 268 nm. Retention time: (*S*) 18.7 min; (*R*) 25.4 min; ee (*S*) 87%. EIMS *m/z* 224; ^{13}C-NMR (150 MHz, $CDCl_3$): Data revised from literature (Dinda *et al.*, 1998; Yue *el al.*, 1997): δ 177.2 (C-1), 36.6 (C-2), 36.9 (C-3), 208.7 (C-4), 18.5 (C-5), 117.8 (C-6), 150.2 (C-7), 145.7 (C-8), 120.4 (C-9), 119.1 (C-10), 120.6 (C-11); ^{13}C-NMR (150 MHz, DMSO): δ 175.1 (C-1), 38.6 (C-2), 38.2 (C-3), 211.8 (C-4), 18.8 (C-5), 120.0 (C-6), 153.0 (C-7), 148.8 (C-8), 122.7 (C-9), 119.8 (C-10), 121.1 (C-11).

***Methyl plumbagate* (5):** white powder, m.p. 93°C; $[\alpha]_D^{25}$: - 24.0° (*C* 0.5, $CHCl_3$); chiral HPLC: mobile phase 99.1% n-hexane and 0.9% EtOH, flow rate 1 ml/min, UV detection at 268 nm. Retention time: (*S*) 23.2 min; (*R*) 33.5 min; ee (*S*) 79% (Fig 2); EIMS *m/z* 238; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 12.46 (1H, bs, O$\underline{H}$ on C-7), 7.40 (1H, dd, J_1 = 8.0 Hz; J_2 = 0.9 Hz, H-11), 7.12 (1H, dd, J_1 = 8.0 Hz; J_2 = 0.9 Hz, H-9), 6.84 (1H, t, $J_1 = J_2$ = 8.0 Hz, H-10), 5.50 (1H, bs, O$\underline{H}$ on C_8), 3.96 (1H, m, H-3), 3.66 (3H, s, $OC\underline{H}_3$), 3.02 (1H, dd, J_1 = 8.6 Hz; J_2 = 16.8 Hz, H-2a), 2.50 (1H, dd, J_1 = 16.8 Hz; J_2 = 5.7 Hz, H-2b), 1.28 (3H, d, J = 7.2 Hz, H-5); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 173.1 (C-1), 37.7 (C-2), 37.7 (C-3), 209.8 (C-4), 19.1 (C-5), 118.6 (C-6), 150.9 (C-7), 146.4 (C-8), 121.0 (C-9), 119.8 (C-10), 121.3 (C-11), 52.5 ($COO\underline{C}H_3$).

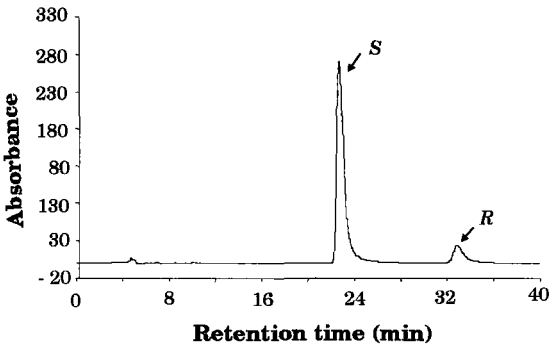

Fig 2.2. Chiral HPLC of **5.** Analysis performed with a Chiralcel OD-H (25 × 4.6 mm, *i.d.*) column, mobile phase n-C_6H_{12}- EtOH (99.1-0.9, v/v), flow rate 1 mL/min and UV detection at 268 nm

(3*R*,4*R*)-Isoshinanolone (6): $[\alpha_D]$ = + 22.39°. ^{1}H-NMR, ^{13}C-NMR and MS: literature (Bhattacharyya *et al.*, 1986; Bringmann *et al.*, 1999; Bringmann *et al.*, 2001).

Plumbagin (7): ^{1}H-NMR, ^{13}C-NMR and MS: literature (Bhattacharyya *et al.*, 1986).

***7-Hydroxy-2,5-dimethyl-chromen-4-one* (8):** Data revised from literature (Kashiwada *et al.*, 1984): ^{1}H-NMR (600 MHz, CD_3OD): δ 6.63 (1H, d, J = 2.7 Hz, H-8), 6.62 (1H, d, J = 2.7 Hz, H-6), 6.00 (1H, d, J = 0.5 Hz, H-3), 2.69 (3H, s, $C\underline{H}_3$ on C-5), 2.31 (3H, d, J = 0.5 Hz, $C\underline{H}_3$ on C-2); ^{13}C-NMR (150 MHz, CD_3OD): δ 166.6 (C-2), 111.4 (C-3), 182.1 (C-4), 143.6 (C-5), 118.1 (C-6), 163.3 (C-7), 101.7 (C-8), 115.6 (C-9), 161.5 (C-10), 19.8 ($\underline{C}H_3$ on C-2), 23.1 ($\underline{C}H_3$ on C-5).

1,7,8-Trimethyl plumbagate (9): colorless gum; $[\alpha]_D^{25}$: + 11.6° (*C* 0.25, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.12 (1H, dd, J_1 = 1.8 Hz; J_2 = 7.8 Hz, H-11), 7.10 (1H, t, $J_1 = J_2$ = 7.8 Hz, H-10), 7.03 (1H, dd, J_1 = 7.8 Hz; J_2 = 1.8 Hz, H-9), 3.90 (3H, s, $OC\underline{H}_3$ on C_7), 3.89 (3H, s, $OC\underline{H}_3$ on C_8), 3.82 (1H, m, H-3), 3.67 (3H, s, $COOC\underline{H}_3$), 2.90 (1H, dd, J_1 = 8.0 Hz; J_2 = 16.5 Hz, H-2a), 2.40 (1H, dd, J_1 = 16.5 Hz; J_2 = 6.1 Hz, H-2b), 1.17 (3H, d, J = 7.3 Hz, H-5); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 173.1 (C-1), 37.7 (C-2), 42.7 (C-3), 206.1 (C-4), 17.4 (C-5), 134.2 (C-6), 148.2 (C-7), 153.5 (C-8), 115.9 (C-9), 124.8 (C-10), 121.5 (C-11), 52.5 ($COO\underline{C}H_3$), 62.5 (OCH_3 on C-7), 56.6 (OCH_3 on C-8). 2nd CD $[\theta]_{192.0}$ + 1.169, $[\theta]_{201.5}$ – 1.138, $[\theta]_{211.5}$ + 0.464, $[\theta]_{220.0}$ – 1.082, $[\theta]_{230.5}$ + 0.687, $[\theta]_{245.5}$ - 0.411, $[\theta]_{262.5}$ – 0.186 (*c* 0.007, MeOH).

6,7-Dedihydroxyl plumbagic acid (10): yellowish gum, $[\alpha]_D^{25}$: - 3.0° (*C* 2.5, $CHCl_3$); ^{1}H-NMR, ^{13}C-NMR and MS: literature (Blanco *et al.*, 1993), chiral HPLC: mobile phase 98.8% n-hexane and 1.2% EtOH, flow rate 1 mL/min, UV detection at 278 nm. Retention time: (*R*) 24.4 min; (*S*) 27.0 min; ee (*S*) 78.0%. 2nd CD $[\theta]_{193.5}$ + 0.886, $[\theta]_{203.5}$ –1.645, $[\theta]_{212.5}$ +0.723, $[\theta]_{221.5}$ – 0.199, $[\theta]_{237.5}$ + 0.341, $[\theta]_{253.5}$ - 0.224, $[\theta]_{265.5}$ – 0.017 (*c* 0.095, MeOH).

6,7-Dedihydroxyl plumbagic acid methyl ester (**11**): colorless gum, $[\alpha]_D$: + 9.2° (*c* 1.25, $CHCl_3$); ^{1}H-NMR, ^{13}C-NMR and MS: literature (Blanco *et al.*, 1997); chiral HPLC: mobile phase 99.1% n-hexane and 0.9% EtOH, flow rate 1 mL/min, UV detection at 278 nm. Retention time: (*R*) 7.5 min; (*S*) 10.0 min; *ee* (*S*) 76.0%. 2nd CD $[\theta]_{193.5}$ + 1.219, $[\theta]_{204.0}$ – 2.520, $[\theta]_{213.0}$ + 1.109, $[\theta]_{222.0}$ – 0.347, $[\theta]_{238.5}$ + 0.601, $[\theta]_{255.0}$ - 0.324, $[\theta]_{268.5}$ – 0.023 (*c* 0.067, MeOH).

Biological Testing

***Assessment of cell viability*:** Three cell lines, K562 (leukemia), MCF7 (breast cancer) and Bowes melanoma (skin cancer) were incubated at 37°C (5% CO_2) in a growth culture medium (500 mL MEM containing 5% heat inactivated FCS, 2% L-glutamin, 2% penicillin-streptomycin and 0.2% gentamicin). The cells were seeded into 96-well plate at a density of 4×10^4 cells per well in culture medium and incubated over night before treating with the extracts and pure compounds.

After addition of base 2 logarithmic dilution of extracts or pure compounds (from 0.001 to 100 µg/mL) to each well, the cells were incubated for 3 days. Then the drug solutions were removed and 100 µl of MTT solution (0.1%) were added. After 4 h incubation, the plates were centrifuged and the supernatant was removed. The crystal blue-formazan was then dissolved in 100 µl DMSO and the absorbance was measured using a Multiskan Ascent (Labsystem) at 570 nm. The cell viability and IC_{50} of the compounds or extracts were assessed as previously reported (Nguyen *et al.*, 2004; Camby *et al.*, 1996).

***Analysis of apoptosis by annexin V-FITC staining* (bdbioscience, 2005):** Briefly, K562 cells were incubated with or without 1, 5 and 10 µg/mL plumbagin solutions (0.5% DMSO in growth culture medium) for 6, 12, 24 and 48 h; cells were collected, washed twice in cold PBS, and then re-suspended in binding buffer (Hepes-buffered saline solution containing 2.5 mm calcium chloride) at a density of 1×10^6 cells/mL. 5 µl fluorescein-labeled annexin V (FITC) and 10 µl propidium iodide (PI) were added to 100 µl of cell suspension. Samples were then incubated for 15 min before being analyzed by flow cytometry on a FACS-Calibur (Becton Dickinson). Annexin V-FITC signals and PI signals were monitored using the FL1-H and FL3-H channels respectively.

***Detection of mitochondrial membrane potential* (trevigen, 2005):** K652 cells were incubated with or without 5 and 10 µg/mL plumbagin solutions for 24 h at 37°C and then stained with a mitochondial probe (DePsipher® kit). For this purpose, the cell suspension was adjusted to a density of 1×10^6 cells/mL and incubated in diluted DePsipher® solution at 37°C, 5% CO_2 for 20 min. The cells were then washed twice in PBS, re-suspended in 1 mL PBS and the dye fluorescence was rapidly analyzed by flow cytometry.

RESULTS AND DISCUSSION

Compound **5,** obtained as a white powder, showed an M^+ peak at *m/z* 238. Its ^{1}H-NMR spectrum was similar to that of plumbagic acid but for an additional signal of methyl protons at δ_H 3.66 (3H, s), which showed lonely correlation to the carboxyl carbon at δ_c 173.11 (C-1) in the ^{1}H-^{13}C long-range correlation spectrum (HMBC). It was therefore identified as methyl plumbagate. This compound might be a natural product because plumbagic acid (**4**) treated in the condition used for isolation process did not afford **5**. Other isolated compounds (**1-3**) and (**6-8**) were identified by detailed analysis of 1D and 2D NMR spectra and by comparison to literature data (see materials and methods). However, the aromatic ^{13}C-NMR assignments of **4** reported by Dinda *et al.* (1998) and by Yue *et al.* (1997) had to be revised. The specific rotation of compound **4** was also different to that reported by Dinda *et al.* (1998). The ^{13}C-NMR assignments of compound **8** were similar to those reported by Kashiwada *et al.* (1984) except for the inversed assignments of C-3 and C-6. In the HMQC spectrum of **8**, the olefinic proton H-3 at δ_H 6.00 correlated to the C-3 at δ_c 111.4 and the aromatic proton at δ_H 6.62 correlated to C-6 at δ_c 118.1. In addition, the HMBC showed long-range correlations of the methyl protons on C-2 to C-3, of H-3 to C-2, of H-6 to C-7 and of the methyl protons at C-5 to C-6.

The chiral chromatography of compounds **4**, **5**, **10** and **11** showed that these are enantiomeric mixtures, which contain mainly one isomer (Fig 2.2).

Compound **9** was obtained as oil by methylation of **4** and **5** (ee 53%). Its ^{1}H-NMR spectrum presents three additional methoxyl proton signals at δ_H 3.90, 3.89, 3.67 (each 3H, s) in comparison to the proton signals of plumbagic acid. In the HMBC of **9,** the methoxyl protons at δ_H 3.67 gave cross peak to the carbonyl carbon at δ_c 173.1 (C-1); the methoxyl protons at δ_H 3.90 gave cross peak to a quaternary aromatic proton at δd_c 148.2 (C-7), which was further correlated to C-9 and C-11; and the methoxyl protons at δ_H 3.89 gave cross peak to a quaternary aromatic proton at δ_c 153.5 (C-8), which was further correlated to C-10. These elements thus proved the location of three methoxy groups on C-1, C-7 and on C-8. Compound **9**, therefore, was identified as 1,7,8-trimethyl plumbagate, which has a structure similar to the known active compound **10** (Blanco *et al.,* 1993) and compound **11** obtained by methylation of **10** using diazomethane (Salmoun, 2002). In order to determine the absolute configuration of **4** and **5**, the 2nd derivative CD spectrum of **9** was compared with that of **10** and of **11**. All chemical transformations and CD spectra similarities (Fig 2.3) point to the same absolute configuration for **4**, **5**, **9**, **10** and **11**. In the plant, **4** and **5** are enantiomeric mixtures with excess 9:1 of most probably the 3*S* enantiomer. These results are in accordance with the absolute configuration of **4** proposed by Dinda *et al.* (1998). The internal hydrogen bond makes the lateral chain less flexible, leading to a preferential 3*S* enzymatic methylation.

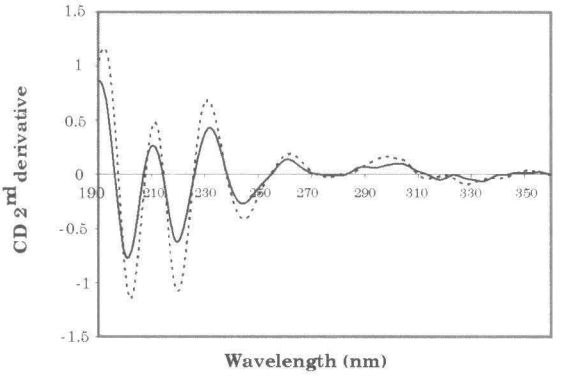

Fig 2.3. CD spectra (2nd derivative) of **9** (dashed line) and both **4** and **5** (trait line)

In vitro cytotoxicity of each isolated compound (**1-8**) was determined using three human cancer cell lines (K562, MCF7 and Bowes) and the MTT assay. Only **7** was active in the three cell lines and compared to adriamycin as a reference (Table 2.1). Other isolated compounds were considered as inactive with IC_{50}>50 µg/mL.

Table 2.1. Cytotoxicity[a] of isolated compounds (**1-8**)

Compound	IC_{50} (µg/mL) Cancer cell lines		
	K562	**MCF7**	**Bowes melanoma**
1-6 and **8**	> 50	> 50	> 50
7	0.23 ± 0.03	0.24 ± 0.01	0.26 ± 0.02
Adriamycin	0.05 ± 0.01	0.08 ± 0.01	0.30 ± 0.04

[a] Data from 3 independent experiments, each in hexaplicate (N=18). Experimental data were fitted to a parametric function by means of an original simplex algorithm: N=N°. $e^{(-k.C)}$, where C=concentration, N=percentage of living cells at concentration C, N°=percentage of living cells at concentration 0 and k=parameter. The IC_{50} were determined from the fitted curves.

Plumbagin Induced Apoptosis on Leukemia Cells K562

Although the cytotoxicity of plumbagin on cancer cells is well known, its mechanism of action is not yet fully understood. We therefore examined the characteristic apoptotic patterns on leukemia cells treated with plumbagin by using a flow cytometry method (Fig 2.4). Staining of the cells with both PI and FITC-labeled annexin V was examined; annexin V binds specifically to the phosphatidylserine exposed at the outer membrane in apoptotic cells and PI can label into necrotic cells, but not viable early apoptotic cells. Untreated K562 cells and 0.5% DMSO-treated cells (control)

did not bind with annexin V and excluded PI (Fig 2.4). The viable cells could be seen as black dots in the *bottom-left* quadrant (all population). The K562 cells treated with 1 µg/mL plumbagin showed early apoptotic change (*bottom-right* quadrant) at 12 and 24 h (annexin-V+PI- increased from 23.7 to 25.1%) as well as increased late apoptotic/necrotic cell population (*top-right* quadrant) at 24 and 48 h (annexin-V+PI+ increased from 8.7 to 16.7%). The K562 cells treated with 5 µg/mL plumbagin clearly showed early apoptosis at 6, 12 and 24 h (annexin-V+PI- increased to 45.4, 52.3 and 59.4%) and late apoptosis/necrosis at 24 and 48 h (annexin-V+PI+ increased to 31.6 and 76.0%). The K562 cells treated with 10 µg/mL plumbagin showed apoptosis and late apoptosis/necrosis at similar rate as the cells treated with 5 µg/mL plumbagin. Moreover, the early apoptosis induced by plumbagin at the same time clearly increases when the plumbagin concentration is increased (from left to right of Fig 2.4).

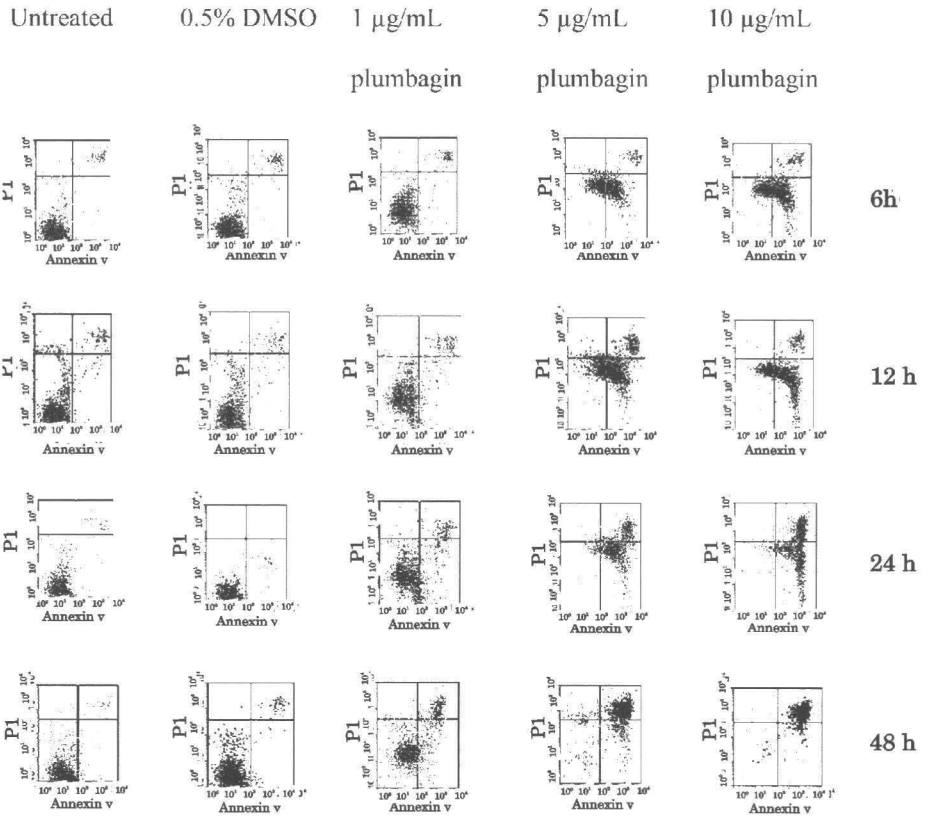

Fig 2.4. K562 cells: Flow cytometry analysis of population of cells at early stage apoptosis and late stage apoptosis/necrosis (annexin-V and PI staining). Each dot plot presents the count of annexin-V+PI+ (cells in late stage apoptosis/necrosis, *top-right* quadrant) and annexin-V+PI-(cells in early stage apoptosis, *bottom-right* quadrant)

Plumbagin Depolarizes the Leukemia Cells K562 Mitochondrial Membrane

Cell energy produced during mitochondrial respiration is stored as an electrochemical gradient across the mitochondrial membrane. This accumulation of energy in healthy cells creates a mitochondrial transmembrane potential, $\Delta\Psi_m$, which enables the cell to drive the synthesis of ATP. Disruption of $\Delta\Psi_m$ has been shown to be one of the first intracellular changes following the onset of apoptosis. In Fig 2.5, plumbagin-treated K562 cells showed lower $\Delta\Psi_m$ than untreated cells or 0.5% DMSO treated K562 (as control) after 24 h (dashed lines). The cells treated with 5 µg/mL plumbagin present a clearly reduced $\Delta\Psi_m$.

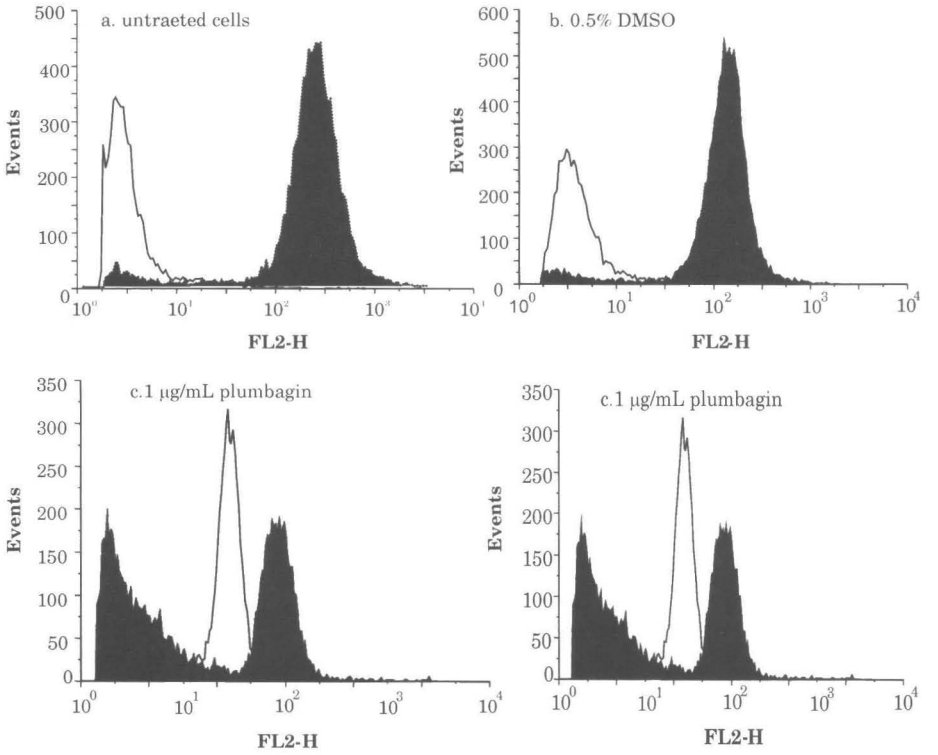

Fig 2.5. K562 cells : plumbagin induced mitochondrial membrane depolarization: The mitochondrial membrane potential was measured with the DePsipher Kit (Trevigen) and the fluorescence of 10^4 cells was recorded using a FACS calibur. Cells were treated with plumbagin concentrations 1 and 5 µg/mL for 24 h (c and d). The appropriate controls were also tested (untreated and 0.5% DMSO: a and b). The red fluorescence (DePsipher aggregates upon membrane polarization, forming a red compound) was measured at 585/590 nm. The green monomers (DePsipher can not access the disturbed potential transmembrane space and reverts to its green monomeric form) were measured at 510/527 nm. (-): K562 cells not treated with DePsipher™ solution, (darked-): K562 cells treated with DePsipher™ solution

CONCLUSIONS

From the bio-active fraction obtained from *Plumbago zeylanica*, eight pure phenolic compounds were isolated. Their structures and absolute configurations were determined using spectroscopic data, chiral HPLC and CD analysis. This is the first report on the natural occurrence of (*R,S*)-methyl plumbagate in the *Plumbago* genus. Among the isolated compounds, plumbagin has a marked activity on cancer cell proliferation. It was found to induce apoptosis in K562 cells and to depolarize the mitochondrial membrane. Our results are in agreement with the literature (Srinivas *et al.,* 2004; Srinivas *et al.,* 2004), in which plumbagin was examined in BRCA1-mutated (ovarian cancer) and in ME-180 (cervical cancer) cell lines. These results confirm that plumbagin induces cytotoxicity through apoptosis with disruption of the mitochondrial membrane potential and is a potential drug for cancer treatment.

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3

Extraction, Characterization and Biological Properties of 4-*O*-Methyl Glucuronoxylan from Hard Wood – A Review

ALINE BARBAT, CHARLOTTE MOINE, PIERRE KRAUSZ AND VINCENT GLOAGUEN[1,*]

ABSTRACT

Xylans are the most common hemicelluloses and account for the major non-cellulosic cell wall polysaccharide fraction of angiosperms where they present many different compositions and structures. 4-O-methyl-β-D-glucuronopyranosyl uronic acid xylan (MGX) is one of the main hemicellulose components of the cell wall of hard wood and hard wood by-products such as sawdust. The presence of close interactions between xylans and lignin within the cell wall reduces their extractability. A classical sodium chlorite preliminary delignification is considered as a first step that aids extraction of cell-wall xylan by alkalis. In a context of a greener chemistry, new procedures are nevertheless needed. In another respect, depending on their botanical origin, the fine structure of glucuronoxylan could differ according to their molecular masses, esterification by acetic acid, the 4-O-Methyl glucuronic acid to xylose ratio and the distribution of 4-O-Methyl glucuronic acid along the xylan backbone. Chemical characterization and structural elucidation of MGX required a combination of liquid chromatography, gas chromatography after derivatization, mass spectrometry and ^{1}H and ^{13}C NMR spectrometry. Plant cell walls are also known to be potential sources of pharmacologically active polysaccharides. Xylan-based therapeutics offers a new class of compounds for controlling various disorders such as cancer, viral infections and immune dysfunctions and we presently know that close structure/function

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relationships determine the biological properties of xylans. The aim of the current review is then to focus on the recent advances on glucuronoxylan extraction, structural characterization and biological valorization. As a case study, we report the results of our recent work concerning the extraction, structural characterization and cytotoxic properties of the 4-O-methyl-β-D-glucuronopyranosyl uronic acid xylan isolated from chestnut tree (Castanea sativa).

Key words : 4-*O*-methyl glucuronoxylan, chestnut tree, extraction, structure, biological properties, structure-function relationship

INTRODUCTION

Xylans, the most common hemicelluloses, account for the major non-cellulosic cell wall polysaccharide fraction of angiosperms (Ebringerová *et al.*, 2000). Cell walls of woody tissues of higher plants, particularly hardwoods and softwoods, are mainly composed of carbohydrate molecules or macromolecules: first of all, lignocellulose, but also pectins and other hemicelluloses. In spite of their ranking, sugars and their derivatives have long been considered as molecules of secondary biological importance, either playing roles in structural support (for example cellulose) or energy storage (starch or sucrose). Scientists are currently re-evaluating the biological importance of carbohydrates and are planning on developing a large number of applications, more particularly in the biomedical field (Hensel *et al.*, 1998). This is the case for polysaccharides, already widely used as starting material in the paper and food industries (Ebringerová, 2006). Xylan type polysaccharides such as glucuronoxylan have attracted much attention as plant constituents (Ebringerová *et al.*, 2000). Attention has been paid to the biosynthesis of xylans (York *et al.*, 2008), along with their constitution and structure in relation to their functional properties in plants (Ebringerová *et al.*, 2000). Recent studies have shown that glucuronoxylans are endowed with potential pharmacological activities (Moine *et al.*, 2007). The stimulating properties of sugars have long been used by traditional pharmacopoeias, in particular for their cytotoxic, anti-tumor, anti-complementary and antimicrobial properties (Moine *et al.*, 2007; Yanaki *et al.*, 1983; Samuelsen *et al.*, 1999; Christakopoulos *et al.*, 2003).

The aim of the current review is to focus on recent advances about hardwood glucuronoxylan extraction, structural characterization and biological valorization. As a case study, we summarize our recent work concerning the extraction, structural characterization and cytotoxic properties of 4-*O*-methyl-β-D-glucuronopyranosyl uronic acid xylan isolated from chestnut tree (*Castanea sativa*).

ORIGIN AND EXTRACTION PROTOCOL OF GLUCURONOXYLANS

Xylan resources are very common, and xylan abundance in a number of plants greatly facilitates their valorization at the industrial level. Xylans are mainly present in the wood of dicotyledons, and apart from *Graminaceae*, in monocotyledons where arabinoxylan and glucuronoxylan-type hemicelluloses account for 25 to 35% of dry weight (Alèn, 2000). Grain contains large amounts of arabinoxylan, glucuronoarabinoxylan and homoxylan-type hemicelluloses up to 30–50% of dry weight (Wilkie, 1979). Corn or wheat by-products represent more conventional resources of xylan type polysaccharides (Ebringerová *et al.*, 1992; Sun *et al.*, 1998; Wang *et al.*, 2005). Other resources easily accessible and abundant also exist. This is for example the case of xylans isolated from the residues of the sunflower (Bazus *et al.*, 1993) or olive oil industries (Coimbra *et al.*, 1995) or extracted from straw by the steam explosion process (Sun *et al.*, 2005). Wood and forestry wood by-products such as sawdust are also rich in xylans (Ebringerová & Heinze, 2000). Hard wood presents a very homogeneous content in xylan.

Experimental procedures for the extraction of biomolecules especially from plant material have to meet two goals: extract a major class of molecules under consideration with a sensible yield, and at the same time limit degradation during the extraction process. There is no universal protocol - *stricto sensu* - for the extraction of xylan. A large number of practices are based on laboratories' know-how, of which some are often reported in the literature. Liberation of xylan from the cell wall matrix of lignified woody tissue is hampered by the presence of a lignin network as well as ester (Takahashi *et al.*, 1988) and ether lignin-carbohydrate linkages (Watanabe, 1989). The close interaction of glucuronoxylan with other cell wall polysaccharides has been reported, for example in the case of xyloglucan (Coimbra *et al.*, 1995), pectin (Hromádková *et al.*, 1996) and type II arabinogalactan (Kwan & Morvan, 1991). The elaboration of easier and more suitable multi-step or, ideally, one-step extraction procedures of glucuronoxylan from hardwood is still under investigation. Various factors affecting xylan extraction have been studied and the results evaluated with regards to yield, purity as well as molecular weight of isolated xylans. In the case of hardwood derivatives such as sawdust, a preliminary delignification is classically considered as a first step that helps extraction of cell-wall xylan (Yamagaki, 1997). Delignification with acidic sodium chlorite is usually performed before 24% KOH alkaline extraction of xylans (Adams, 1965). A dilute 24% KOH pre-treatment, inducing the swelling of cell wall fibers, can contribute to improve the extractability of cell wall xylans (Carpita, 1984). Ebringerová and coworkers substituted the hazardous and expensive $NaClO_2$ step by a two-step delignification procedure that involved NaOH and H_2O_2 (Ebringerová *et al.*, 1989). In these conditions, the structure of alkali-extracts of woody tissue was thought to be quite similar to that of the native polysaccharide, except that, due to the alkaline conditions,

O-acetyl groups have been removed. Thus, in order to obtain acetylated 4-*O*-methylglucuronoxylan, wood must be pre-treated prior to its extraction. Acetylated hardwood xylans could be obtained from holocellulose by extraction with dimethyl sulfoxide (Timell, 1967; Reicher *et al.*, 1984; Teleman *et al.*, 2002). Extraction of acetylated 4-*O*-methylglucuronoxylan could also be achieved after subjecting wood to steam-water, for a short time at high temperature. This principle gave rise to various procedures: steam treatment with and without explosion (Korte *et al.*, 1991; Palm *et al.*, 2004; Krawczyk *et al.*, 2008), thermomechanical treatment in water (Khan *et al.*, 1990) and microwave treatment (Junel, 1999; Teleman *et al.*, 2000; Jacobs *et al.*, 2002; Lundqvist *et al.*, 2002; Chen *et al.*, 2005). Even if the majority of the acetyl substituents of 4-*O*-methylglucuronoxylan actually withstands to such treatments (Korte *et al.*, 1991; Khan *et al.*, 1990), microwave treatment generally led to the partial depolymerisation of xylans producing xylosyl-oligosaccharides with degrees of polymerization ranging from 5 to 35 (Teleman *et al.*, 2000). As an alternative, physical pre-treatment of plant material has been proposed. Due to their sonomechanical effects whose impact on plant tissues have already been investigated (Toma *et al.*, 2001), it was demonstrated that application of ultrasound could facilitate the extraction of xylan from *Salvia officinalis* L. (Hromádková *et al.*, 1999), wheat straw (Sun *et al.*, 2002), corn cob (Hromádková *et al.*, 1999), buckweat hulls (Hromádková *et al.*, 2003), apple pomace (Caili *et al.*, 2006) or Chinese jujube (Li *et al.*, 2007) without substantial modification of their structural and molecular properties (Hromádková *et al.*, 2003). In the case of hardwood glucuronoxylan, and from our experience, water extraction yields remain unfortunately very low. In a context of a greener chemistry, the utilization of enzymes has been proposed for lignin degradation and improvement of xylan extraction. Mainly secreted by white-rot fungi, enzymes as laccase, manganese peroxidase and lignin peroxydase are known to be associated with the degradation of lignin in wood (Kondo *et al.*, 1994; Hammel & Cullen, 2008). Thanks to a low redox potential, these enzymes are able to catalyse single-electron oxidation steps (Baiocco *et al.*, 2003) in presence of oxygen and a mediator (often N-hydroxybenzotriazole or 2,2'–azinobis(3-ethylbenzothiazoline-6-sulfonate)) (Fig 3.1). Several reports showed effective lignin oxidation and bleaching of kraft pulp by enzymatic processes. Applied to crude sawdust, the laccase-mediator system, unfortunately, could not significantly degrade lignin and could only lead to partial extraction of xylan.

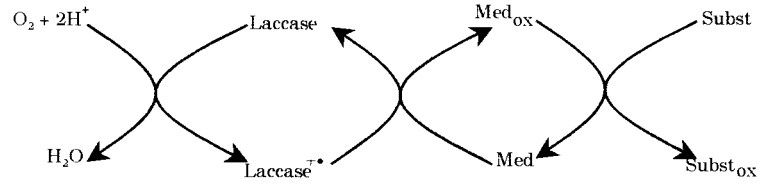


Fig 3.1. Proposed scheme for the role of mediator in laccase activity

We recently proposed a successful chemical alternative to enzyme delignification in which 4-*O*-methyl glucuronoxylan were directly obtained from delignified or non-delignified woody tissues (Barbat *et al.*, 2008). *Prior* to a hot aqueous extraction, chestnut sawdust or chestnut holocellulose were subjected to radical delignification making use of a H_2O_2 / phthalocyanin (FePcS) system (Fig 3.2). Repeated with different times and temperatures of extraction, this methodology led - *with good extraction yield* - to the selective extraction of native and homogeneous acetylated 4-*O*-methylglucuronoxylan.

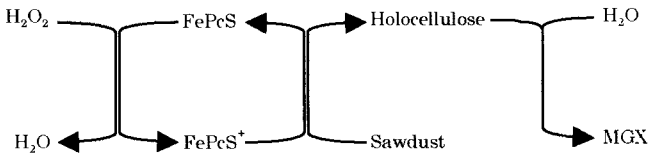


Fig 3.2. Proposed mechanism for phthalocyanin delignification of sawdust

To conclude this section, the most common extraction procedure used at the laboratory scale is based on the alkaline extraction of holocellulose obtained from $NaClO_2$- delignified woody tissue (Fig 3.3).

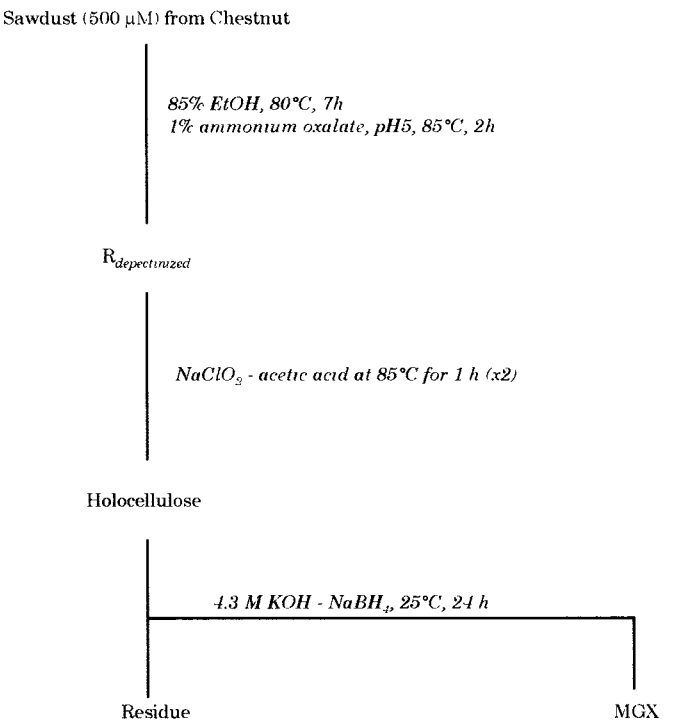


Fig 3.3. Extraction and purification of glucuronoxylans from chestnut sawdust

4-*O*-METHYL GLUCURONOXYLAN STRUCTURAL ANALYSIS AND HYDROLYSIS

Chemical structures of the main classes of hemicelluloses have already been elucidated and their descriptions can be found in early reviews by Aspinall (1959) and Timell (1965). Technological advances realized during the last decades in the area of oligo- and polysaccharide analysis, namely GC-MS, MS and ^{1}H /^{13}C-NMR contributed to specify the fine structure of these molecules. In-depth analysis of the literature pictures xylans as polysaccharides bearing a variety of chemical substituents. Such a diversity can be associated with the various functions played by xylans and could justify their distribution in the plant kingdom (Ebringerová & Heinze, 2000; Mellerowicz & Sundberg, 2008; Poper, 2008).

Among the xylan family, the structure of 4-*O*-methylglucuronoxylan (MGX) extracted from hardwood has already been reported and fully characterized (Moine *et al.*, 2007). The most representative structure is probably a linear backbone of 200 Xyl*p* β-(1→4) bearing 4-*O*-methylglucuronic acid residues linked to C-2 of Xyl*p* units. The 4-*O*-MeGlcA to Xyl*p* ratio value is commonly comprised between 1 and 10. The C-2 and/or the C-3 of Xyl*p* residues can also be esterified by acetic acid (Fig 3.4).

β-*D*-Xyl*p*(1→4)β-*D*-Xyl*p*(1→4)β-D-Xyl*p*(1→4)β-D-Xyl*p*(1→4)β-D-Xyl*p*(β-D-Xyl*p*(1→4)β-D-Xyl*p*(1→

2	3	3	2	3	2
↑	↑	↑	↑	↑	↑
Ac	Ac	Ac	Ac	Ac	4-*O*-Me-D-Gi*ep*A

Fig 3.4. General structure of 4-*O*-methylglucuronoxylan (MGX) extracted from aspen wood (Teleman *et al.*, 2000)

These substitutions account for the large variability of 4-*O*-methylglucuronoxylan from different biological origins. To fully characterize the fine structure of these molecules, many parameters have to be specified:

- The 4-*O*-methylglucuronic acid substitution rate of the xylosyl backbone in hardwoods: from 4:1 to 16:1 (Ebringerová *et al.*, 2005).
- The 4-*O*-methylglucuronic acid distribution along the xylosyl backbone: random or regular distribution (Jacobs *et al.*, 2001).
- The occurrence of acetic acid esterification of the xylosyl backbone. The precise location of acetyl groups on xylose residues is still a matter of discussion since spontaneous C-2 ↔C-3 migrations have been observed. The MGX acetyl group rate is generally comprised between 3 and 13 weight percent (Ebringerová & Heinze, 2000) that means a degree of substitution between 0.13 and 0.54.

The structural characterization of polysaccharides often required their fragmentation into oligosaccharidic units that were more easily analyzed. In the case of polysaccharides containing uronic acids, the stability of uronosidyl linkages limited the use of classical methodologies. Nevertheless, the linkage that associated one or more hexuronic acids could constitute a specific cleavage point.

Specific glycosidases have been successfully used to produce representative oligosaccharidic repeating units. This method is particularly interesting in the case of homogeneous polysaccharides such as MGX which are composed of a β(1→4) Xyl_p backbone. A total degradation of MGX could be achieved by a combination of endo- and exoenzymes that not only cleave the xylosyl backbone of MGX but also its substitutions (Fig 3.5). Enzymes that cleave the xylosyl backbone are named xylanases, while those that cleave the lateral parts are called accessory enzymes (Bonnin *et al.*, 1997). Xylanases are widespread and have been isolated from bacteria, yeasts and fungi. The following have been widely used for the degradation of MGX:

- 1,4-β-D-xylan-4-xylanohydrolase (E.C. 3.2.1.8), an endoxylanase that produces xylo-oligosaccharides with various degrees of polymerization. The presence of a 4-*O*-methylglucuronic acid substitution generally inhibits endoxylanase activity. A very useful exception is presented by the glucuronoxylan xylanohydrolase extracted from *Bacillus subtilis* (Nishitani & Nevins, 1991) that recognizes monomeric glucuronyl side chains attached to the xylan backbone and cleaves the β(1→4) xylosyl linkage of the adjacent unsubstituted xylosyl unit.
- 1,4-β-D-xylan-4-xylohydrolase (E.C. 3.2.1.37) otherwise known as xylosidase, an exoxylosidase that produces xylose residues from xylo-oligosaccharides of low degree of polymerisation.
- Accessory enzymes among which:
    - Xylan-α-D-1,2-glucuronohydrolase (E.C. 3.2.1.131) or glucuronidase which releases glucuronic acid and/or its methylated derivative α(1→2) linked to the xylosyl backbone (Siika-Aho *et al.*, 1994). This activity is required for a complete depolymerization of MGX.
    - Xylan acetylesterase (E.C. 3.2.1.6) which releases acetic acid from the C2 and/or the C3 of the acetylated native MGX. This enzyme is essential for the total hydrolysis of native MGX, since acetyl groups inhibit xylanase adsorption on MGX (Biely *et al.*, 1986).

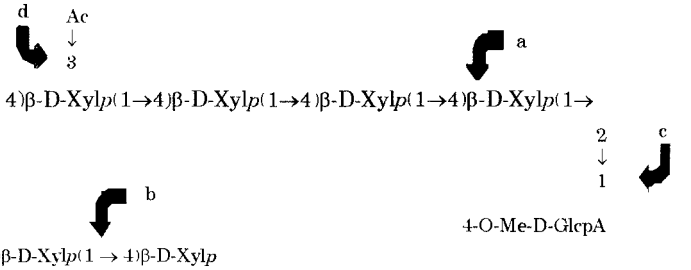


Fig 3.5. Enzymatic hydrolysis of MGX (Bonnin *et al.*, 1997)
a : *1,4 β D xylan-4-xylanohydrolase, E.C. 3.2.1.8 ;*
b : *1,4 β D xylan-4-xylohydrolase, E.C. 3.2.1.37 ;*
c : *xylan α D 1,2 glucuronohydrolase, E.C. 3.2.1.131 ;*
d : *acetyl-esterase (E.C. 3.2.1.6).*

The non selective mild acidic hydrolysis could sometimes be considered as an alternative and complementary approach to enzymatic hydrolysis of MGX. The chemical resistance of uronosidic linkages could confer them a good resistance to a strong acid such as HCl or TFA, even at high temperature. In such conditions and in the case of MGX, the (4-*O*-methylglucurono acid α(1→2) xylose) aldobiuronic acid is often characterized as a hydrolysis product. The relative stability of this disaccharide to acidic hydrolysis is explained by the hydrogen bond contracted between the C-6 carboxyl proton and the ring oxygen of the glucuronosyl unit which, because of the inductive effect, increases the energy barrier to acid hydrolytic cleavage of the adjacent glycosidic bond (Fig 3.6).

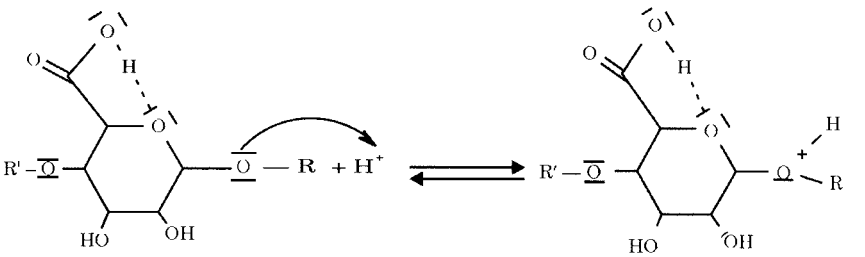


Fig 3.6. Uronosidic linkage stability to acidic hydrolysis (Timell *et al.*, 1965)

Lastly, native MGX could be directly analyzed by ^{1}H- and ^{13}C- NMR spectroscopies. Combination of these two techniques can provide all the information required to establish the fine structure of the analyzed molecules. It was thus possible to assay and identify each monosaccharide (homonuclear COSY), to identify their branching points and anomeric nature (Heteronuclear Multiple-Quantum Coherence (HMQC) COSY), and to determine the sequence of monomers (Heteronuclear Multiple Bond

Correlation (HMBC) COSY). In the case of MGX, the substitution ratio of the xylosyl backbone by 4-*O*-methylglucuronic acid as well as the ratio and location of acetyl substitution could be determined.

Typical ^{1}H and ^{13}C NMR spectra are given in Figs 3.7 and 3.8 respectively, for the MGX alkali extract of chestnut tree. Complete assignment of the proton and carbon spectra (chemical shifts reported in Table 1) was achieved by performing 2D COSY and 2D HMQC experiments. Examination of data relative to ^{1}H NMR analysis revealed three important groups of protons: major signals corresponding to the non-substituted D-xylose backbone units and two groups of minor signals, the first group corresponding to the 4-*O*-Me-D-GlcA residues and the second one assigned to D-Xyl units substituted with 4-*O*-Me-D-GlcA. From the fact that the coupling constants of the anomeric protons of xylose units, either substituted (at 4.5 ppm) or non-substituted (at 4.6 ppm) were larger than 7 Hz, the xylose residues were shown to be linked via β-glycosidic bonds while the anomeric proton of 4-*O*-Me-α-D-GlcA appeared as a doublet with a coupling constant less than 2 Hz, corresponding to an α-configuration. In addition, the presence of the methyl group of MeGlcA was confirmed by a corresponding sharp singlet at 3.46 ppm. The linkage via (1→2) glycosidic bonds between 4-*O*-MeGlcA and Xyl was confirmed by the deshielding of the H-2 signal of the substituted Xyl (3.44 ppm), by comparison with the non-substituted one (3.29 ppm). Complete ^{1}H and ^{13}C assignment of chestnut MGX could be achieved by performing a HMQC 2D experiment (chemical shifts reported in Table 1).

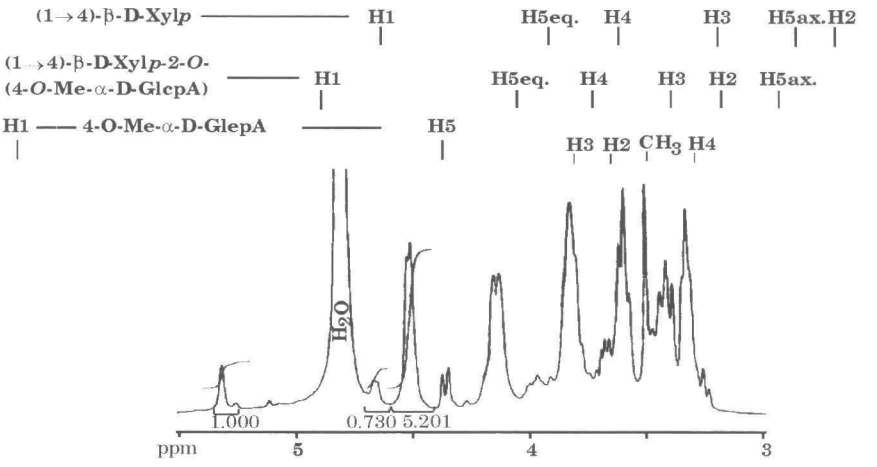


Fig 3.7. ^{1}H NMR spectrum of chestnut 4-*O*-methylglucuronoxylan (MGX). In D_2O, T= 300 K, δ in ppm relative to TMS (Moine *et al.*, 2007)

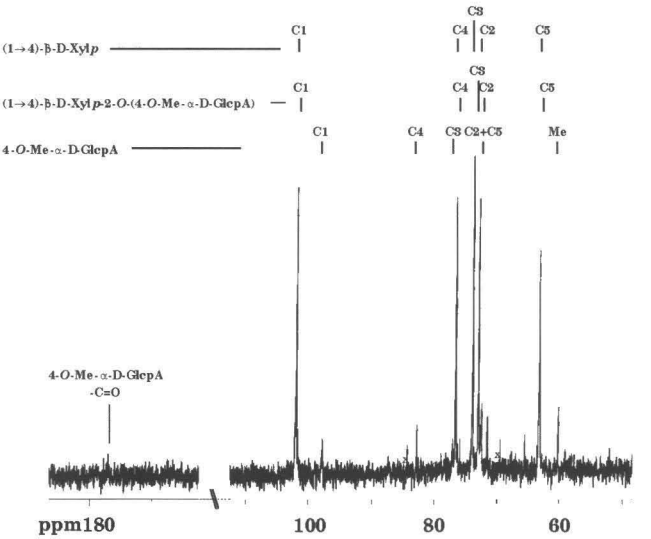


Fig 3.8. ^{13}C NMR spectrum of chestnut 4-*O*-methylglucuronoxylan (MGX). In D_2O, T = 300 K, δ in ppm relative to TMS (Moine *et al.*, 2007)

Table 3.1. ^{1}H and ^{13}C NMR chemical shift (ppm) assignments for residues of chestnut xylan (MGX), $^{3}J_{H,H}$ (Hz). [a] ax = axial, eq = equatorial ; [b] = assignments may be interchanged (Moine *et al.*, 2007)

	(1→4)-β-D-Xyl*p*		**(1→4)-β-D-Xyl*p*-2-*O*-(4-*O*-Me-Glc*p*A)**		**4-*O*-Me-α-D-Glc*p*A**	
	^{1}H	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**	**^{13}C**
Position	**δ (ppm) (*J* Hz)**	**δ (ppm)**	**δ (ppm) (*J* Hz)**	**δ (ppm)**	**δ (ppm) (*J* Hz)**	**δ (ppm)**
1	4.48 d (7.5)	102.09	4.63 d (7.2)	101.79	5.29 d (2.0)	97.94
2	3.29 t (8.2)	73.11	3.44 m	76.03	3.60 m	72.64 [b]
3	3.55 t (9.0)	74.07	3.62 m	71.67 [b]	3.76 m	77.22
4	3.79 m	76.76	3.81 m	74.21	3.22 t (9.7)	82.89
$5_{ax.}$	4.10 dd (4.5, 11.5)	63.38	4.15 m	65.64	4.33 d (10.1)	72.76 [b]
$5_{eq.}$	3.38 t (11.0)		3.42 m			
6	-	-	-	-	-	177.21
O-CH_3	-	-	-	-	3.46 s	60.29

The ^{13}C NMR spectrum contained five major signals corresponding to those of a (1→4) -linked-β-xylan. The signal at δ 102.09 ppm corresponds to the anomeric region in a β-configuration, as confirmed by the ^{1}H NMR spectrum, while the signals at δ 76.76, 74.07 and 73.11 ppm correspond to C-4, C-3 and C-2, respectively, and the 63.38 ppm signal arose from C-5.

Minor differences were observed for ^{13}C NMR chemical shifts of substituted xylose units, especially at the C-2 substituted position. Concerning the glucuronic unit, the signal of the methoxylated C-4 appeared at 82.89 ppm and the carbon signal of the methoxyl group was found at 60.29 ppm. Lastly, carboxyl signal was observed at 177.21 ppm. The relative amounts of Xyl and 4-*O*-Me-α-D-GlcA were determined by integration of the corresponding anomeric protons, and the ratio Xyl/4-*O*-MeGlcA subsequently calculated; integration results gave an approximate value of 5.9:1, in agreement with GC analysis. This value obtained for this xylan from chestnut wood examined here is typical of hardwood xylans. Employing ^{1}H and ^{13}C NMR, and using homonuclear and heteronuclear two-dimensional techniques, the structure of acetylated xylan can be easily determined (Teleman *et al.*, 2000). Native acetylated xylan extracted in water from chestnut shows a ^{1}H NMR spectrum close to that of Fig 3.9. The signals around δ 2.2 ppm indicate a high acetyl substitution rate with an average degree of acetylation (DS_{AC}) of the xylose residues of 0.45.

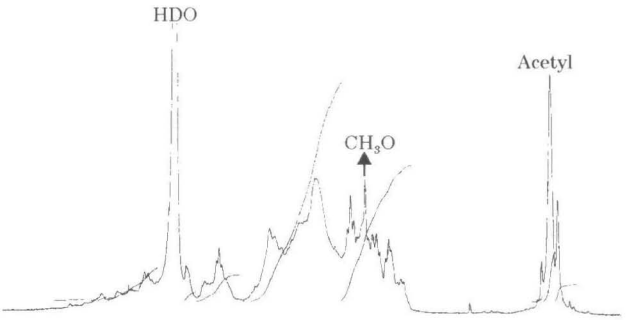
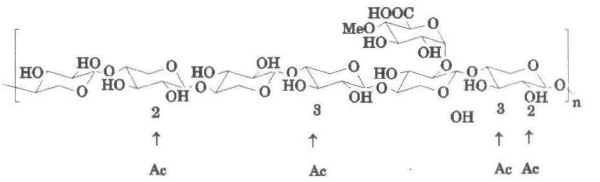


Fig 3.9. ^{1}H NMR spectra of acetylated 4-*O*-methylglucuronoxylans from chestnut wood

Based on the experimental data obtained from GC and NMR analyses, a theoretical structural model can be proposed for the MGX repeating unit of chestnut as follows:



The repeating unit consists of a linear backbone of six β(1→4) linked xylopyranosyl residues. At least one of the xylose residues is monosubstituted at C-2 by a 4-*O*-methyl-glucuronic acid, giving for chestnut tree a typical ratio of 4-*O*-methyl glucuronic acid to Xyl of 1 to 6 with an average DS_{AC} of 0.45. Owing to its characteristic large content of carboxyl functions, MGX from chestnut wood can be classified as an acidic xylan. Precaution must be

taken about the attachment of the *O*-acetyl groups (2-*O*-acetylated, 3-*O*-acetylated or 2,3-di-*O*-acetylated) on the xylose backbone since these groups can spontaneously migrate (Reicher *et al.*, 1984).

BIOLOGICAL PROPERTIES OF GLUCURONOXYLANS

A Survey of MGX Biological Properties

Plant cell walls are known as potential sources of pharmacologically active polysaccharides (Hensel *et al.*, 1998). Even though there is an abundant literature regarding the biological properties of plant xylans, a few data have been published on the potential antitumor activity of 4-*O*-methyl-glucuronoxylans from wood. Hashi and Takeshita (1979) reported the inhibition of sarcoma-180 and other tumor systems by Japanese beechwood MGX. The authors explained this phenomenon by the indirect stimulation of the non-specific immunological host defence. Recently, Ebringerová *et al.* (2002) used the comitogenic thymocyte test in order to check the properties of MGXs extracted from beech-wood and three medicinal herbs, *Rudbeckia fulgida*, *Althea officinalis* and *Mahonia aquifolium*. These polymers have different molecular weights (between 17000 and 37000 Da), compositions (ratio Xyl:MeGlcA between 6.4:1 and 8.4:1), and distributions (random or regular) of MeGlcA substituents. Since they reflect the fine structure of the macromolecular chains, these parameters may justify the intermolecular interactions between xylan molecules in solution creating networks or microgels, as well as interactions with the thymocyte cell coat (Ebringerová *et al.*, 2002). Comparison of the biological responses to the tested acidic xylans did not reveal any unequivocal relation either to molecular weight, MeGlcA content, or distribution patterns. Experimental clues leading to the identification of the individual structural parameters having an influence on the immunological effects are yet to come.

In vitro Studies of the Anti-Tumoral Activity of Native Chestnut MGX

Chestnut MGX was also shown to exhibit various *in vitro* proliferation-inhibiting effects on five human tumor cell lines: A 431 squamous cell carcinoma (vulvar epidermoid carcinoma), breast carcinoma (MDA-MB-231), prostate carcinoma (DU145), fibrosarcoma (HT1080) and hepatocarcinoma (HuH7). To investigate the effects of MGX on cell proliferation, these tumor cells were treated with increasing doses of xylan ranging from 0.8 to 50 μM (Fig 3.10, Moine *et al.*, 2007).

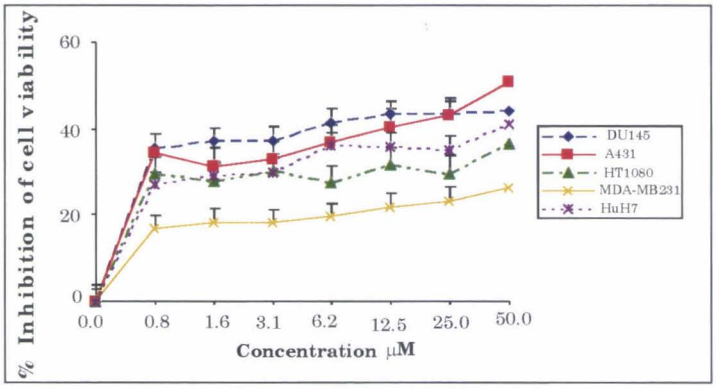


Fig 3.10. Dose-dependent effects of chestnut MGX on DU145, A431, HT1080, MDA-MB-231 and HuH7 cell viability. DU145, A431, HT1080, MDA-MB-231 cells were treated with increasing concentrations (0.8 µM to 50 µM) of this xylan for 72 h and HuH7 cells were treated in a similar manner for 144 h. Results are mean ± SEM of three independent experiments (Moine *et al.*, 2007)

Chestnut MGX inhibited tumor cell proliferation and the concentration inducing 50% of maximal inhibition (IC_{50}) was determined. The IC_{50} value was 50 µM for A431 cells. IC_{50} of the four other cell lines could not be evaluated and the maximum inhibitions observed at 50 µM were 45%, 27%, 26% and 41% for DU145, HT1080, MDA-MB-231 and HuH7 cell lines respectively. As the A431 cell line proved the most sensitive to cell proliferation inhibition by MGX, we then specifically focused our attention on these cells. A431 human squamous cell carcinoma cells represent a good model of an aggressive, highly angiogenic and invasive tumor (Di Benedetto *et al.*, 2003; Hamma-Kourbali *et al.*, 2003). A431 cells display an increase of epidermal growth factor receptors (EGFR) and produce large amounts of vascular endothelial growth factor (VEGF) (Myoken *et al.*, 1991), promoting neovascularization (Melnyk *et al.*, 1996). Increased EGFR expression renders A431 cells less dependent upon an exogenous source of epidermal growth factor (EGF) and enhances the EGF-induced mitogenic responses of squamous cell carcinoma cell lines compared with human epidermal keratinocytes, contributing to the invasiveness of malignant cells (Malliri *et al.*, 1998). In the presence of a chemotatic stimulus (FCS) in the lower part of the Boyden migration chamber, A431 cells migrated through the pores to the lower surface of the membrane (Fig 3.11). MGX from chestnut significantly reduced cell migration. Compared with untreated control cells, migration of A431 was significantly decreased by 68% ($p<0.05$) and 99% ($p<0.05$) in the presence of 5 µM and 50 µM MGX, respectively.

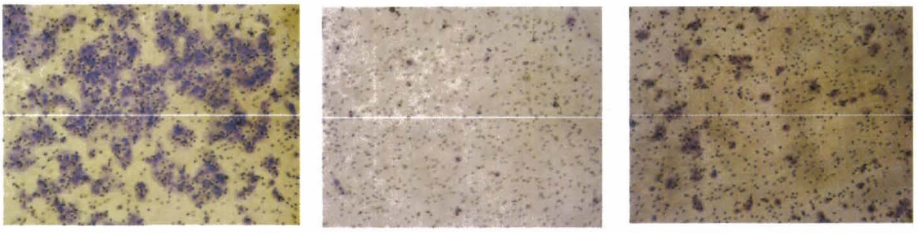


Fig 3.11. Effects of chestnut MGX on the migration of A431 cells seeded on a fibronectin matrix in the upper chamber: 10% FCS was added to the lower chamber. Fewer cells migrated to the lower chamber in presence of this xylan. Migration of A431 cells was inhibited by 68% and 99%, in presence of 5 and 50 µM MGX, respectively. Original magnification x 200 (Moine *et al.*, 2007)

A Matrigel invasion assay was performed to study the effect of MGX (5 µm and 50 µM) on the invasive ability of A431 cells. MGX at 5 µM did not reduce the invasion of tumor cells, while at 50 µM (corresponding to the IC_{50}) this xylan reduced the invasion of A431 cells by 55% as compared with untreated control cells (Fig 3.12). Cell migration that takes place during angiogenesis requires the degradation of the extracellular matrix by proteases as matrix metalloproteases (MMP) (Hessig *et al.*, 2003). Since MGX inhibited migration and the invasion of A431 cells, zymography was used to determine whether this compound affects the secretion of MMP2 and MMP9 gelatinases by A431 cells. As shown by the zymogram in Fig 3.13, untreated A431 cells secreted ProMMP2, ProMMP9 and active forms of MMP9. Exposure of these cells to 12.5 µM MGX resulted in a significant time-dependent inhibition of ProMMP2 and ProMMP9. Analysis by quantitative zymography indicated that the amounts of ProMMP secreted in the medium (normalized to cell number) after 48 h and 72 h of treatment, respectively decreased by 17% and 56% (ProMMP2), and by 50% (ProMMP9). Expression of MMP9 was totally abolished in 24 h treated cells as compared to control cells. The anti-migration and anti-proliferative effects of MGX can therefore be explained, as least in part, by a decrease of MMP2 and MMP9 expressions.

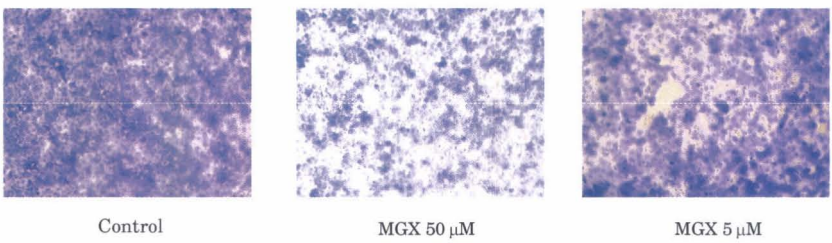


Fig 3.12. Effects of chestnut MGX on the invasion of A431 cells seeded on a Matrigel basement membrane matrix in the upper chamber: 10% FCS was added to the lower chamber. Fewer cells migrated to the lower chamber in presence of MGX. Invasion by A 431 cells was inhibited by 55% with MGX at 50 µM. No effect of MGX at 5 µM on cell invasion was demonstrated. Original magnification x 200 (Moine *et al.*, 2007)

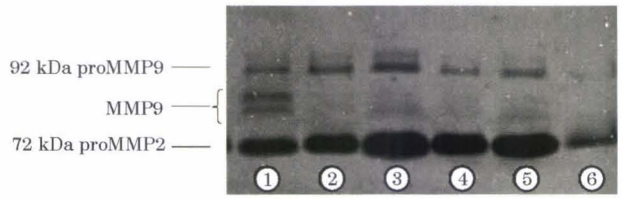


Fig 3.13. Effects of chestnut MGX on ProMMP9, MMP9 and ProMMP2 secretion by A431 cells. Gelatin zymography of serum-free conditioned media from A431 cell cultures. Conditioned media from untreated cell cultures (lanes 1, 3, and 5) or cell cultures treated with 12.5 µM MGX (lanes 2, 4, and 6) were collected after incubation, normalized to cell number, and subjected to gelatin zymography. Incubation times: 24 h (lanes 1 and 2), 48 h (lanes 3 and 4), 72 h (lanes 5 and 6); from Moine *et al.*, 2007

To study the cellular mechanism underlying chestnut MGX-induced A431 cell proliferation inhibition, we then performed cell-cycle analysis and apoptosis detection. MGX at different concentrations (50, 75 and 100 µM) did not exhibit any inhibitory effect on the different phases of cell cycle as compared with untreated control cells. Incubation of A431 cells for 72 h with different concentrations of MGX (50, 75 and 100 µM), induced apoptosis in a dose-dependent manner (respectively 12, 19, and 21%), as compared to untreated cells (5% of apoptotic cells). After 3 days of treatment, additional apoptosis features were observed in A431-treated cells, such as peripheral cytoplasmic budding and the appearance of apoptotic bodies. Then, inhibition of A431 cell proliferation would be most likely related to an induction of apoptosis rather than to cell cycle inhibition.

These results demonstrate that MGX from chestnut tree inhibits proliferation, invasion and migration of highly invasive A431 tumor cells and possibly kills tumor cell by induction of apoptosis. Taken together, our results suggest that this MGX could be very efficient in eradicating aggressive tumors but the exact mode of interaction between glucuronoxylans and tumor cells remains unknown. A number of suggestions have been proposed to explain the biological properties of pectins (Paulsen & Barsett, 2005) and β–(1→3) glucans (Kulicke *et al.*, 1997). These proposals refer to the type of glycosidic linkages of the polymer backbone and/or its ramifications, along with their degree of branching, molecular mass, polyanionic characteristics, or helical structure (Gloaguen & Krausz, 2004). It is now accepted that not only the primary structure but also the whole set of chemical and physicochemical properties, as well as supramolecular structural features may altogether contribute to the expression of the biological properties of these polymers. To amplify this structure-function relationship, we initiated a study on their three-tridimensional structure, from which xylans appeared as highly organized helical macromolecules (Fig 3.14) (Mazeau *et al.*, 2005).

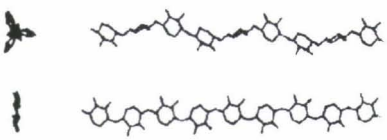


Fig 3.14. Conformational analysis of xylans. Side and top view of a xylan helix (Mazeau *et al.*, 2005)

The role played by 4-*O*-methylglucuronic acid substitutions in the establishment of tri-dimensional structure of 4-*O*-methylglucuronoxylans remains to be specified and additional work is needed in order to identify the carbohydrate sequence(s) and/or the chemical group(s) responsible for the observed biological properties. To this end, we undertook a study with the aim to correlate the structural characteristics of a few MGX - in term of their 4-*O*-methylglucuronic acid content and distribution as well as their molecular mass – to their influence on A431 cell proliferation, invasion and migration; two MGX were extracted from chestnut sawdust (MGX C_1 and C_2, non-impregnated and impregnated extracts, respectively) and one another from the pericarp of argan (*Argania spinosa*) seeds called MGX A. Homoxylan (HX) extracted from the pericarp of argan tree seeds was considered as a neutral model.

Comparison of the Biological Properties of MGX from Different Sources: Seeking Bio-Active Patterns

Mass polydispersity of both MGX and HX extracts were studied by means of SEC-HPLC (Fig 3.16). In the chromatographic conditions used for filtration, chestnut and argan MGX as well as argan HX were eluted as narrow peaks - giving evidence of good molecular mass homogeneity (Fig 3.15). Based on colorimetric estimation, the ratio of reducing sugar to total carbohydrate suggests that average degrees of polymerisation (DP) of MGX range from 182 (chestnut) to 340 (argan). HX from argan with a DP value of 360, presented the highest molecular mass.

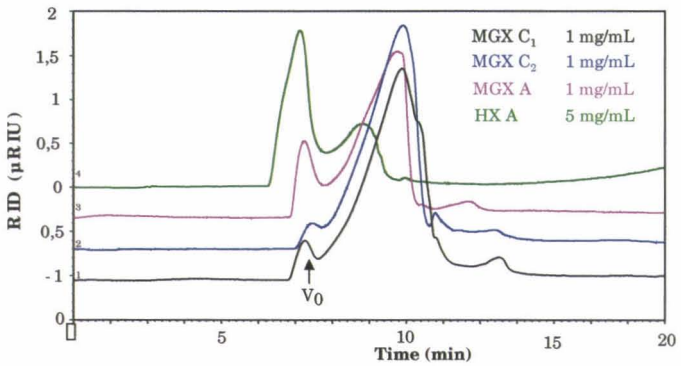


Fig 3.15. Superposition of HPLC elution profiles of MGXs and HX. V_0: Void volume (Barbat *et al.*, 2008)

From a quantitative point of view, xylans were isolated according to the procedure described in Fig 3.3 in mass yields ranging from 12 (MGX C_2) to 19% (MGX C_1) (Table 2). The water insoluble fraction from argan pericarp, with a xylosyl molar ratio higher than 98.6% (Table 2), presents the typical composition of a homoxylan-type polysaccharide (HX). With their xylosyl and 4-*O*-methylglucuronic acid contents ranging from 76% to 86% and 13% to 14.6%, respectively (Table 2), KOH extracts from argan pericarp (MGX A) and chestnut sawdust (MGX C_1 and MGX C_2) were characteristic of 4-*O*-methylglucuronoxylan-type polysaccharides. The percentage of uronic acid was also confirmed by ^{1}H NMR spectroscopic analysis (Barbat *et al.*, 2008). Traces of Rha, Ara, Man, Glc, Gal and GalA were also detected and could be considered as contaminants.

To investigate the effects of xylans on cell proliferation, A431 tumor cells were treated with xylan extracts at 0.7 and 50 µM (Table 2), the chestnut MGX extracts significantly inhibited tumor cell proliferation. Nevertheless, the concentration inducing 50% of maximal inhibition (IC_{50}) was reached with MGX C_1 only. With respectively 29 and 19% inhibition of tumoral cell proliferation, MGX and HX extracts from argan presented lower bioactivity.

In the presence of a chemotatic stimulus (FCS) in the lower part of the Boyden migration chamber, A431 cells migrated through the pores to the lower surface of the membrane; this migration was found to be strongly inhibited by C_1 and C_2 MGX from chestnut. Compared with untreated control cells, migration of A431 was significantly decreased up to 99% ($p<0.05$) in the presence of 50 µM of MGX C_1 (Table 2). A Matrigel invasion assay was performed to study the effect of these MGX extracts on the invasive ability of A431 cells. At a concentration of 50 µM (corresponding to the IC_{50}), with up to 72% of inhibition (case of MGX C_2), both extracts significantly reduced the invasive ability of A431 cells as compared with untreated control cells (Table 2).

Since MGX inhibited migration and invasion of A431 cells, we tested the influence of this compound on the secretion of MMP9 and MMP2 gelatinases by A431 cells. Exposure of these cells to 12.5 µM MGX C_1 resulted in a significant inhibition of ProMMP9 and ProMMP2. Analysis by quantitative zymography indicated that the amount of ProMMP secreted in the medium and normalized to cell number, decreased by 56% for ProMMP2 and by 50% for ProMMP9. Expression of MMP9 was totally abolished in 24 h treated cells as compared to control cells. Therefore, the anti-migration and anti-proliferative effects of MGX C_1 can be explained, as least in part, by a decrease of MMP9 and MMP2 expression. On the opposite, MGX C_2 extract at the same concentration induced lower or non-significant inhibition of MMP9, ProMMP2 and ProMMP9 expressions (10, 39 and 0%, respectively).

Ebringerová and co-workers (2002) suggested that the structural variability of xylans may affect their biological properties. Such structural variability may originate from the degree of polymerization (DP) of the polysaccharide (hence its molecular mass), the monosaccharidic composition (and especially the typical Xyl/MeGlcA ratio) as well as the random or regular distribution of MeGlcA substituents along the xylosyl backbone. The latter feature, reflecting the fine structure of the macromolecular chains, may affect the intermolecular interactions between xylan molecules in solution, creating networks or microgels, as well as interactions with biopolymers located on the surface layers of A431 cell coat (Ebringerová *et al.*, 2002). This led us to investigate the distribution of MeGlcA units in MGX C_1, MGX C_2 and MGX A by means of MALDI-MS analysis, after their degradation by autohydrolysis. Applied to chestnut and argan MGX extracts, autohydrolysis led to their degradation into characteristic oligosaccharides comprising of 4-*O*-methylglucuronic acid (GA) and xylosyl (X) residues whose molecular masses, from MALDI mass spectra, could be assigned to typical X_n, X_nGA or X_nGA_2 (n=1 to 14) oligosaccharides (Fig 3.16). Fig 3.16, the mass data are represented as graphs, in which the height of a given species is deduced from the relative abundance of the corresponding m/z ion (about 250 laser shots were averaged in each mass spectrum, in order to reach a total ion current of about 2.5 10^3 and to get a representative view of the spot). The mass profiles of glucurono-xylooligosaccharides obtained from MGX C_2 and MGX A were quite similar: a Gaussian distribution was observed, centered on the DP 5 to 7 species. Two series of abundant species were observed in both cases, corresponding to non-substituted (X_n) or mono-substituted (X_nGA) forms. Additionally, the presence of large amounts of 4-*O*-methylglucuronic acid di-substitutions mainly of the X_2GA_2 types as well as X_n mainly of the X_2 to X_{12} forms reveals an irregular distribution of GA already suggested by Jacob (2001) for hardwood MGX. In contrast, in the case of MGX C_1 obtained from non-impregnated chestnut sawdust, glucurono-xylooligosaccharides were found to be significantly shorter: X_1, X_2, X_3, XGA and X_2GA being the most abundant species. The $XnGA_2$ series is also present but in very low amount suggesting in this case a more regular distribution of GA units along the xylan backbone. Of interest is the structural difference deduced from the MALDI-MS spectra of MGX C_1 and MGX C_2 obtained from autohydrolysis of non impregnated or impregnated chestnut sawdust respectively. Such differences might be explained by the typical extraction protocols used. As suggested by Dahlman *et al.* (2003), xylan molecules are distributed from the inner to the outer layers of hardwood pulps and their structural characteristics (molar mass, uronic acids content) differ according to their location and also to the pulping process used (cooking, bleaching, delignification). From our results, it could be assumed that a preliminary KOH impregnation step favors the extraction

Table 3.2. Extraction yields and monosaccharide composition of alkaline extracts (Barbat *et al.*, 2008)

	Extraction yields%W/W	Rha	Ara	Xyl	Man	Glc	Gal	GalAU	GlcAU	4-O-Me GlcA
MGX C_1	19	1.8	0.8	**76.3**	0.8	1	2	2.1	0.6	**14.6**
MGX C_2	12	2	1.3	**78.4**	0.1	0.1	2.8	2.3	0	**13**
MGX A	19	0.9	0	**85.8**	0	0	0	0	0	**13.3**
HX	18	0.7	0	**98.6**	0	0	0	0	0	**0.7**

Table 3.3. Effects of chestnut and argan xylans on proliferation, migration and invasion of A431 cells and on MMP9, ProMMP2 and ProMMP9 expression by A431 cells. nd: not determined (Barbat *et al.*, 2008)

	Proliferation inhibition (%)		Migration inhibition (%)		Invasion inhibition (%)		12,5 µM Zymography		
	0.7 µM	50 µM	5 µM	50 µM	5 µM	50 µM	MMP9 inhibition (%)	ProMMP2 inhibition (%)	ProMMP9 inhibition (%)
MGX C_1	35	51	68	99	0	55	100	56	50
MGX C_2	18	47	50	55	72	72	10	39	0
MGX A	29	29	nd	nd	nd	nd	nd	nd	nd
HX	0	19	nd	nd	nd	nd	nd	nd	nd

of a second class of MGX - *with an irregular distribution of 4-O-methylglucuronic acid units* - closely associated with the cell wall through ester linkage with residual phenolic compounds (Takahashi & Koshijima, 1988).

Comparison of biological responses obtained from the tested neutral HX and acidic MGX revealed some interesting relation between DP, xylosyl to MeGA ratio, MeGA distribution pattern through the xylosyl backbone and cytotoxic properties. The argan neutral HX, characterized by a high DP and absence of MeGA substituent, is devoid of cytotoxic activity towards A431 cells (Table 3.4). This is also the case for argan MGX acidic form. Then, the presence of MeGA could not be considered as a key determinant. The case of MGX C_1 and MGX C_2 extracted from chestnut tree is of particular interest (Table 4). With similar DP values and xylosyl to MeGA ratios, these extracts did not present the same level of A431 cytotoxic activity. IC_{50} value could only be reached with the non-impregnated MGX C_1 chestnut sawdust extract. The latter is the one that presents a regular distribution of MeGA substituents through the xylosyl backbone. In another direction, the monosaccharidic compositions obtained from argan MGX A and impregnated chestnut MGX C_2 extracts as well as their characteristic MeGA distribution through the xylosyl backbone are quite similar. Nevertheless MGX A which presents a lower A431 cytotoxic activity is also characterized by a higher DP value. In this case, the high DP value seems to have a negative influence on its biological activity.

Table 3.4. Contents and distribution of 4-*O*-methylglucuronic acids values, degrees of polymerization and IC_{50} of extracted xylans. Nr: not reached (Barbat *et al.*, 2008)

	Xyl/MeGlcA ratio (^{1}H NMR)	Distribution of acids	DP	IC_{50}
MGX C_1	5.9/1	regular	200	50 µM
MGX C_2	6.1/1	random	182	Nr
MGX A	4.8/1	random	340	Nr
HX	/	/	360	Nr

From our data, we could speculate that the distribution of MeGA and the degree of polymerization are key structural determinants of the biological activity of xylans. As we said before, the degree of polymerization could influence the three-dimensional structure of xylans and especially their helical organization. The influences of higher DP, variability of MeGA substitution pattern along the xylan helical conformation as well as the identification of carbohydrate sequence responsible for the A431 cytotoxic properties are the next steps that could improve our understanding of the biological properties of xylan-type hemicelluloses.

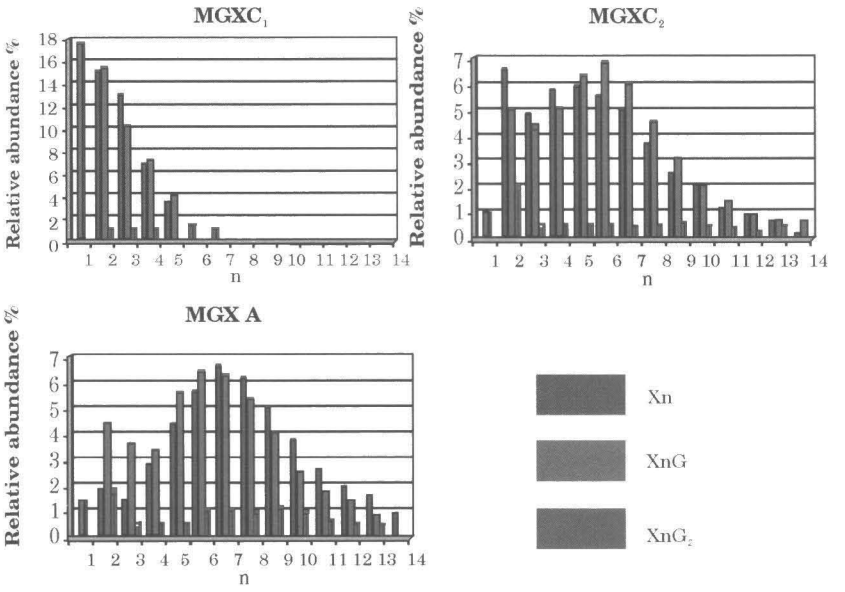


Fig 3.16. Distribution of uronic acids in xylans from chestnut and argan after autohydrolysis and MALDI mass spectrometry analysis. Mass data are represented as graphs, where bar height corresponds to the relative abundance of the corresponding ion (Barbat *et al.*, 2008)

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4

Biologically Active Naphthaquinones from Nature

VINOTHKUMAR S.P.[1] AND GUPTA JAYANTA KUMAR[1,*]

ABSTRACT

Secondary metabolites having 1,4- and 1,2-naphthoquinone moieties are widespread in nature and many of those play important and interesting role in numerous physiological and pharmacological activities in plants and animals which include cellular respiration, photosynthesis, cellular defence against bacteria, fungi and parasites etc. in one hand and antitumour, antineoplastic, trypanocidal, anti-inflammatory, antiviral, antifungal on the other. Thus, the naphthaquinone juglone (5-hydroxy-1,4-naphthaquinone), is known to be involved in pathogenic defence mechanisms in plants, but it may also take part in normal developmental processes. Lapachol (2-hydroxy-3-prenyl-1,4-naphthaquinone) is active against certain carcinoma and sarcoma whereas β-allyl lapachone, a 1,2-naphthaquinone derivative of Lapachol exhibits trypanocidal activity. Certain naturally occurring biquinones and higher quinone oligomers having two or more quinone units linked together at the quinone double bonds possess a diverse array of biological activities. Thus, Conocurvone, isolated from the Western Australian smoke bush, acts as a dual inhibitor of HIV integrase, an enzyme essential for the specific viral life cycle and HIV mediated cell fusion. A varied group of naphthaquinones and some other related quinone derivatives from plants have been presented.

Key words : Antibacterial, anticancer, cytotoxic, ichthyotoxic, leishmanicidal, naphthoquinone, plumbagin, topoisomerase

INTRODUCTION

Compounds containing quinone moiety, as per literature, are of varied nature which include derivatives of benzoquinone, 1,2- and 1,4-naphthoquinone,

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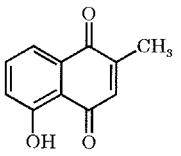
anthraquinone, binaphthaquinone, terpenoid type quinone and many others. They are widely distributed as secondary metabolites in plants, animals, organisms of marine origin like algae, sponges etc. and also in various microbial sources even. Quinones and their easily hydrogenated hydroquinone form, as a class are biochemically important compounds functioning as oxidizing and reducing agents and they play vital roles in the biochemical reactions in side the living cells. While the K series of vitamins are probably the best known quinones from nature, the Vitamin E is a hydroquinone derivative acting as antioxidant while ubiquinones are compounds which are involved in electron transport in mitochondria.

A substantial proportion of the collection of quinones from literature include derivatives of 1,4-naphthaquinones mostly. This class of quinones have been found to be frequently studied because of their wide spectrum of biological activities such as antitumor, anti-inflammatory, antiparasitic, wound healing, antimalarial etc. which have been attempted to be exploited. In this review, information on 1,4- naphthaquinones only have been included in a tabular form.

S. No.	Structure	Plant name \Family	Biological activity
1.	 Plumbagin		*In vitro* leishmanicidal activity Oliver *et al.* (2000)
		Dionacea muscipula	Topoisomerase II-mediated DNA cleavage activity. Cytotoxicity, Antimicrobial activity Noboru *et al.* (1992)
		Diospyros maritima Blume (Ebenaceae)	Lchthyotoxicity, germination inhibition activity Matsutake *et al.* (2002)
		Plumbago zeylanica. L. (Plumaginaceae)	Anti-*Helicobacter pylori*, Bacterial activity Ynan-Chuen Wang *et al.* (2005)
		Diospyros maritima Blume (Ebenaceae)	Cytotoxicity against Lul, LNCap, HuVEC. Antimicrobial activity Jian-Qiao Gu *et al.* (2004)
		Plumbago scandens (*Plumbago*) species	Antimicrobial activity Selma Ribeiro de *et al.* (2003)

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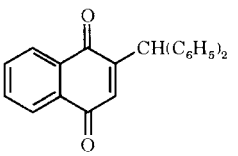
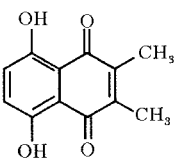
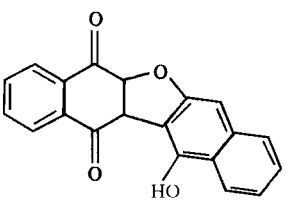
		Plumbago zeylanica. L. (Plumaginaceae)	Cytotoxic activity against Raji, Calul, Hcla & wish tumor cells Lie-Chwen Lin *et al.* (2003)
		Drosera rotundifolia.L.	—— Terttu Kamarainen *et al.* (2003)
		Diospyros maritima Blume (Ebenaceae)	Cytotoxic and Antimicrobial activity Jian-Qiao Gu *et al.* (2004)
		Plumbago scandens	Antimicrobial activity Selma Ribeiro de *et al.* (2003)
		Plumbago zeylanica Linn. (*Plumbaginaceae*)	Cytotoxicity of Raji, Calu-1, HeLa, and wish tumor cell lines Lie-Chwen Lin *et al.* (2003)
		Nepenthes. rafflesiana Jack	—— Heiko Rischer *et al.* (2002)
2.	 2-(Diphenyl methyl)-naphthoquinone		*In vitro* leishmanicidal activity Oliver *et al.* (2000)
3.	 2,3-Dimethylnaphthazarin		*In vitro* leishmanicidal activity Oliver *et al.* (2000)
4.	 Brasanquinone		*In vitro* leishmanicidal activity Oliver *et al.* (2000)

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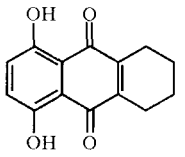
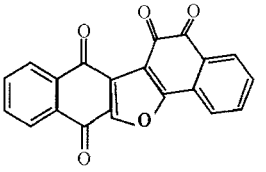
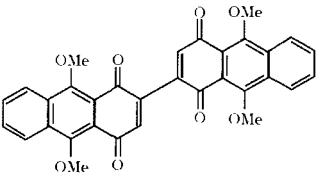
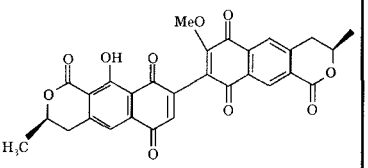
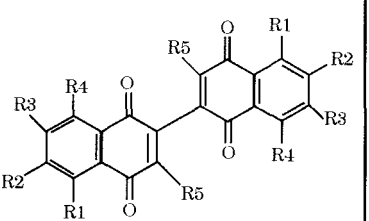
5.	 5,6,7,8-Tetrahydroquinizarin		*In vitro* leishmanicidal activity Oliver *et al.* (2000)
6.	 Dinaphtho(2,3,1', 2-d) furan-5,13,11,12-diquinone		*In vitro* leishmanicidal activity Oliver *et al.* (2000)
7.	 9,9',10,10'-tetramethoxy (2,2' bianthry) -1,4,1'4,-diquinone		*In vitro* leishmanicidal activity Oliver *et al.* (2000)
8.	 Xanthouegnin		*In vitro* leishmanicidal activity Oliver *et al.* (2000)
9.	 Dimeric naphthoquinone		*In vitro* leishmanicidal activity Oliver *et al.* (2000)

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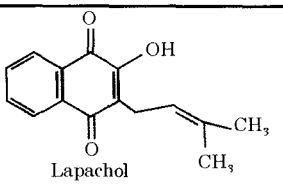
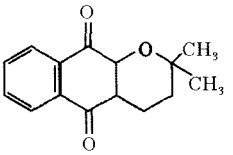
10.	 Lapachol	*Tabebuia avellanedae*	Anti microbial activity Eliezer Menezes Perira *et al.* (2006)
11.	 α Lapachone	*Dionacea muscipula*	Topoisomerase II-mediated DNA cleavage activity, Cytotoxicity, Antimicrobial activity Noboru Fujii *et al.* (1992)
		Species of *Tabebuia*	Active against walker 256 carcinoma yoshida sarcoma Alcides J.M.da Silva *et al.* (2002)
		Avicennia alba Blume and *Avicennia rumphiana* Hall.f. (Avicenniaceae)	Cancer chemopreventive activity Masataka Itoigawaa *et al.* (2001)
		Tabebuia avellanedae	Anti microbial activity Eliezer Menezes Pereira *et al.* (2006)
		Species of *Tabebuia*	Active against walker 256 carcinoma yoshida sarcoma Alcides J.M. da Silva *et al.* (2002)
		Tabebuia avellanedae	Induction of DNA topoisomerase-II mediated DNA cleavage Benjamin Frydman *et al.* (1997)
		Tabebuia avellanedae (Bignoneaceae)	Antimicrobial activity against multiresistant bacteria T.B. Machado *et al.* (2003)
		Ekmanianthe longiflora (Griseb.) Urb. (Bignoniaceae)	Significant cytotoxicity in a panel of human cancer Cells Sergio R. Peraza-Sa' nchez *et al.* (2002)

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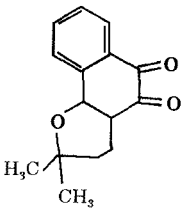
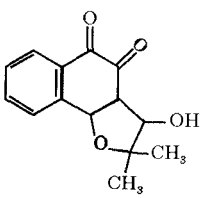
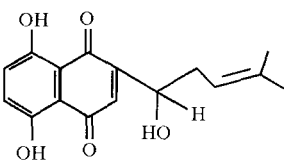
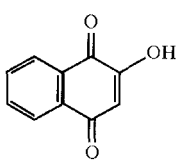
12.	 βlapachone β-allyl lapachone	*Tabebuia avellanedae*	Anti microbial activity Eliezer Menezes Perira *et al.* (2006)
		Diospyros morrisiana	Prototypic human topoisomerase I Chun-Yuan *et al.* (2003)
		Species of *Tabebuia*	Active against walker 256 carcinoma yoshida sarcoma, Trypanocidal activity Alcides J.M. da Silva *et al.* (2002)
		Tabebuia avellanedae	Induction of DNA topoisomerase -II mediated DNA cleavage Benhamin Frydman *et al.* (1997)
		Lantana involucrata	Cytotoxic activities against various human tumor cell lines Ken-ichiro Hayashi *et al.* (2004)
13.	 3-hydroxy-beta-N-lapachone	*Tabebuia avellanedae*	Anti microbial activity Eliezer Menezes Pereira *et al.* (2006)
14.	 Shikonin	*Dionacea muscipula*	Topoisomerase II-mediated DNA cleavage activity, Cytotoxicity, Antimicrobial activity Noboru Fujii, L. *et al.* (1992)
		Lithospermum erythrorhizon	Anti-inflammatory Antifungal activity Kenroh Sasaki *et al.* (2002)
15.	 Lawson	*Dionacea muscipula*	Topoisomerase II-mediated DNA cleavage activity, Cytotoxicity, Actimicrobial activity Noboru Fujii, L. *et al.* (1992)

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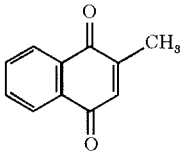
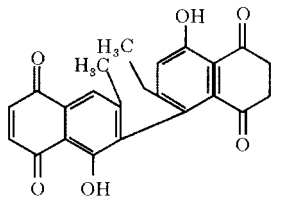
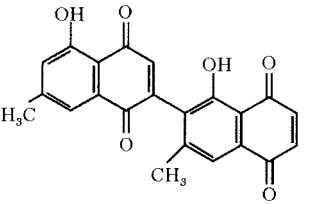
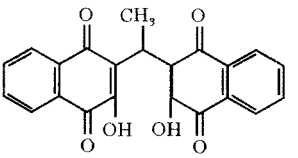
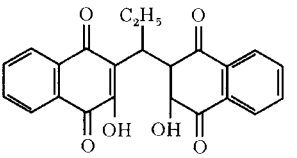
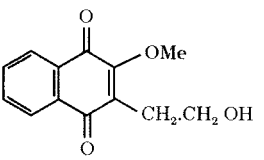
16.	 Vitamin K_3	*Dionacea muscipula*	Topoisomerase II-mediated DNA cleavage activity, Cytotoxicity, Actimicrobial activity Noboru Fujii, L. *et al.* (1992)
17.	 Isodiospyrin	*Diospyros morrisiana*	Prototopic human topoisomerase I Chun-YuanTing *et al.* (2003)
18.	 Diospyrin	*Diospyros morrisiana*	Prototypic human topoisomerase I Chun-Yuan Ting *et al.* (2003)
		Diospyros montana Roxb.,	Antimycobacterial activity N. Lall *et al.* (2003)
19.	 2,2'-methylenebis(3-hydroxy1,4-naphthoquinone)	*Impatiens balsamina*	Antipruritic effects Hisae OKU *et al.* (2002)
20.	 2,2'-ethylidenebis(3-hydroxy1,4-naphthoquinone) (impatienol)	*Impatiens balsamina*	Antipruritic effects Hisae OKU *et al.* (2002)
21.	 Balsaquinone	*Impatiens balsamina*	Antipruritic effects Hisae OKU *et al.* (2002)
		Impatiens balsamina L. (Balsaminaceae).	Cyclooxygenase-2 Inhibitory activity Hisae OKU *et al.* (2002). 25(5)

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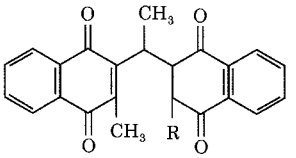
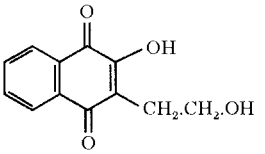
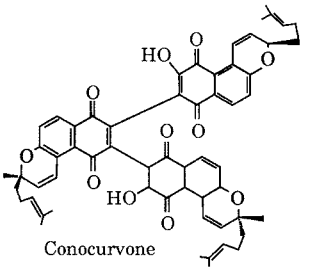
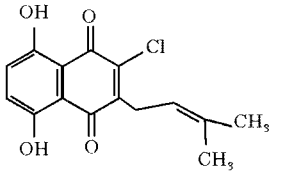
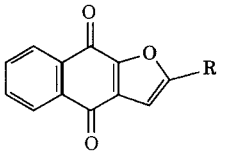
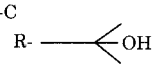
22.	 Impatienolate	*Impatiens balsamina* L. (Balsaminaceae).	Cyclooxygenase-2 Inhibitory activity Hisae OKU *et al.* (2002). 25(5)
23.	 2-hydroxy-3-(2-hydroxy ethyl)-1,4 -naphthoquinone	*Impatiens balsamina* L. (Balsaminaceae).	Cyclooxygenase-2 Inhibitory activity Hisae OKU *et al.* (2002). 25(5)
24.	 Conocurvone	Western Australian smoke bush	HIV inhibitory activity Kenneth W. *et al.* (2006)
25.	 Chlorosesamone (2-chloro-5,8-dihydroxy-3,3 (3-methyl-2-butenyt) 1,4-naphthoquinone	*Sesamum indicum* L. (Pedaliaceae)	——— A.F.M. Feroj Hasm *et al.* (2000)
26.	 Avicequinone-B (R-H) Acicequinone-C 	*Avicennia alba* Blume and *Avicennia rumphiana* Hall.f. (Avicenniaceae)	Cancer chemopreventive activity Masataka Itoigawaa *et al.* (2001)

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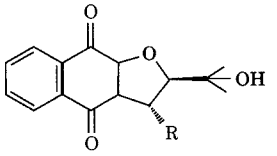
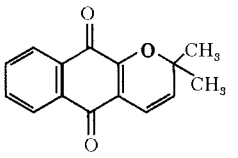
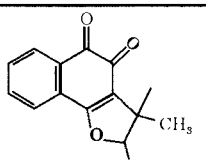
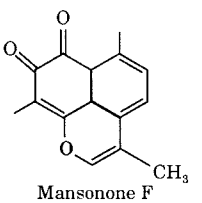
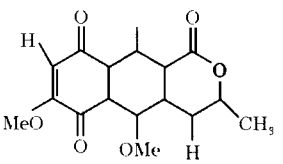
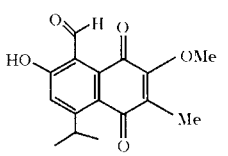
27.	 Stenocarpoquinone-B (R-H) Avicequinone (R-OH)	*Avicennia alba* Blume and *Avicennia rumphiana* Hall.f. (Avicenniaceae)	Cancer chemopreventive activity Masataka Itoigawaa *et al.* (2001)
28.	 Dehydro-alpha-lapachone	*Avicennia alba* Blume, *Avicennia rumphiana* Hall.f. (Avicenniaceae) and *Tabebuia avellanedae*	Cancer chemopreventive activity Masataka Itoigawaa *et al.* (2001) Induction of DNA topoisom erase-II mediated DNA cleavage Benjamin Frydman *et al.* (1997)
29.	 Dunnione	*Streptocarpus dunnii* (the Cape primrose)	Induction of DNA topoisomerase -II mediated DNA cleavage Benjamin Frydman *et al.* (1997)
30.	 Mansonone F	*Ulmus davidiana* *Ulmus Pumila.* L.	Potent anti-MRSA activity Young-Ger Suh *et al.* (2000) Cytotoxic effects, Antiproliferative effects. Dong Wang *el al.* (2004)
31.	 3.4.3',4'-bisdehydroxanthomegnir	*Paepalathus latipes*	Evaluated the *in vitro* cytotoxicity of the 1,4-naphthoquinone on McCoy cells using the microculture MTT-tetrazolium assay Rodrigo Rezende *et al.* (2004)
32.	 8-farmyl-7-hydroxy-5-isopropyl-2-methoxy -3-methyl1,4-naphthoquinone	*Bombax malabaricum* DC. (Bombacaceae)	—— Vijaya Bhaskar *et al.* (2003)

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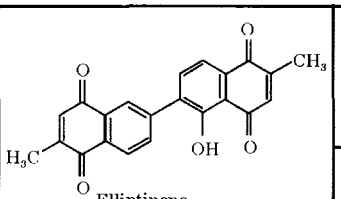
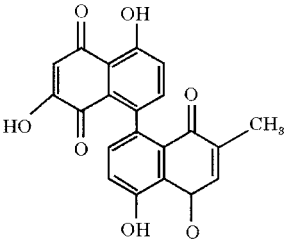
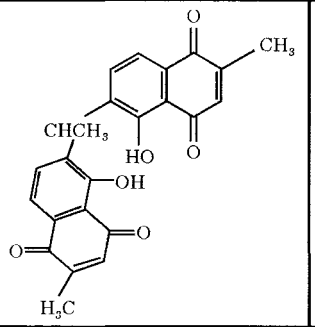
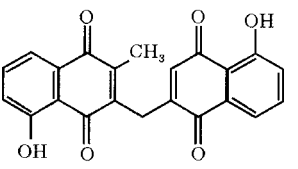
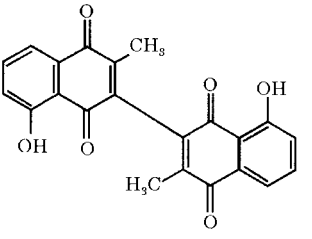
33.	 Elliptinone	*Diospyros maritima* Blume (Ebenaceae)	Ichthyotoxic activity germination inhibitory activity Matsutake *et al.* (2002)
		Plumbago zeylanica (Plumbaginaceae)	Cytotoxicity of Raji, Calu-1, HeLa, and Wish tumor cell lines
34.		*Diospyros maritima* Blume (Ebenaceae)	Ichthyotoxic activity germination inhibitory activity Matsutake HIGA *et al.* (2002)
35.		*Diospyros maritima* Blume (Ebenaceae)	Ichthyotoxic activity germination inhibitory activity Matsutake HIGA *et al.* (2002)
36.	 Isozeylanone	*Diospyros maritima* Blume (Ebenaceae)	Ichthyotoxic activity germination inhibitory activity Matsutake HIGA *et al.* (2002)
37.	 3,3'-biplumbagin	*Diospyros maritima* Blume (Ebenaceae)	Ichthyotoxic activity germination inhibitory activity Matsutake HIGA *et al.* (2002)

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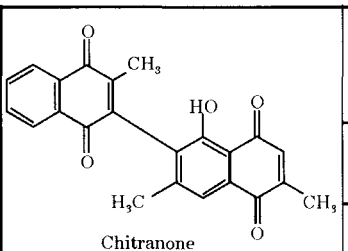
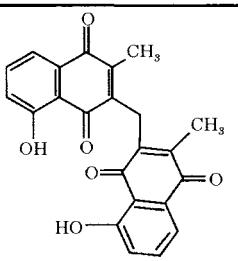
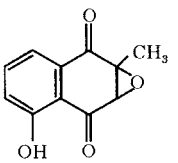
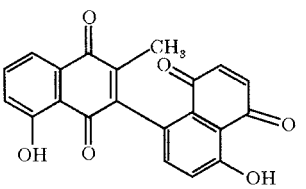
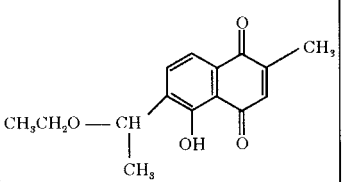
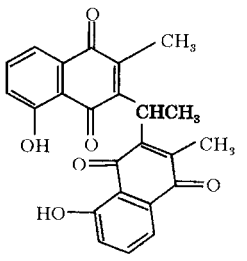
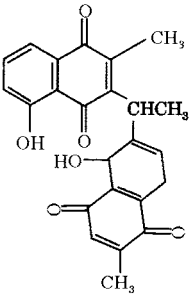
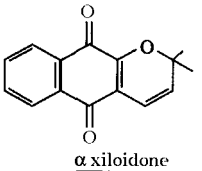
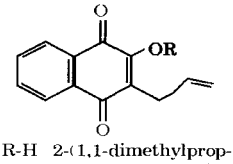
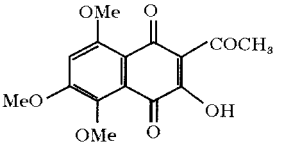
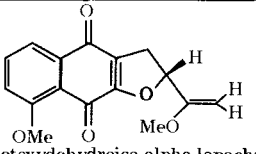
38.	 Chitranone	*Diospyros maritima* Blume (Ebenaceae)	Ichthyotoxic activity and germination inhibitory activity Matsutake HIGA *et al.* (2002)
		Diospyros maritima Blume (Ebenaceae)	Cytotoxic and Antimicrobial activity Jian-Qiao Gu *et al.* (2004)
		Plumbago Zeylanica Linn. (Plumbaginaceae)	Cytotoxicity of Raji, Calu-1, HeLa, and Wish tumor cell lines Lie-Chwen Lin *et al.* (2003)
39.	 Methyl-ene-3.3'-biplumbagin	*Diospyros maritima* Blume (Ebenaceae)	Ichthyotoxic activity germination inhibitory activity Matsutake *et al.* (2002)
40.	 2,3-epoxyplumbagin	*Diospyros maritima* Blume (Ebenaceae)	Ichthyotoxic activity germination inhibitory activity Matsutake *et al.* (2002)
41.	 3',8'-biplumbagin	*Diospyros maritima* Blume (Ebenaceae)	Ichthyotoxic activity germination inhibitory activity Matsutake *et al.* (2002)
42.	 6-(1-ethoxyetyl)plumbagin	*Diospyros maritima* Blume (Ebenaceae)	Ichthyotoxic activity and germination inhibitory activity Matsutake *et al.* (2002)

43.	 Ethylidene-3,3'-biplumbaglin	*Diospyros maritima* Blume (Ebenaceae)	Ichthyotoxic activity and germination inhibitory activity Matsutake *et al.* (2002)
44.	 Ethylidene-3,6'-biplumbaglin	*Diospyros maritima* Blume (Ebenaceae)	Ichthyotoxic activity and germination inhibitory activity Matsutake *et al.* (2002)
45.	 α xiloidone	*Tabebuia avellanedae* Bignoneaceae	Antimicrobial activity against multiresistant bacteria T.B. Machado *et al.* (2003)
46.	 R-H 2-(1,1-dimethylprop-2-enyl)-3-hydroxy-1,4-naphthoquienone R-COCH3 2-acetoxy-3-(1,1-dimethylprop-2-enyl)-1,4-naphthoquinone	*Calceolaria andina* L. (Scrophulariaceae)	Activities against homoptern and acarine species (pesticides) Bhupinder P.S. *et al.* (1999)
47.	 5(2-acetyl-3-hydroxy-5,6,8-trimethoxy-1,4-r	*Sideritis taurica.* (Lamiaceae)	Analgesic, Anti-inflammatory, Antiulccerogenic Antihyperglycaemic activities Aboutabl, M.I. Nassar *et al.* (2002)
48.	 8-metoxydehydroiso-alpha lapachon	*Catalpa ovata G.* Don (Bignoniaceae),	Exhibited significant inhibitory activity against 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells Aki Fujiwara *et al.* (1998)

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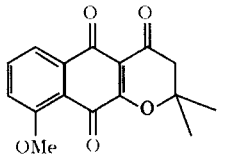
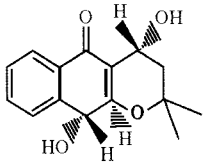
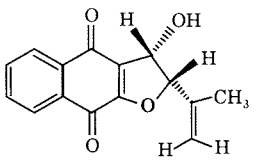
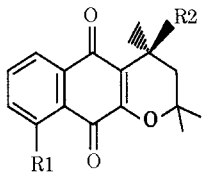
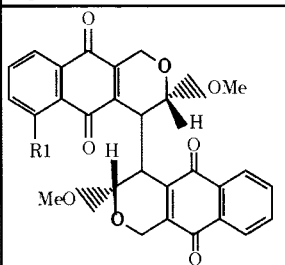
49.	 9-metoxy, 4-oxo-alpha lapachone	*Catalpa ovata G.* Don (Bignoniaceae),	Exhibited significant inhibitory activity against 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells Aki Fujiwara *et al.* (1998)
50.	 (4*S*,4a*R*,10*R*,10a*R*)-4,10-dihydroxy-2, 2-dimethyl-2,3,4,4α,10,10α hexahydrobenzo[*g*]chromen-5-one	*Catalpa ovata G.* Don (Bignoniaceae),	Exhibited significant inhibitory activity against 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells Aki Fujiwara *et al.* (1998)
51.	 3-Hydroxydehydroiso-alpha-lapachone	*Catalpa ovata G.* Don (Bignoniaceae),	Exhibited significant inhibitory activity against 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells
52.	 (R1, R2 = OH) 4,9-dihydroxy-α-lapachone (R1=H;R2=OH) 4-hydroxy-α-lapachone (R1=OCH3;R2=H)9-methoxy-α-lapachone	*Catalpa ovata G.* Don (Bignoniaceae),	Aki Fujiwara *et al.* (1998) Exhibited significant inhibitory activity against 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells
53.	 [(3α, 3□ α,4β,4□β)-3,3□]-dimethoxycis-[4,4□-bis(3,4,5,10-tetrahydro-1H-naphtho[2,3-c]pyran)]-5,5□,10,10□-tetraone	*Pentas longiflora* Oliver (Rubiaceae)	Aki Fujiwara *et al.* (1998) Samir El-Hady *et al.* (1999)

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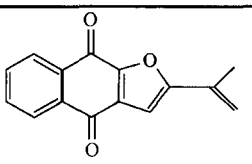
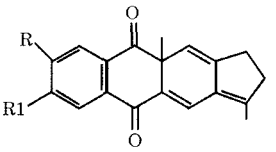
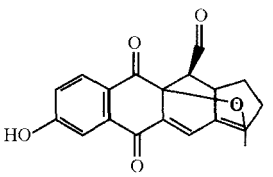
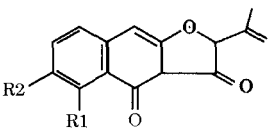
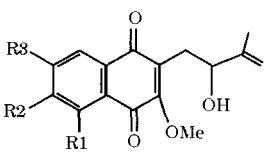
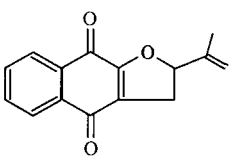
54.	 2-(1-hydroxyethyl)naphtho (2,3-b)furan4,9-dione	*Kigelia pinnata* (Bignoniaceae)	Antiplasmodial Drug Assay. *In vitro* drug activity against *P. falciparum* Cytotoxicity Assay using KB cells Claudia R. Weiss *et al.* (2000)
55.	 Kigelinol R-OH-R1-H)Kigelinol R-H;R1-OH)Iso kigelinol	*Kigelia pinnata* (Bignoniaceae)	Antiplasmodial Drug Assay. *In vitro* drug activity against *P.falciparum* Cytotoxicity Assay using KB cells Claudia R. Weiss *et al.* (2000)
56.	 Isopinatal	*Kigelia pinnata* (Bignoniaceae)	Antiplasmodial Drug Assay. *In vitro* drug activity against *P. falciparum* Cytotoxicity Assay using KB cells Claudia R. Weiss *et al.* (2000)
57.	 Lantalucratin A-R1-OCH3;R2-H, B-R1-OH;R2-H, C-R1-H:R2-OH, dehydroiso-β-laachone	*Lantana involucrata*	Cytotoxic activites against various human tumor cell lines Ken-ichiro Hayashi *et al.* (2002)
58.	 D-R1-OCH3,R2-H,R3-H,R4-H, E-R1-OCH3,R2-H,R3-H,R4-OH, F-R1-H,R2-OCH3,R3-OH,R4-OH	*Lantana involucrata*	Cytotoxic activies against various human tumor cell lines Ken-ichiro Hayashi *et al.* (2002)
59.	 Dehydroiso-alpha-lapachone	*Lantana involucrata*	Cytotoxic activies against various human tumor cell lines Ken-ichiro Hayashi *et al.* (2002)
		Ekmanianthe longiflora (Griseb.) Urb. (Bignoniaceae)	Significant cytotoxicity in a panel of human cancer Cells Sergio R. Peraza *et al.* (2000)

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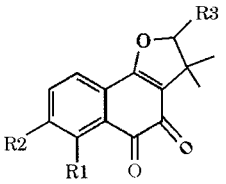
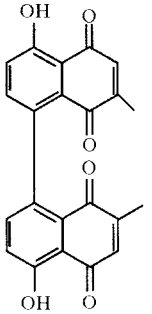
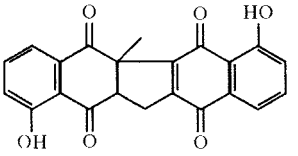
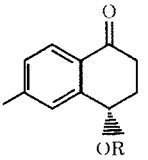
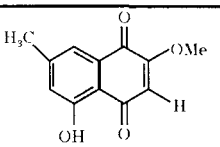
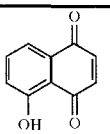
60.		*Lantana involucrata*	Cytotoxic activies against various human tumor cell lines Ken-ichiro Hayashi (2004)
61.	 Maritinone	*Diospyros maritima* Blume (Ebenaceae)	Cytotoxic and Antimicrobial activity Jian-Qiao Gu *et al.* (2004)
		Plumbago zeylanica Linn. (Plumbaginaceae)	Cytotoxicity of Raji, Calu-1, HeLa, and Wish tumor cell lines Lie-Chwen Lin *et al.* (2003)
62.	 Zeylanone	*Diospyros maritima* Blume (Ebenaceae)	Cytotoxic and Antimicrobial activity Jian-Qiao Gu *et al.* (2004)
63.	 Shinanolone	*Diospyros maritima* Blume (Ebenaceae)	Cytotoxic and Antimicrobial activity Jian-Qiao Gu *et al.* (2004)
		Plumbago zeylanica Linn. (Plumbaginaceae)	Cytotoxicity of Raji, Calu-1, HeLa, and Wish tumor cell lines Lie-chwen Lin *et al.* (2003)
		Nepenthes. rafflesi and jack	Heiko Rischer *et al.* (2002)
64.	 2-methoxy-7-methyyl juglone 3-methoxy-7-methyl juglone 7-methyljuglone	*Diospyros maritima* Blume (Ebenaceae)	Cytotoxic and Antimicrobial activity Jian-Qiao Gu *et al.* (2004)
65.	 Juglone 7-methyl juglone	———	Pathogenic defence mechanisms in plant Laurent Duroux *et al.* (1998)
		Drosera rotundifolia. L.	—— Terttu Kamarainen *et al.* (2003)

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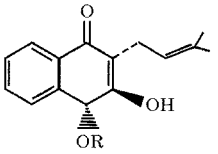
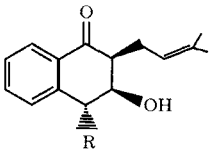
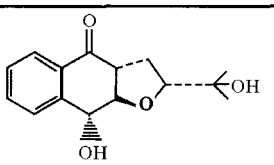
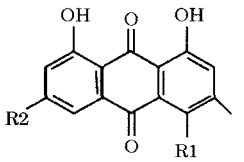
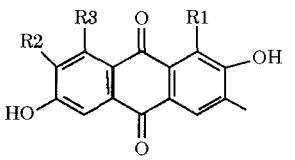
66.	 (2*R*,3*R*,4*R*)-3,4-dihydro-3,4-dihydroxy-2-(3-methyl-2-butenyl)-1(2*H*)-naphthalenone	*Ekmanianthe longiflora* (Griseb.) Urb. (Bignoniaceae)	Significant cytotoxicity in a panel of human cancer cells Sergio R. Peraza *et al.* (2000)
67.	 (2*S*,3*R*,4*R*)-3,4-dihydro-3,4-dihydroxy-2-(3-methyl-2-butenyl)-1(2*H*)-naphthalenone	*Ekmanianthe longiflora* (Griseb.) Urb. (Bignoniaceae)	Significant cytotoxicity in a panel of human cancer cells Sergio R. Peraza *et al.* (2000)
68.	 (2*R*+,3aR+,9*R**,9a*R**)-9-hydroxy-2-(1-hydroxy-1-methylethyl)-2,3,3a,4,9,9a-hexahydronaphtho [2,3-b]furan-4-one	*Ekmanianthe longiflora* (Griseb.) Urb. (Bignoniaceae)	Significant cytotoxicity in a panel of human cancer cells Sergio R. Peraza *et al.* (2000)
69.	 Islandicin-4-methylether **R1 R2** Chrysophanol H H Islandicin OH H Parictin OH OCH_3 Emodin H OH Catenarin OH OH	*Ventilago leiocarpa* Benth. (Rhamnaceae)	The cytotoxicity against HeLa, Vero, K562 Raji, Wish, and Calu-1 tumor cell lines Lie-Chwne Lin *et al.* (2001)
70.	 1,2,6-trihydroxy-7,8-dimethoxy, 3-methylanthraqe	*Ventilago leiocarpa* Benth. (Rhamnaceae)	The cytotoxicity against HeLa, Vero, K562 Raji, Wish, and Calu-1 tumor cell lines Lie-Chwne Lin *et al.* (2001)

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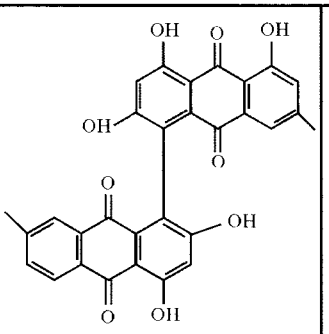
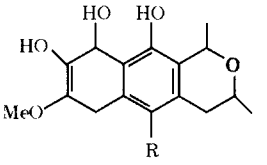
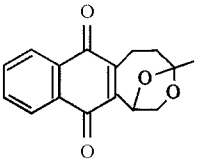
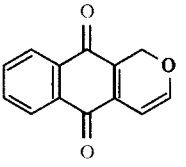
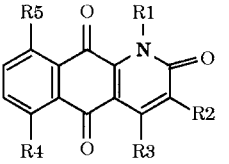
71.	 Skyrin	*Ventilago leiocarpa* Benth. (Rhamnaceae)	The cytotoxicity against HeLa, Vero, K562 Raji, Wish, and Calu-1 tumor cell lines Lie-Chwne Lin *et al.* (2001)
72.	 Ventilloquinone R= OMe-VentiloquiononeK R=H-Ventiloquinonel	*Ventilago leiocarpa* Benth. (Rhamnaceae)	The cytotoxicity against HeLa, Vero, K562 Raji, Wish, and Calu-1 tumor cell lines Lie-Chwne Lin *et al.* (2001)
73.	 Isagarin	*Pentas longiflora* Oliv. (Rubiaceae)	—— Luc Van Puyvelde *et al.* (1998)
74.	 Pentaiongin	*Pentas longiflora* Oliv. (Rubiaceae)	—— Luc Van Puyvelde *et al.* (1998)
75.	 **R1 R2 R3 R4 R5** Marcanin A H H CH_3 H H Dielsiquinone H OCH_3 CH_3 H H Marcanin B CH_3 OCH_3 CH_3 H H Marcanin C CH_3 OCH_3 CH_2OH H H Marcanin D H OCH_3 CH_3 OH H Marcanin E CH_3 OCH_3 CH_3 H OH	*Goniothalamus marcanii* Craib (Annonaceae)	Cytotoxicity against several human tumor cell lines, A-549, HT-29, MCF7, RPMI, and U251 Noppamas Soonthornchar-ecoon *et al.* (1999)

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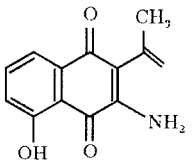
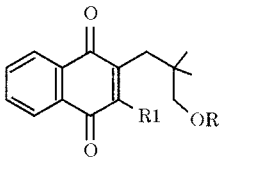
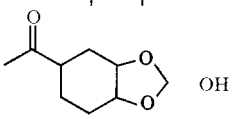
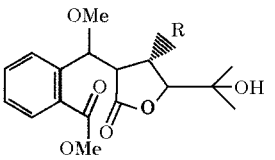
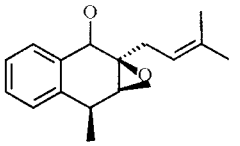
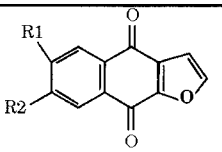
76.	 5-hydroxy-3-amino-2-aceto-1, 4-nahthoquinone	*Goniothalamus marcanii* Craib (Annonaceae)	Cytotoxicity against several human tumor cell lines, A-549, HT-29, MCF7, RPMI, and U251 Noppamas Soonthornchar-eonnon *et al.* (1999)
77.	 **R** **R1** Rhinacanthin-C OH  OH Rhinacanthin-D	*Rhinacanthus nasutus* (L). Kurz (Acanthaceae)	Exhibit inhibitory activity against cytomegalovirus Jian Lu Chen *et al.* (1996)
78.	 Avicennone Avicennone A = **R** - H Avicennone B = **R** - OH	*Avicennia marina* (Forsk.) Vierh., (Verbenaceae)	Strong antiproliferative and moderate cytotoxic activities, as well as antibacterial effects Li Han, Xueshi Huang *et al.* (2007)
79.	 Avicennone-C	*Avicennia marina* (Forsk.) Vierh., (Verbenaceae)	Strong antiproliferative and moderate cytotoxic activities, as well as antibacterial effects Li Han, Xueshi Huang *et al.* (2007)
80.	 Avicennone D Avicennone D R1 – OH, R2-H Avicennone E R1 – H, R2-OH	*Avicennia marina* (Forsk.) Vierh., (Verbenaceae)	Strong antiproliferative and moderate cytotoxic activities, as well as antibacterial effects Li Han, Xueshi Huang *et al.* (2007)

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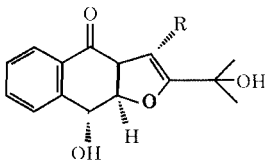
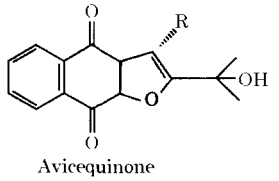
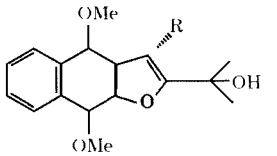
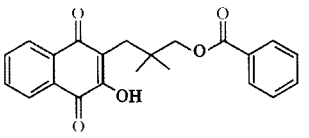
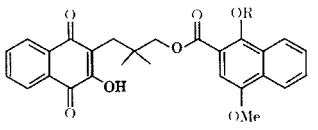
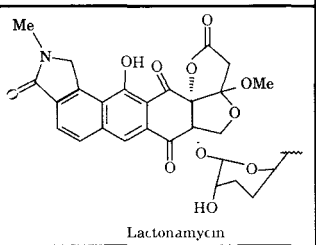
81.	 Avicennone - F Avicennone F R - H Avicennone G R - OH	*Avicennia marina* (Forsk.) Vierh., (Verbenaceae)	Strong antiproliferative and moderate cytotoxic activities, as well as antibacterial effects Li Han, Xueshi Huang *et al.* (2007)
82.	 Avicequinone Avicequinone A = R-OH Stenocarpoquinone B = R-H Avicequinone C = R-H (Δ^{23})	*Avicennia marina* (Forsk.) Vierh., (Verbenaceae)	Strong antiproliferative and moderate cytotoxic activities, as well as antibacterial effects Li Han, Xueshi Huang *et al.* (2007)
83.	 Avicenol	*Avicennia marina* (Forsk.) Vierh., (Verbenaceae)	Strong antiproliferative and moderate cytotoxic activities, as well as antibacterial effects Li Han, Xueshi Huang *et al.* (2007)
84.	 Rhinacanthin-M	*Rhiacanthus nasutus* (Acanthaceae)	Cytotoxicity against the cancer cell lines KB, HeLa, and HepG2 as well as against the normal Vero cell line Ngampong Kongkathip *et al.* (2004)
85.	 Rhinacanthin-N	*Rhiacanthus nasutus* (Acanthaceae)	Cytotoxicity against the cancer cell lines KB, HeLa, and HepG2 as well as against the normal Vero cell line Ngampong Kongkathip *et al.* (2004)
86.	 Lactonamycin	*Streptomycies rishirienes*	Cytotoxicity against cell lines Christopher Cox *et al.* (2001)

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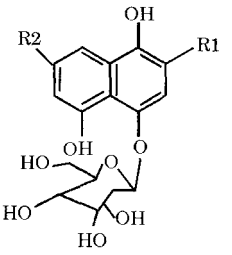
87.	 Rossoliside Rossoliside (7-methlhydrojuglone 4-O-glucoside) hydroplumbagin 4-O-glucoside 7-methyljuglone Plumbagin Plumbaside A = (R-H,R2-Me)	*Drosera rotundifolia* *Drosera spathulata* *D. intermedia* *Dionaea muscipula* (Droseraceae)	—— Jaromir budzianows *et al.* (1996)
		Nepenthes rafflesiana Jack	—— Heiko Risher *et al.* (2002)

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5

Biological Function of Glycoproteins

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ABSTRACT

It has been established that glycoproteins in the living system play an important role in the intracellular and extracellular membrane. Every molecule of glycoprotein has a dual function e.g. a protective function for itself and an attack function against other living systems. We speculate that their functions may be related to signals related to the survival or to attack functions. The plant-originated glycoproteins do not have any cytotoxic ability, but they have bioactive potential in respect of animal cells and edible nutritional values. In particular, their biofunctional activity is not related to their molecular weight, or to the component ratio between the carbohydrate moiety and the protein moiety, but varies on an individual basis. Nevertheless, many questions remain about the function of the whole molecule, the protein moiety, and the carbohydrate moiety separately. Also, are there different functions between O-linked and N-linked glycoproteins? Such different functions should be elucidated with glycoproteomics. Specifically, the bioactivity of glycoprotein may depend on the ratio between the carbohydrate moiety and the protein moiety in the whole molecule.

Key words : Plant glycoprotein, anti-oxidative effect, bioactivity

INTRODUCTION

The glycoprotein is a protein that contains carbohydrate moiety from <1% to >90% by weight. The carbohydrate portion of the glycoprotein can be strictly divided into two groups *e.g.* glycoprotein and proteoglycan.

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Glycoproteins are defined as conjugated proteins that contain, as a prosthetic group, one or more saccharides lacking a serial repeat unit and bound covalently to a peptide chain. That is, glycoproteins usually contain proportionally less carbohydrate than protein by weight. The proteoglycan contains as much as 95% or more carbohydrate, with properties that may resemble those of polysaccharides, rather than protein. Possible carbohydrates of glycoproteins include glucose, glucoseamine, galactose, galactoseamine, mannose, fucose, and sialic acid. The carbohydrate moieties of glycoproteins play important roles in protein folding in the endoplasmic reticulum, in the targeting of proteins for delivery to the appropriate intracellular components, and are recognition sites in the cell-cell interactions. The proteins in the glycoprotein to which carbohydrate chains have been added are usually secreted or localized to the cell surface, although some nuclear and cytosolic proteins are also glycosylated. Many other proteins secreted from cells into extracellular fluids are glycoproteins, such as hormones found in blood [follicle-stimulating hormone, luteinizing hormone, thyroid stimulating hormone, alpha-fetoprotein, and chorionic gonadotropin] and plasma proteins [orosomucoids, ceruloplasmin, plasminogen, prothrombin, and imunoglobulins], IgG, molecule of the histocompatibity complex (MHC), and zona pellucida of oocyte.

On Linkage of Saccharide to the Residue of Peptide, proteins in the natural glycoproteins are covalently linked oligosaccharide groups. Carbohydrate groups may be linked to polypeptide chains either via the hydroxyl groups of serine, threonine, or hydroxylysine residues (O-linked saccharide) or via the amide nitrogen of an asparagines residue (N-linked saccharide). O-linked saccharides are often found in the cell surface glycoprotein and also in mucins that coat and protect mucous membranes in the respiratory and gastrointestinal tracts. Certain O-linked saccharides in the intracellular plasma play a role of signal transmitter as nucleoplasmic glycoprotein. N-linked saccharides can affect the physical properties and functions of a protein, such as to help proteins fold, IgG, IgM, peptide hormones. O-linked oligosaccharides are synthesized in the Golgi apparatus by serial addition of monosaccharide unites to a completed polypeptide chain and post translationally formed. For example, in order for a protein modification to play an active role in signal transduction, O-GlcNAc needs to have certain key features. First, the modification needs to be dynamic. For the proteins that have been examined to date, the O-GlcNAc half-life is much shorter than that of the modified polypeptide chain (Hart, 1977). Second, the removal or attachment of the modification should be inducible by certain stimuli. O-GlcNAc modification of certain proteins is known to change in response to T cell activation, insulin signaling, glucose metabolism, and cell cycle progression (Roquemore *et al.*, 1996). Thus, O-GlcNAc displays features essential for a role in signal transduction. All animals and plants dynamically attach and remove O-linked β-*N*-acetylglucosamine (*O*-GlcNAc) at serine and threonine residues on myriad nuclear and cytoplasmic proteins.

O-GlcNAc cycling, which is tightly regulated by the concerted actions of two highly conserved enzymes, serves as a nutrient and stress sensor. On some proteins, *O*-GlcNAc competes directly with phosphate for serine/threonine residues. Glycosylation with *O*-GlcNAc modulates signalling, and influences protein expression, degradation and trafficking (Gerald *et al.,* 2007).

N-linked glycoproteins are synthesized in the endoplasmic reticulum and further processed in the Golgi apparatus. They occur in all forms of life and have functions that span the entire spectrum of protein activities including those of enzymes, transport proteins, receptors, hormones and structural proteins. Inside cells, they are found in specific organelles such as Golgi complexes, secretory granules, and lysosomes. They are rich in information, forming highly specific site for recognition and high-affinity binding by other proteins. The functions of glycoprotein in the cell membrane may have a critical role in the group behavior of cells and other important biological functions of the membrane. On the other hand, proteoglycans are mostly high molecular weight polyanionic compounds consisting of many different glycosaminoglycan chains linked covalently to protein core. They are predominantly the components of the extracellular matrics and cell surface, and play more dynamic roles in the cell adhesion and signaling. Proteoglycans modulate processes in cells and organisms *e.g.* to involve binding to other proteins, cell growth processes, and making cartilage flexible and resilient.

Here, we discuss the character of glycoprotein without consideration of the protein and carbohydrate moieties in the whole glycoprotein molecule. We are only interested in the functions of glycoprobins that already exist in the cells of living systems (animal and plant).

SOLUBLE GLYCOPROTEINS OFTEN SHOW A HIGH VISCOSITY

The absorption of glycoprotein is achieved by the following mechanism. Amino acid, peptide, and glucose are absorbed from intestinal lumen into the blood stream using a specific carrier because they have either a charge or polarity. Therefore, they can not permeate the membrane of intestinal wall against a concentration gradient and their vectorial transport is the combined result of several separate membrane events. A possible explanation of the mechanism of glycoprotein absorption is that it is firstly divided by proteoglycosylase into two parts, protein and carbohydrate, which are absorbed by carrier-mediated transport from the lumen into the blood stream in the small intestine. Although amino acid transport and peptide transport in the small intestine have different characteristics, they both use a carrier-mediated transporter cross the intestinal wall, such as ASCT-1, EEAT-3, and pep T1. On the other hand, glucose also uses carriers such as SGLT (sodium glucose transporter) and GLUT (glucose transporter) (Hediger & Rhoads, 1994). After permeation across the intestinal wall, amino acids and

glucoses are brought together into the entire molecule of glycoprotein. Then, the glycoprotein molecules are moved to the outside of liver cell membrane through the blood stream. The glycoprotein interacts with an extracellular receptor on the outside of the liver cell membrane. Their interaction consequently results in the transmission of a signal to the cytoplasm. Such a signal is further transmitted to activate a downstream signal transduction cascade resulting in the activation of specific transcriptional factors to express anti-inflammation. Generally speaking, glycoproteins in their natural form have a high hygrosopic character in the air, because of the air's high viscosity. Glycoproteins with a high molecular weight have a more biologically active function than proteins with a low molecular weight. The reason for this phenomenon is that the glycoprotein with the large carbohydrate component, such as lactose, fructose, galactose and mannose, binds to proteins that provide biological functions to the cell.

GLYCOPROTEIN FUNCTION

Glycoproteins have functions that span the entire range of protein activities, although the roles of their carbohydrate moieties are just beginning to be understood. Glycoproteins are also important mediators of cell-cell recognition and in many cases, are the receptors for bacterial attachment, via adhesins, in the initial stages of infection. The functions of glycoproteins in humans are of great interest. The glycoprotiens have critical roles in biological system such as cancer marker, hormone, and bioactive substance and so on.

Glycoprotiens as Markers of Colon Cancer

Carcinoembryonic antigen (CEA), known as tumor marker, is first described as a 180 kDa tumor-associated cell surface glycoprotein in colon cancer cells. A number of closely related, cross-reacting antigens have been found in normal cells. The CEA gene belongs to a family of at least 20 closely related genes which belong to the immunoglobulin gene superfamily (Thompson & Zimmermann, 1988). The CEA gene family consists of two major subgroups. The CEA subgroup contains gene that encode CEA, non-specific cross reacting antigen (NCA), and biliary glycoprotein (BGP), while the other subgroup consists of genes that encode the pregnancy-specific cross reacting antigen. Also, CEA is heavily glycosylated with 28 consensus sites for the addition of asparagine-linked carbohydrate structures, leading to a molecule with a bottle brush-like structure (Thompson & Zimmermann, 1988). CEA functions in several biological roles including homotypic and heterotypic (with other CEA family members) cell adhesion. The CEA can also inhibit the differentiation of several different cell types and contribute to tumorigenesis through the CEA-CEA interactions. Cell-cell interaction can be modulated by different factors, *e.g.* post-translational modifications

such as glycosylation. Recently, it has been shown that post-translational modifications of its hydrophobic carboxy-terminal region can block CEA-CEA binding via anchoring of a glycosyl-phosphatidylinositol linkage (Charbonneau & Stanners, 1999). Therefore, the modulation of glycosylation of CEA, such as the degree of glycosylation and the strength of adhesion by an agent, means that it can act as a potent inhibitor of CEA-mediated tumorigenesis in colon cells (Charbonneau & Stanners, 1999).

On the other hand, CEACAM1 (also known as biliary glycoprotein, C-CAM or CD66a) is a cell adhesion molecule of the CEA family that has been implicated in a number of physiological processes (*e.g.* tumor inhibitor in colon, prostate and breast cancer cells, and potent angiogenic factor in microvessel formation, and microbial receptor in epithelial cells) (Bamberger *et al.,* 2002). CEACAM1 expression is downregulated in colorectal carcinomas, indicating that loss of normal expression of CEACAM1 might play an important role in the pathogenesis of this tumor type. These findings have been recently corroborated by the finding that BGP is one of the 20 genes most frequently downregulated in colonic cancer (Zhang *et al.,* 1997). Furthermore, immunohistochemical studies have indicated that the CEACAM1 expression pattern is changed in malignant lesions of the human mammary gland (Riethdorf *et al.,* 1997). Recently, it has been reported that the association between the potential tumor-suppressor gene CEACAM1 and the cell-cycle inhibitory proteins Rb, Rb2, and p27 indicate the possibility of a functional link between cell adhesion/tissue architecture and cell-cycle regulation (Bamberger *et al.,* 2002). Loss of this link might lead to dysregulation of the proliferation process of breast cells, especially regarding regulation of proliferation by means of cellular contact and tissue architecture, leading to potentially aggressive proliferation. Early characterization of these parameters might thus prove to be a useful additional prognostic marker of tumor behavior (Bamberger *et al.,* 2002).

Glycoprotein as a Marker of Prostate Cancer

The Chromogranin A (CgA), one of the most abundant acidic glycoproteins ubiquitously present in neuroendocrine/endocrine cells, is a member of the secretogranin/chromogranin class of proteins present in the electron-dense granules of a variety of endocrine tissues and neurons. The CgA has been proposed to play a role in the packaging and the processing of hormones and neuropeptides, in the organization of the granular matrix and to act as a precursor of hormones and neuropeptides such as vasostatin I and II, chromostatin, pancreastatin and parastatin (Nagakawa *et al.,* 1999). CgA has also been found in neuroendocrine cells in several organs like breast, lung and the prostate gland. CgA is used as a general neuroendocrine marker to histochemically classify neuroendocrine tumors. Recently, it has been shown that the C-terminal of CgA peptides (322–364, 367–391) inhibited the growth of PC-3 (prohormone-converting enzymes) prostate cancer cells,

whereas the N-terminal of chromogranin A peptides increased it (Deftos & Granin, 1998). The plasma CgA level is the most useful marker to identify neuroendocrine differentiation during the progression of advanced prostate cancer. In addition, it has been reported that all patients with elevated CgA had aggressive hormone-resistant disease (Kadmon *et al.,* 1991). Therefore, these results indicate that the plasma CgA level was the most useful marker to identify neuroendocrine differentiation during the progression of advanced prostate cancer (Nagakawa *et al.,* 1999).

Zinc-α 2-glycoprotein: Zinc-α 2-glycoprotein (Znα2gp or ZAP) was initially purified from plasma; it can be precipitated by adding zinc ions and it displays electrophoretic mobility in the -region of the plasma globulins, hence its name. It has been detected in most body fluids, in the secretory epithelia of various human glands, and in the epidermis (Lei *et al.,* 1997). The molecular weight is in the range of 35–41 kDa, depending on variations in glycosylation among different tissues (mostly about 12–18% carbohydrate except that seminal plasma is unglycosylated). The amino acid sequence consists of a single polypeptide chain of 278 amino acids. The nucleotide sequence, ascribed to a single active gene and one or two pseudogenes, differs only in post-translational modifications for prostate, breast, and epidermis. ZAG accumulates in breast cyst fluids to 30- to 50-fold plasma concentrations (Sanchez *et al.,* 1997) and is overexpressed in 40–50% of breast carcinomas. Serial analysis of gene expression (SAGE) and microarray analysis have confirmed the overexpression of ZAG in breast cancer relative to normal mammary epithelium. In breast carcinomas, ZAG expression was found to correlate with tumor differentiation and did not independently affect prognosis. Recently, it was reported that zinc α-2-glycoprotein is expressed by malignant prostatic epithelium and may serve as a potential serum marker for prostate cancer (Hale *et al.,* 2001).

Glycoprotein as a Marker of Breast Cancer

Apolipoprotein D (apoD) is a 29-kDa glycoprotein that is primarily associated with high density lipoproteins in human plasma. It is an atypical apolipoprotein and based on its primary structure, apoD is predicted to be a member of the lipocalin family. Lipocalins adopt a beta-barrel tertiary structure and transport small hydrophobic ligands. Although apoD can bind cholesterol, progesterone, pregnenolone, bilirubin and arachidonic acid, it is unclear if any, or all of these, represent its physiological ligands. The apoD gene is expressed in many tissues, with high levels of expression in spleen, testes and brain. ApoD is present at high concentrations in the cyst fluid of women with gross cystic disease of the breast, a condition associated with increased risk of breast cancer. It also accumulates at sites of regenerating peripheral nerves and in the cerebrospinal fluid of patients with neurodegenerative conditions, such as Alzheimer's disease. ApoD may, therefore, participate in maintenance and repair within the central and

peripheral nervous systems. While its role in metabolism has yet to be defined, apoD is likely to be a multi-ligand, multi-functional transporter. It could transport a ligand from one cell to another within an organ, scavenge a ligand within an organ for transport to the blood or could transport a ligand from the circulation to specific cells within a tissue (Rassart *et al.*, 2000).

Recently, it has been shown that breast cancer cells are surrounded by a modified extracellular matrix (ECM) composed of a complex meshwork of collagens, fibrillar glycoproteins and proteoglycans which intercommunicate with the cell interior and thus modulate cell adhesion, proliferation and differentiation. Tenascin (TN) and fibronectin (FN) are glycoprotein components of the ECM which seem to have competitive functions. It can be speculated that this competitive relationship between these molecules is important for cellular functions. TN is a protein of the ECM that contains 14 repeats of the epidermal growth factor (EGF)-like domain. It is produced by stromal fibroblasts and also by epithelial cells of normal and malignant breast tissues and is expressed transiently during embryogenesis, inflammation and malignancy. FN is regarded as the major mesenchymal ECM glycoprotein involved in cell–matrix and cell–cell adhesion, cell migration, morphogenesis, differentiation and oncogene transformation. Tissue FN is found in the connective tissue in close apposition to the BMs. Studies of FN in breast carcinomas showed a strong expression and different distribution compared to normal breast parenchyma (Ioachim *et al.*, 1997).

In addition, Procathepsin D (pCD) is a major secreted glycoprotein in some human breast and other cancer cell lines. Several groups proposed that pCD served as a growth factor for these cell lines. Secreted pCD has been demonstrated in tissue section, tissue culture supernatants, carcinoma cytosols, and nipple aspirates. Moreover, several clinical studies suggested a potential role for this molecule in metastasis because its concentration in primary tumors correlated with an increased incidence of tumor metastases (Ioachim *et al.*, 2002).

P-glycoprotein for Multidrug Resistance

P-glycoprotein (P-gp) was first studied in the context of cancer research where its overexpression in tumor cells has been associated with the multidrug resistance (MDR) phenotype. In cancer cells, P-gp acts as an efflux pump that extrudes chemotherapeutic agents out of the cells, decreasing their intracellular concentration. This ATP-dependent transporter also exports a wide variety of structurally unrelated compounds such as vinka alkaloids, antibiotics, anthracyclines, etoposides, cytokines, opioids and steroids. P-gp is also expressed in normal tissues. High levels of P-gp expression have been observed in the endothelial cells of brain capillaries, in kidney and in adrenal glands, while moderate expression has been detected

in lung, liver and intestines (Demeule *et al.,* 2001). P-gp is involved in organism detoxification by excreting toxic compounds into the bile, urine and gastrointestinal tract (Ernest & Bello-Reuss, 1998; Trambas *et al.,* 1997). Moreover, it seems to play an important role in brain protection at the blood-brain barrier (Schinkel, 1999). Localized at the luminal side of endothelial cells in brain capillaries, P-gp prevents the passage of many drugs into the brain (Beaulieu *et al.*, 1997). Recently, many groups have studied the beneficial effects of natural products in cancer prevention or treatment. Studies have suggested that polyphenols from green tea could have chemopreventive, antiatherogenic, anticarcinogenic and antioxidant proprieties (Brown, 1999). Epidemiological studies suggest a chemopreventive effect of green tea against breast, prostate, esophagus, stomach, pancreas and colon cancers (Brown, 1999; Kim & Masuda, 1997; Mukhtar & Ahmad, 1999). Moreover, green tea consumption in animal models inhibits cancer angiogenesis (Cao & Cao, 1999) and metastasis (Chu & Juneja, 1997), and reduced tumor formation in skin, lung, liver, pancreas and the gastrointestinal tract (Brown, 1999; Kim & Masuda, 1997). However, nothing is known about the effects of polyphenols from green tea on the activity of P-gp. Green tea contains many polyphenolic compounds. Flavanols, also called catechins, are the major polyphenols found in green tea. Six catechins are present in green tea, the most abundant being (-)-epigallocatechin gallate (EGCG) followed by (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epicatechin (EC), (-)-catechin gallate (GC) and (+)-catechin (C) (Chu & Juneja, 1997).

Glycoproteins as Hormones

Reproduction and metabolism are under hormonal control. Human Glycoprotein hormones include thyrotropin (TSH), lutropin (LH), chorionic gonadotropin (hCG), follitropin (FSH), also referred to as the pregnancy hormone. These hormones bind to receptors on reproductive organs and the thyroid of men and women, regulating fertility and metabolism respectively. Mutations in the genes that code for glycoprotein hormones and their receptors can result in infertility and thyroid dysfunction (Smits *et al.*, 2003; Layman, 2000). Glycoprotein hormone receptors [thyrotropin (TSHr), luteinizing hormone/chorionic gonadotropin (LH/CGr), follicle stimulating hormone (FSHr)] are rhodopsin-like G protein-coupled receptors with a large extracellular N-terminal portion are responsible for hormone recognition and binding. In structural models, this ectodomain is composed of two cysteine clusters flanking nine leucine-rich repeats (LRRs). The LRRs form a succession of β-strands and α-helices organized into a horseshoe-shaped structure. It has been proposed that glycoprotein hormones interact with residues of the β-strands making the concave surface of the horseshoe. Gain-of-function homology scanning of the β-strands of glycoprotein hormone receptors allowed identification of the critical residues responsible for the

specificity towards human chorionic gonadotropin (hCG). Substitution of eight or two residues of the LH/CGr into the TSHr or FSHr, respectively, resulted in constructs displaying almost the same affinity and sensitivity for hCG as wild-type LH/CGr. Molecular dynamics simulations and additional site-directed mutagenesis provided a structural rationale for the evolution of binding specificity in this duplicated gene family (Dias & Van Roey, 2001; Ascoli *et al.*, 2002; Szkudlinski *et al.*, 2002; Cornelis *et al.*, 2001; Remy *et al.*, 2001; Schmidt *et al.*, 2001).

Glycoprotein as a Molecular Chaperone

Calnexin and calreticulin are molecular chaperones that are involved in the protein folding, assembly, and retention/retrieval. Calnexin (also known as IP 90 or p88) is a calcium-binding, endoplasmic reticulum (ER)-resident transmembrane protein which acts as a molecular chaperone for glycosylated and oligomeric proteins. Newly synthesized, incompletely folded, glycosylated soluble protein, calreticulin acts as a chaperone until they are correctly processed and folded. Mutant proteins, partially assembled oligomers in cell lines in which a component of the complex is not expressed, or proteins misfolded due to the incorporation of azetidine-2-carboxylic acid, display prolonged association with calnexin and their rates of secretion are slowed.

Calreticulin is a Ca2+-binding chaperone and a component of the calreticulin/calnexin pathway. Both calreticulin and calnexin act as lectins and molecular chaperones (Ellgaard *et al.*, 1999; Saito *et al.*, 1999; Ihara *et al.*, 1999), and they bind monoglucosylated proteins and associate with the thiol oxidoreductase ERp57, which promotes disulfide formation/ isomerization in glycoproteins (High *et al.*, 2000). Folding substrates associate transiently with calnexin and calreticulin and enter cycles of de-glucosylation/ re-glucosylation which plays an important role in their association with the chaperones. Calreticulin-deficiency is lethal in utero due to impaired cardiac development (Mesaeli *et al.*, 1999; Guo *et al.*, 2002).

Recently, it has been shown that Calreticulin and calnexin share extensive molecular homology, and there is considerable overlap between the substrate glycoproteins of calnexin and calreticulin, and they can associate with the same protein simultaneously or sequentially. Some proteins which associate with calnexin are: MHC class 1 molecules, T-cell receptor complex, the cystic fibrosis transmembrane conductance regulator and integrin chains beta-1.

Glycoprotein as a Maker of Renal Damage

Tamm-Horsfall protein (THP), the most abundant glycoprotein in normal human urine, is produced by the kidney (Oleczak, 1999). Tamm The Horsfall glycoprotein (THP)-1 which has since been characterized as a 616-amino

acid glycoprotein, is synthesized specifically by cells of the thick ascending limb of the loop of Henle. About 30% of the molecular weight of THP is carbohydrate. THP is heavily glycosylated by N-linked glycans, which are responsible for most of its properties. The sugar moiety is also modified in some diseases. Although the physiologic significance of THP remains undefined, this protein plays an integral role in the pathologic condition known as cast nephropathy. Cast nephropathy, or "myeloma kidney," is a common complication of multiple myeloma (Huang & Sanders, 1997).

Also, THP plays an important role in ion transport and in maintaining water impermeability of the TALH and also protects the urinary system from uropathogens. It is also involved in immunoregulatory processes. THP was reported to influence many pathological conditions, such as formation of kidney stones, the development of interstitial nephritis and tubular blockade. Recently, It has been shown that urinary THP may be a useful marker for renal damage and may be useful in evaluating urinary THP in post-menopausal diabetic women (Below *et al.*, 1999).

Glycoprotein as a Cell-cycle Regulatory Protein

The p75 neurotrophin receptor (p75NTR) is a 75-kDa cell-surface receptor glycoprotein that shares both structural and sequence homology with the tumor necrosis factor receptor superfamily of proteins. The expression pattern of p75NTR is widespread, extending outside of the nervous system to numerous peripheral organs and tissues, including bladder urothelium (Vaidyanathan *et al.*, 1998), where it may regulate cell survival, proliferation, and growth. The p75NTR binds to the neurotrophin (NTR) family of growth factors, including the prototypic ligand nerve growth factor (NGF) (Djakiew, 2000; Krygier & Djakiew, 2001). NGF is abundantly expressed by smooth muscle cells of the human bladder (Tanner *et al.*, 2000; Clemow *et al.*, 2000). Recently, p75NTR was identified as a tumor suppressor and metastasis suppressor of both bladder and prostate cancer cells (51). This tumor suppressor function of p75NTR was associated with the retardation of cell-cycle progression by inducing the accumulation of cancer cells in the G1 phase with a concomitant reduction of cells in the S phase of the cell cycle (Krygier & Djakiew, 2001). A dose-dependent increase in p75NTR protein expression was associated with a decrease in cell proliferation. This p75NTR-dependent suppression of proliferation was rescued with NGF. In the absence of ligands, a dose-dependent increase in p75NTR protein expression was associated with the reduced expression of cyclin D1, cyclin E, and cyclin-dependent kinase 2 (cdk2) as well as decreased cdk2 activity. There was also a decrease in the expression of hyper-phosphorylated retinoblastoma protein, the transcription factor E2F1, and proliferating cell nuclear antigen, and there was an increase in expression of hypophosphorylated Rb and the cdk inhibitor p16Ink4a with increasing p75NTR expression. Treatment of tumor cells with NGF ameliorated p75NTR-dependent changes in the levels of cell-cycle regulatory

proteins and rescued the tumor cells from p75NTR-dependent inhibition of proliferation. Hence, it can be concluded that p75NTR inhibits proliferation by altering the expression of cell-cycle regulatory proteins and that NGF ameliorates this effect (Khwaja & Djakiew, 2003).

OTHER GLYCOPROTEINS

Fibrillin-1

Fibrillins are large cysteine-rich glycoproteins (~350kDa) which form the molecular scaffold of a class of beaded microfibrils that are key structural elements of dynamic connective tissues. These microfibrils are extensible polymers which act as a structural lattice for elastin deposition during elastic fibre formation. Mutations in the human fibrillin-1 gene (FBN-1) cause the connective tissue disease Marfan syndrome and related disorders, which are characterized by defects in the skeletal, cardiovascular and ocular systems of the body (Handford, 2000).

Fibrillin molecules have a cysteine-rich multidomain organization dominated by calcium-binding epidermal growth reactor-like domains (cbEGF- like domains) interspersed with eight-cysteine-containing motifs. The contiguous arrays of cbEGF-like domains form rod-like structures in the presence of calcium (Reinhardt *et al.,* 1997). Each isoform contains a unique hydrophobic sequence towards the amino terminus which may act as a potential molecular hinge; in fibrillin-1 this sequence is proline-rich sequence, and in fibrillin-2 it is glycine-rich. Amino and carboxy-terminal fibrillin sequences contain furin/PACE proprotein convertase tetrabasic consensus sequences, and processing at these sites may be important regulatory steps in fibrillin assembly (Raghunath *et al.,* 1999; Ritty *et al.,* 1999).

1-Acidic-Glycoprotein (AAG)

Tumor cells can gain either lymphatic or blood vessels and adhere to their walls through the expression of carbohydrate chains which facilitate cancer cell migration (Nakamori *et al.,* 1997). The presence of theses carbohydrate ligands have been described in AAG molecules, which is also consistent with the association of AAG with tumor spreading. Serum from patients with different malignancies contains an abnormal concentration of α1-acidic-glycoprotein (AAG) and also, increased levels of AAG are associated with the presence of tumor mass. Recently, serum levels of AAG were measured by radial immunodiffusion in squamous cell carcinoma of the head and neck (SCCHN) patients taking into account disease status parameters such as tumor localization, stage and extension of disease (Croce *et al.,* 2001). In addition, the mRNA for the AAG was expressed not only in hepatoma cells, but also in non-hepatic cancer cells. The expression of the AAG mRNA in

HT-29 human colon carcinoma cells is induced by cytokines, IL-6, IL-1, and TNF-alpha, in a manner characteristic of the acute phase response, and the expression of AAG mRNA was up-regulated in differentiated HT-29 cells (Lee *et al.,* 2001).

β2-Glycoprotein I (β2GPI)

β2-Glycoprotein I (β2GPI) is a 50-kDa molecule that is present in the circulation at a concentration of approximately 200 μg/mL and acts as an anticoagulant in *in vitro* assays. Although the role of β2GPI has not yet been elucidated, several properties have been observed: its ability to bind negatively charged phospholipids or influence phospholipid-expressing cells (apoptotic cells, activated platelets); its ability to scavenge modified cellular surfaces such that they may engulf macrophages (Thiagarajan *et al.,* 1999); and its role as an important target for binding of autoimmune antiphospholipid antibodies (aPLs) (Schwarzenbacher *et al.,* 1999). With regard to the latter property, it should be stated that β2GPI has to undergo structural alteration in order to be recognized by aPLs. Such change can be brought about, for example, by binding to negatively charged phospholipids or high binding plates, but also *in vivo* by binding apoptotic cells that express 'phophatidylserine. Whereas previous reports pointed toward the presence of five 'sushi'-like domains, recent crystalographic data suggest that β 2GPI more likely forms a 'J' (hook-like) structure (Schwarzenbacher *et al.,* 1999).

Glycoprotein Derived Grom Virus

Rabiås virus is an enveloped virus with a single type I glycoproteinG (RVG) of 65 kDa inserted in its membrane. It is a lyssavirus that belongs to the rhabdovirus family. It is a neurotropic virus usually transmitted through the bite of a rabid animal. RV penetrates either directly into nerve endings at the site of inoculation or after a limited multiplication in myotubes; it is then transported along axons to the cell body of motor and sensory neurons, where replication takes place. Viral budding is observed mostly in internal compartments of infected neurons and the virus is transported to synapses in vesicles. Within the nervous system (NS), propagation of RV between connected neurons occurs exclusively at the synapse. Late in infection, the virus eventually spreads to a few categories of non-neuronal differentiated tissues, such as submaxillary salivary glands, taste buds, adrenal glands, pancreas, kidney, hair fonlicles and brown fat tissue. Recently, it has been shown that RVG is a specific high affinity ligand for a non-neurotrophin binding site on the p75NTR. The existence of a specific high affinity trimeric ligand for p75 will be useful for future work on the pharmacology and physiology of this receptor (Langevin *et al.*, 2002).

Glycoprotein Derived from Strain

Chlorella, a unicellular green algae, can divide into four cells every 16–20 h, utilizing sunlight for photosynthesis. Chlorella cells contain 55–67% protein,

1–4% chlorophylls, 9–18% dietary fiber and large amounts of minerals and vitamins (Hasegawa *et al.,* 2000). At present, Chlorella is widely sold as a health supplement in Japan, the US and other countries. Recently, it has been reported that a glycoprotein-rich substance was released from *Chlorella vulgaris* strain CK-22 cells into the culture medium. The substances, designated as *C. vulgaris* culture supernatant (CVS), is a glycoprotein with an approximate molecular weight of 63100 amu and contains 6-linked β-(1–6) galactopyranose-rich carbohydrate (66.9%) and protein (35.2%) (Tanaka *et al.*, 1998). A glycoprotein prepared from CVS is a biological response modifier (BRM) which exhibits protective activities against tumor metastasis, 5-fluorouracil-induced immunosuppression and psychological stress-induced apoptosis in mice (Hasegawa *et al.,* 2000; Tanaka *et al.*, 1998). Also, CVS is known to stimulate macrophages to produce pro-inflammatory cytokines such as IL-1β, TNF-α and IL-12, of which transcriptions are required for NF-κB activation (Hasegawa *et al.*, 1997). In addition, hot water extract of Chlorella vulgris (CVE) is thought to promote Th1-biased response and to enhance resistance to *L. monocytogenes*, and *Escherichia coli* infections via augmentation of IL-12 production. Induction of endogenous IL-12 and IFN γ by CVE may result not only in promotion of the Th1-type immune response, but also in inhibition of the Th2-type immune response. Thus, It has been reportedly shown that oral administration of CVE in mice suppressed the production of immunoglobulin (Ig)E against casein antigen accompanied by increased IFNγ and IL-12 mRNA expression (Hasegawa *et al.,* 1990). Oral administration of CVE enhanced Th1 response to casein in the spleen of casein immunized mice. CVE may be useful for the prevention of allergic diseases with a predominant Th2 response (Hasegawa *et al.*, 1999; Hasegawa *et al.,* 1997).

GLYCOPROTEIN ISOLATED FROM PLANTS

First, we prepared glycoprotein from plants that have traditionally been used in Korean herbal medicine and studied bioactive functions either *in vitro* or *in vivo* system.

UDN Glycoprotein

Ulmus davidiana Nakai (UDN, 116 kDa) consists of a carbohydrate component (78.65%) and a protein component (21.35%) (Lee *et al.,* 2006a), and has pharmacological activities such as strong antioxidative activity against •OH, anti-apoptotic activity through inhibition of caspase-3 activation, and hepatoprotective activity against toxic metabolite, indicating UDN glycoprotein is a functional substance with multiple biological activities (Lee *et al.,* 2006b; Oh *et al.,* 2006a).

SNL Glycoprotein

Solanum nigrum Linne (SNL, 150 kDa) consists of a large carbohydrate (69.74%) and a protein (30.26%). This glycoprotein has a strong scavenging

effect against reactive oxygen radicals, and growth inhibition effects against JA221 and XL1-Blue (Lim *et al.,* 2002). Moreover, it has been reported that SNL glycoprotein has a cytotoxic effect against MCF-7, HCT-116 and HT-29 cells, and induction of apoptosis, even at low concentrations (Heo *et al.,* 2004; Lim, 2005; Lee & Lim, 2006a).

GJE Glycoprotein

Gardenia jasminoides Ellis (GJE glycoprotein, 27kDa) consists of a carbohydrate component (58.65%) and a protein component (42.35%), has scavenging activity against oxygen radicals and inhibits the oxygen radical-induced protein kinase C alpha and nuclear factor-kappa B in NIH/3T3 cells (Lee *et al.,* 2006c). It has hepatoprotective and hypolipidemic effects in glucose/glucose oxidase (G/GO)-treated BNL CL.2 cells, as well as in CCl_4, Triton WR-1339 and corn oil-treated mice. In G/GO-treated BNL CL.2 cells, the results showed that GJE glycoprotein has an inhibitory effect on G/GO-induced cytotoxicity and intracellular reactive oxygen species production. In addition, GJE glycoprotein has an anti-oxidant effect against the lipid peroxidation process in the Fe^{2+}/ascorbic acid system. In CCl_4 (1.0 mL/kg)-treated mice, pretreatment with GJE glycoprotein (80 mg/kg) blocked lactate dehydrogenase release and the formation of thiobarbituric acid-reactive substances. In addition, in these mice, GJE resulted in increased nitric oxide production and the activation of anti-oxidant enzymes, accompanied by the inhibition of the cytotoxic-related signals hepatic cytochrome c, nuclear factor-kappaB and activator protein-1. In both Triton WR-1339 (400 mg/kg) and corn oil (1.0 g/kg)-treated mice, pretreatment with GJE glycoprotein (80 mg/kg) lowered the levels of plasma lipoproteins (triglyceride, total cholesterol and low-density lipoprotein) (Lee *et al.,* 2006d).

OFI Glycoprotein

Glycoprotein of *Opuntia ficus-indica* var. saboten MAKINO (OFI, 90KDa) has antioxidative and hypolipidemic effects in Triton WR-1339-induced A/J mice and inhibits the production of reactive oxygen species (ROS) generated by glucose/glucose oxidase (G/GO) in BNL CL.2 cells. It resulted in a significant decrease of plasma lipid levels in Triton WR-1339-treated mice such as total cholesterol (TC), triglyceride (TG), and low-density lipoprotein (LDL). Interestingly OFI glycoprotein resulted in a decrease of thiobarbituric acid-reactive substances (TBARS) level and in an increase of nitric oxide (NO) amount in the presence of Triton WR-1339 treated mice, while the activities of antioxidant enzyme [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] were augmented (Oh & Lim, 2006c). Also the glycoprotein isolated from OFI glycoprotein has an antioxidative effect and protects NIH/3T3 cells from glucose/glucose oxidase (Oh *et al.,* 2004).

RVS Glycoprotein

Several earlier studies indicated that ethanol extracts of RVS have pharmacological activities such as antimicrobial, antioxidative and antiapoptotic effects (Lee & Lim, 2000). They also have an inhibitory activity of human cancer cell proliferation and an enhancing activity of detoxifying enzymes in hepatocytes (Lee & Lim, 2002; Ko *et al.,* 2006). RVS glycoprotein (36.0kDa) also has protective activity on carbon tetrachloride induced liver injury in mice (Oh *et al.*, 2006b), decreases cholesterol levels in plasma, inhibits CT 26-induced tumor growth and has a scavenging effect on reactive oxygen radicals (Ko *et al.,* 2006).

DBD Glycoprotein

Glycoprotein of *Dioscorea batatas* Decne (DBD, 30kDa) in the hydroxyl radicals generated by glucose/glucose oxidase (G/GO) has remarkable scavenging effects in cell-free systems and DBD glycoprotein (200 μg/mL) significantly inhibiting intracellular ROS amounts and protects from cytotoxicity in primary mouse splenocyte culture treated with GO (30 mU/mL) (Oh and Lim, 2008). DBD glycoprotein (200 μg/mL) has an inhibitory effect on the production of intracellular hydrogen peroxide (H_2O_2), on the phosphorylation of p38 mitogen-activated protein (MAP) kinase, on DNA binding activity of activator protein-1 (AP-1) and on c-Jun and c-Fos protein expression respectively. In addition, DBD glycoprotein treatment markedly suppressed the interleukin (IL)-1beta, IL-6, and inducible nitric oxide synthase (iNOS) expression and the production of nitric oxide (NO) in LPS-stimulated RAW 264.7 cells. Interestingly, IL-1beta, IL-6, and iNOS expressions were significantly attenuated by treatment with protein kinase C (PKC) inhibitor (staurosporine) as well as p38 MAP kinase inhibitor (SKF86002) in LPS-stimulated RAW 264.7 cells (Lee & Lim, 2008a).

ZPDC glycoprotein

Glycoprotein isolated from *Zanthoxylum piperitum* DC fruit (ZPDC glycoprotein, 24kDa) consists of a carbohydrate component (18%) and a protein component (82%). It has a strong scavenging activity against DPPH, superoxide anion and hydroxyl radicals without any pro-oxidant activity in the cell-free system. In hepatocyte cell lines (Chang liver and BNL CL.2 cells), the results showed that ZPDC glycoprotein has an inhibitory effect on hypoxanthine/xanthine oxidase- or glucose/glucose oxidase induced cytotoxicity in a dose dependent manner. In addition, administration of ZPDC glycoprotein (20 mg/kg) lowers the levels of lactate dehydrogenase, alanine transaminase and thiobarbituric acid reactive substances, whereas increases the level of nitric oxide, accompanying the normalizing effects on the activity

of hepatic anti-oxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) in mouse models of carbon tetrachloride stimulated acute liver injury (Lee & Lim, 2008b).

Glycoprotein preparation from all above mentioned herbal plants was less than 1% of each raw material. The glycoproteins were obtained in 0.10, 0.43, 0.61, 0.05, 0.63, 0.02, 0.07, 0.06, 0.81% from UDN, SNL, GJE, OFI, RVS, DBD, ZPDC, CT and GSL respectively. In general, the bands of glycoproteins as displayed on the gel after staining were broad not sharp (data not shown). This is a distinctive characteristic of glycoprotein, because the number of carbohydrate residues from whole glycoprotein molecules was not fixed. One of the reasons for this phenomenon is that some carbohydrate residue can be lost during the preparation of glycoprotein and electrophoresis. The other reason is that glycoprotein loses its activity after it is treated with deactivating agents (pronase E for protein and $NaIO_4$ for carbohydrate in whole glycoprotein molecule). Glycoprotein has been confirmed to change its activity after treatment with deactivating agents.

The few characteristics of each glycoprotein, a and their biological functions, are summarized (Table 5.1). Thus, glycoproteins can function to have antioxidative activity, induce apoptosis, protect hepatocytes and inhibit inflammations. When glycoprotein is treated with pronase E or $NaIO_4$, reagents, respectively, it loses its scavenging activity of radicals using DPPH assay. However, the radical scavenging activities of glycoprotein (100 μg/mL) in the absence of deactivation agents increased. The values of the results obtained from the DPPH assay are indicated at 50 and 100 μg/mL glycoprotein (Table 5.2). For example, the values of DPPH radical scavenging activity were 85.9, 47.0, 90.0, 73.9, 71.6, 44.3, 92.0, 28.7 and 89.3% at 100 μg/mL for UDN, SNL, GJE,OFI, RVS, DBD, ZPDC, CT and GSL glycoprotein respectively, while they were less than 5% after treatment with either pronase E or $NaIO_4$ (data not shown). This means that glycoprotein needs both parts (protein and carbohydrate) in order to have scavenging activities of radicals. However, the deformation of glycoprotein caused by treatment with pronase E or $NaIO_4$ prohibits the glycoprotein from acting as a scavenger due to changes of the three-dimensional structure of the glycoprotein. Glycoprotein should be joined in covalent chemical linkage between both protein and carbohydrate in order to produce an optimal activity. It is still unknown whether or not the attached carbohydrate has several effects.

Analysis of glycoprotein components (carbohydrate and protein moiety) was carried out according to the method of Dubois *et al.* (1951) for carbohydrate moiety and Lowry *et al.* (1951) for protein moiety from whole glycoprotein molecule. Data represent the mean values of triplicates and are significantly difference between experiments, $p<0.05$.

Table 5.1. The components and bioactivities of glycoproteins

Abbreviated name	Nomenclature	Components of glycoprotein			Bioactive functions
		Carbohydrate (%)	Protein (%)	Ratio (C/P)	
UDN	*Ulmus davidiana* Nakai	78.65	21.35	3.68	Antioxidative effect and protection of liver
SNL	*Solanum nigrum* Linne	69.74	30.26	2.30	Antioxidative effect and induction of apoptosis
GJE	*Gardenia jasminoides* Ellis	58.65	42.35	1.38	Antioxidative and hypolipidemic effect
OFI	*Opuntia ficus-indica var. saboten* MAKINO	37.54	62.46	0.60	Antioxidative effect and protection of liver
RVS	*Rhus verniciflua* Stokes	31.0	69.0	0.44	Antimicrobial, antioxidative effect, and protection of liver
DBD	*Dioscorea batatas* Decne	83.75	16.25	5.15	Antioxidative and anti-inflammatory effect
ZPDC	*Zanthoxylum piperitum* DC	18.0**	82.0**	0.21	Antioxidative effect and protection of liver
CT	*Cudrania tricuspitata* Bureau ex Lavalle'e	72.50	27.50	2.63	Enhancing immunity
GSL	*Geranium sibiricum* Linne	10.45	89.55	0.11	Ant-inflammatory effect

Table 5.2. Scavenging activity determinations of glycoproteins by deoxyribose assay

Abbreviated name	Glycoprotein (MW, kDa)	Scavenging activity (DPPH assay, %)		Source
		50 μg/mL glycoprotein	100 μg/mL glycoprotein	
UDN	116	81.0	85.9	Lee *et al.*, 2006b; Oh *et al.*, 2006a
SNL	150	25.0	47.0	Lim *et al.*, 2002; Heo *et al.*, 2004; Lim, 2005; Lee & Lim, 2006a
GJE	27	75.0	90.0	Lee *et al.*, 2006c; Lee *et al.*, 2006d
OFI	90	60.2	73.9	Oh & Lim, 2006c; Oh *et al.*, 2004
RVS	36	46.7	71.6	Lee & Lim, 2000; Lee & Lim, 2002; Ko *et al.*, 2006; Oh *et al.*, 2006b
DBD	30	24.9	44.3	Oh & Lim, 2008; Lee & Lim, 2008a
ZPDC	24	94.0	92.0	Lee & Lim, 2008b
CT	75	18.0	28.7	Unpublished
GSL	18	88.4	89.3	Unpublished

The activity of glycoprotein was evaluated with DPPH assay (Maffei *et al.,* 1999) at 50 and 100 μg/mL after treatment with either Pronase E (Shan *et al.,* 1999) or $NaIO_4$ (Oka *et al.,* 1987). Data represents the mean values of triplicates are significantly difference between treatments and the control ($p<0.05$) respectively.

Generally speaking, glycoproteins in their natural forms have a high hygrosopic character in the air, because of the air's high viscosity. We cannot say with certainty that the biological active function of glycoproteins depends on the molecular weight, because they have distinctive functions individually. Furthermore, the bioactivity of glycoprotein is not related to the ratio between the carbohydrate moiety and the protein moiety in the whole molecule. The difference in biological function stems back to the amounts of carbohydrates and proteins, or different kinds of components, such as lactose, fructose, galactose and mannose or amino acids. Glycoprotein binds to extracellular membrane proteins, which results in different biological functions to the cell. Possible carbohydrates of glycoproteins include glucose, glucoseamine, galactose, galactoseamine, mannose, fucose and sialic acid. It has been reported that the sugar group of glycoprotein assists in protein folding or improving its stability. It may help the protein to fold into the proper geometry, stabilize the protein and affect physical properties such as solubility or viscosity (Nelson & Cox, 2000). Natural antioxidants, found in various kinds of plant sources protect cells against oxidative damage and may provide an exciting preventative and therapeutic prospect for degenerative diseases (Lin *et al.,* 2003). Since glycoprotein has a polar character and a potential as electron acceptors, it has an anti-oxidative and/or anti-cancer effect, and enhanced biological active functions as shown in Table 1. Taken together, glycoproteins in the living system play an important role in the intracellular and extracellular membrane. We speculate that glycoprotein molecule has dual functions, *e.g.* a protective function for itself and an attacking function against other living systems in order to survive. Nevertheless, many questions remain unanswered about their function as whole molecule, protein moieties and carbohydrate moieties separately. Again, is there a difference between O-linked and N-linked glycoproteins? Such different function should be elucidated by way of glycoproteomics. The bioactivity of glycoprotein may depend on the ratio between the carbohydrate and the protein moieties in the whole molecule. The carbohydrate and protein moieties have several important biological roles respectively but in many cases their functions remain enigmatic. It is clear that protein and carbohydrate parts in the glycoprotein molecule may not have any biological functions separately but the whole molecule consisting of protein and carbohydrate together elicit bioactive functions.

SUMMARY

It is well established that glycoproteins in the living system play an important role in the intracellular and extracellular membrane. They have dual functions within one molecule *e.g.* a protective function for itself and an attacking function against other living system in order to survive. We speculate that their functions may be related to signals related to the survival or attack functions. Plant-originated glycoproteins do not have any cytotoxic ability, but they have bioactive potential in respect of animal cells and edible nutritional values. Their biofunctional activity does not relate to their molecular weight, or the ratios between carbohydrate and protein moieties, but varies on an individual basis.

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6

Chemical Constituents and Pharmacology of the Neotropical Burseraceae

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ABSTRACT

Burseraceae family comprises over 700 species distributed among 18 genera. In the Neotropic region, where several species are observed, particularly from Bursera and Protium genera, pharmacological and chemical studies are widespread. Exuded from one of the most important resiniferous families, Burseraceae oleoresins have p-cymene rich essential oils and triterpenic rich resins. Several other secondary metabolites, such as coumarins, flavonoids and lignans are isolated from stems and bark of trees. Some of these substances have displayed potent biological activities, besides, anti-inflammatory and anti-tumor. This article reviews the literature related to Neotropical Burseraceae ethnopharmacology, chemistry and pharmacology.

Key words : Burseraceae, *bursera*, lignans, pharmacology, phytochemistry, *protium*, review, triterpenes

INTRODUCTION

Burseraceae is one of the most important resiniferous botanic families. Its oleoresins have several applications, including medicinal, perfumery, paints and varnishes (Pernet, 1972). More than 700 species of this family are divided in 18 genera, trees and shrubs, commonly found in all over tropical and subtropical regions (Weeks *et al.*, 2005).

The origin of this family is assumed to have occurred in North America, at the Neocen Period, when migratory streams distributed it not only to Central and South America but to Africa, Asia and Oceania also (Weeks *et al.*, 2005).

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In the Neotropic region 230 species are described, belonging to eight genera from Bursereae (*Beiselia*, *Bursera* and *Commiphora*), Canarieae (*Dacryodes* and *Trattinnickia*), and Protieae (*Crepidospermum*, *Protium* and *Tetragastris*) tribes (Weeks *et al.*, 2005).

In the folk medicine, this family has been utilized to treat blenorragy and pulmonary or stomach injuries. It is utilized also as a tonic, stimulant, tea, analgesic, haemostatic, contraceptive, laxative, and in the odontology, among many other applications (Costa, 1975; Pio Correa, 1994).

The ethnobotanic indications are not limited to ethnopharmacology only; there are several other uses in painting and varnish industries, in cosmetics production, medicinal adhesives and to make wooden boat impermeable. Some authors relate the burning of the oleoresin to repel insects, to illuminate small cities and to produce incenses in religious rituals (Costa, 1975; Ribeiro & Daly, 1999).

In the burseraceae family, the oleoresin is exudated not only from the trunk of the trees, but from leaves and roots too. This oleoresin is naturally exudated in very little amounts from trunk and leaves (having the appearance of a fungus) or by natural healings at the trunk that the weight of the leaves and fruits can produce. In several species, a very superficial scratch made with a knife (not deeper than 0.5 cm) can promote the release of more than 1 mL of oleoresin in few minutes.

Another factor commonly associated to the oleoresin exudation is the action of some insects that use the trees of Burseraceae species for depositing their larvae. In the *Protium* genus, *Alipumilio, Cheilosia* (Diptera: Syrphidae) and *Sternocoelus* (Coleoptera: Curculionidae) are closely related (Plowden, 2001), and reportedly *Blepharida*, that is the only insect genus that deposit its larvae at the trunk of the trees of *Bursera* species, in Mexico and Guatemala (Evans *et al.*, 2000; Becerra, 1997). This study tried to correlate the essential oil produced together with the resin in the *Bursera* oleoresin with the attraction of *Blepharida* insects (Evans *et al.*, 2000).

PHYTOCHEMISTRY

The phytochemical research in the Neotropic region is focused in two genera: *Protium* and *Bursera*, in South and North America, respectively. Besides these two genera, *Crepidospermum*, *Dacryodes*, *Tetragastris* and *Trattinnikia* have few studies. *Commiphora* and *Beiselia* are the other two genera found in this region but they were never chemically analyzed.

The oleoresin phytochemical composition in this family shows several types of terpenoids, mainly triterpenes from oleane, ursane and lupane

skeletons. Some tirucalane, taraxane and friedelane skeletons are described too, but not so frequently (Costa, 1975; Pernet, 1972; Khalid, 1983; Rüdiger *et al.*, 2007).

The triterpenes α-amyrin and β-amyrin, together with lupeol are the main constituents identified in the Burseraceae oleoresin, belonging to urs-12-ene, olean-12-ene and lup-20(29)-ene skeletons, respectively. All of them have a hydroxy group at position 3, in the A ring, usually β configuration. Other commonly cited substances are the ketones β-amyrone, β-amyrone and lupenone, together with maniladiol and brein diols, both with another 16a hydroxy group (Fig 6.1).

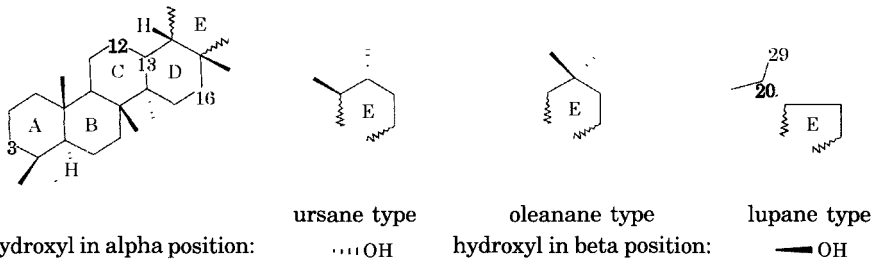


Fig 6.1. Triterpenes ursane, oleanane and lupane

Essential Oils

The very uncommon, attractive and delicate aroma of the oleoresin characterizes the Burseraceae family and helps to locate the trees in the rainforest. This aroma is the volatile part of the oleoresin, the essential oil that can be isolated by steam distillation. The fresh oleoresin has about 30% of essential oil. After some time, the volatile amount decreases to about 7% (Costa, 1975). The main constituents belong to menthane (monocyclic), cymene (aromatic), and pinane (bicyclic), monoterpenes skeletons (Fig 6.2).

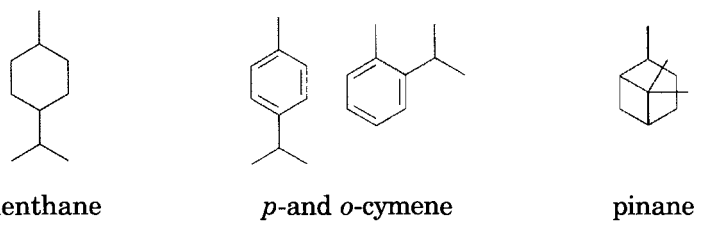


Fig 6.2. Monoterpene types in Burseraceae

Amazon is the Brazilian Region where number of species are described, mainly at Campinarana. *Protium heptaphyllum* is the Burseraceae specie found in all over Brazil, the most known and widely studied. Two studies show a different essential oil obtained from the oleoresin composition pattern, both very rich in terpinolene. From Amazon Region was obtained an essential

oil with 21% of terpinolene, together with *p*-cymene (36%), α- and γ-terpinene (18% and 12%, respectively) and terpinen-4-ol (11%) (Siani *et al.*, 1999a, 1999b). The essential oil obtained from this specie found at the Brazilian Northeast Region showed 28% of terpinolene, together with α-pinene (10%), α-phelandrene (17%) and limonene (17%) (Bandeira *et al.*, 2001).

Similar to *P. heptaphyllum*, *P. strumosum* essential oil from the oleoresin is rich in *p*-cymene (27%) and α-terpinolene (22%), together with β-phelandrene (17%) and *p*-cimen-8-ol (12%). In *P. spruceanum*, this percentual was 31% to *p*-cymene and 42% to β-phelandrene, observed together with α-pinene (16%), α-phelandrene (18%) and *p*-menth-3-ene (21%) (Ramos *et al.*, 2000).

Even higher concentration of *p*-cymene was observed in two varieties: *P. paniculatum* v. *Nova* (69%) and *P. paniculatam* v. *riedelanum* (90%) (Ramos *et al.*, 2000).

Another *p*-cymene rich oleoresin (75%) can be found in *P. hebetatum*, that appears together with α-pinene (16%) and β-phelandrene (11%). *Protium altsoni*, a taxonomically and phylogenetically related specie has an oleoresin essential oil with 33% of *p*-cymene, 61% of α-pinene and 22% of α-phelandrene. At the other hand, *P. nitidifolium*, known as breu-vermelho (red-breu), shows a large taxonomic and phyllogenetic difference from the other species, with oleoresin essential oil comprising 31% of α-pinene and 35% of *p*-cymene (Ramos *et al.*, 2000). The whole profile is hard to analyze and shows a very challenging chemistry work, searching for pattern identification, as these oils have a huge commercial interest depending on the aroma produced.

In the *Trattinnickia* genus, the oleoresin essential oil pattern is very difficult to analyze based published literature. In the essential oil obtained from the oleoresin of *T. burserifolia*, a higher amount of α-phelandrene (44%) and β-phelandrene (19%) were observed (Lima, 2000), with only 16% of *o*-cymene. In the *T. rhoifolium*, *p*-cymene (49%), α-pinene (25%), β-phelandrene (8%), α-phelandrene (8%), *t*-α-dehydroterpineol (6%) and α-terpinene (6%) were observed, but no *o*-cymene (Ramos *et al.*, 2003).

The essential oil obtained from leaves is surprisingly less studied and with a diverse composition, very rich in sesquiterpenes of muurulane, selinane, humulane, and caryophyllane types (Fig 6.3).

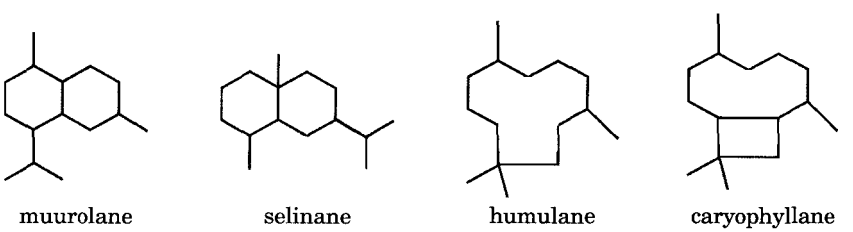


Fig 6.3. Main sesquiterpenic types found in Burseraceae

The very first chemical study with essential oil from leaves was probably performed in 1951 by Bradley and Haagensmit (1951). They reported the

monoterpenes α- and β-phelandrene in *Bursera microphylla*, together with tetrahydrocumic acid (Bradley & Haagensmit, 1951). Recently, in a phytochemical investigation of the essential oils from the leaves of *B. simaruba*, the monoterpene limonene (47%) and the sesquiterpenes β-caryophyllene (15%), α-humulene (13%), and germacrene D (8%) were reported as the majoritary compounds (Sylvestre *et al.*, 2007).

From the leaves essential oil from *Protium heptaphyllum* the monoterpenes β-phelandrene (9%) and (Z)-β-ocymene (2%) were observed in very low concentrations. The sesquiterpenes derived from caryophyllene, as t-*epi*-9-caryophyllene (21%), 9-*epi*-(E)-caryophyllen-14-ol (16%) and t-*iso*-longifolanone (10%) were the main compounds (Pontes *et al.*, 2007). A similar result, but with higher monoterpene concentration (18% of mircene), was observed by Bandeira *et al.* (2001), with 18% of β-caryophyllene.

In the *P. grandifolium* and *P. hebetatum* essential oils obtained from the leaves a similar pattern was observed, a low content of *p*-cimenene (1% & 8%) and higher contents of δ-cadinene (11% & 7%) and β-caryophyllene (24% & 12%), respectively (Siani *et al.*, 1999a).

For *P. strumossum*, the essential oil from the leaves showed a diverse content on sesquiterpenes, with α- and β-selinene (15% & 17%, respectivelly) and very low content of β-caryophyllene (2%) (Siani *et al.*, 1999a).

The *Protium icicariba* showed a different profile, with 23% of monoterpenes, α-terpinene (6%) and α-terpinolene (12%) as the major compounds from this class of terpenoids, and about 10% of bicyclogermacrene, observed together with α-copaene (7%) and γ-elemene (6%) (Siani *et al.*, 2004).

P. unifoliolatum and *P. lewellynii* showed their leaves essential oil with both monoterpene (24% & 18% of limonene) and sesquiterpene (37% & 32%, respectively) as major compounds (Zoghbi *et al.*, 1993; Siani *et al.*, 1999a).

In 1964, a phytochemical analysis of the *Bursera graveolens* essential oil of stems showed the monoterpenes (+)-limonene (57%), besides (+)-β-terpineol (18%) and (±)-carvone (5%) (Crowley, 1964). A study recently published showed a very similar profile, with 59% of limonene and 11% of α-terpineol (Young *et al.*, 2007), in a total of 78% of monoterpenes.

Yukawa *et al.* (2005) isolated the sesquiterpenes 1-acetyl-4-isopropenyl-1-cyclopentene, 10-hydroxy-6,10-epoxy-7(14)-isodaucane, 2-methyl-5-isopropenyl-1-cyclopenten-1-carboxaldehyde, and 6,10-epoxy-7(14)-isodaucane from the essential oil from the wood of *B. graveolens*.

Another essential oil study was published with the *Protium icicariba*, from the fruits, identifying the monoterpenes α-terpinene (30%), *p*-cymene (8%), γ-terpinene (12), terpinen-4-ol (6%) and α-terpiniolene (35%), and sesquiterpenes at very low concentration (Siani *et al.*, 2004).

The Oleoresin

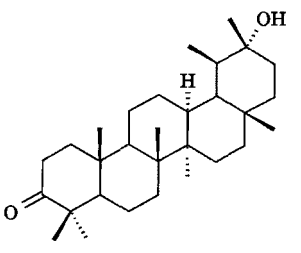
The triterpenes are the major constituents from the Burseraceae oleoresin. They are described in several species, generally with very few variations. The triterpenes from oleane and ursane skeletons are the most common, mainly the 3-hydroxy derivatives, α-amyrin and β-amyrin, have been recently reported in the Neotropical specie *Dacryodes hopkinsii* (Lima *et al.*, 2004).

A review study recently published showed that several Burseraceae oleoresin species have an extremely complex mixture of minor triterpenes, suggesting that new studies must be performed in order to discover possible unidentified compounds (Rüdiger, 2008).

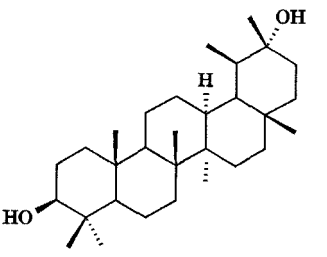
Since the 1960's, phytochemical studies of Burseraceae oleoresin have been describing triterpenes such as *epi*-lupeol, α-amyrin, α-amyrone, lupanol, lupanone, lupine and lupenone (Tursch & Tursch, 1961) as their common compounds. Recently published papers showed the presence of α-amyrin, 3β-acetoxyurs-11-en-28,13-olide, acetyl ursolic acid, ursonic acid, ursolic acid, 11-oxo-acetyl ursolic acid, 11-oxo-ursolic acid and the lactones 3β-acetoxy-11α,12α-epoxyurs-28,13-olide and 3β-acetoxy-12β-hydroxyurs-28,13-olide in the *Bursera delpechiana* oleoresin (Syamasundar *et al.*, 1991; Syamasundar *et al.*, 1995).

In the *B. simaruba* oleoresin α-amyrin and β-amyrin were isolated together with lupeol, *epi*-lupeol, *epi*-glutinol, lup-20(29)-en-3β,23-diol (Perazasanchez *et al.*, 1995), all of then with ursane, oleanane and lupane triterpene skeleton.

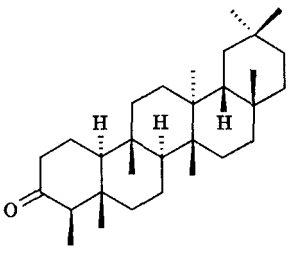
In the 1990's, from the *Protium heptaphyllum* oleoresin the triterpenes 3-oxo-20*S*-hydroxytaraxastane, 3β,20*S*-dihydroxytaraxastane and friedelin, from taraxastane and friedelane skeleton, and the dihydroxylated ursane 3β,24-dihydroxyurs-12-ene (Susunaga, 1996; Susunaga *et al.*, 2001) were identified. Maia *et al.* (2000), identified the pentacyclic triterpenes β-amyradienol (3β-hydroxyurs-9(11),12-diene) and β-amyradienol (3β-hydroxyolean-9(11),12-diene), together with the tetraciclic acids from tirucallane type: 3α-hydroxytirucalla-7,24-dien-21-oic acid and α-elemolic acid (3α-hydroxytirucalla-8(9),24-dien-21-oic acid) (Fig 6.4).



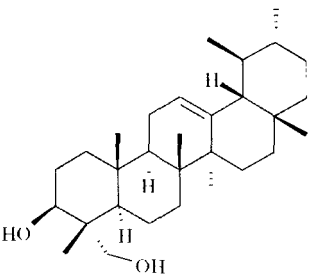
3-oxo-20*S*-hydroxytaraxastane,



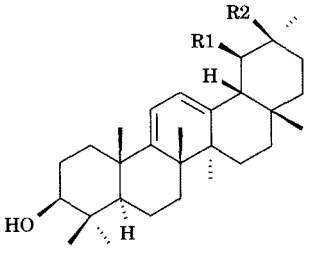
3β,20*S*-dihydroxytaraxastane

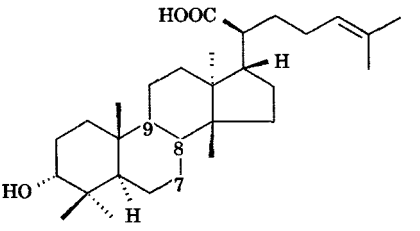


friedelin



3β,24-dihydroxyurs-12-ene

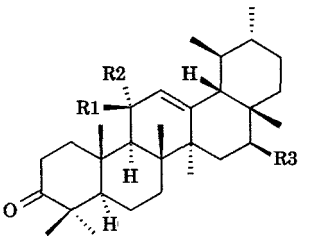




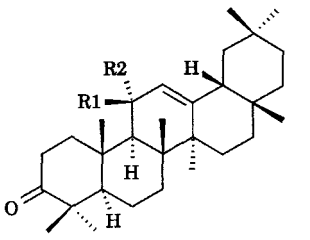
	R1	R2		C=C
α-amyradienol	CH_3	H	3α-hydroxytirucalla-7,24-dien-21-oic acid	Δ7
β-amyradienol	H	CH_3	α-elemolic acid	Δ8

Fig 6.4. Other triterpenes from Burseraceae family

Not many Burseraceae oleoresins were phytochemically studied in subtropical regions. From the *P. kleinii* oleoresin, more oxidized oleanane and ursane triterpenes were identified, as shown in figure 6: 3-oxo-11β,16β-dihydroxyurs-12-ene, 3-oxo-11β-hydroxyurs-12-ene, 3-oxo-11α-hydroxyurs-12-ene, 3-oxo-11β-hydroxyolean-12-ene and 3-oxo-11α-hydroxyolean-12-ene (Lima *et al.*, 2005) (Fig 6.5).



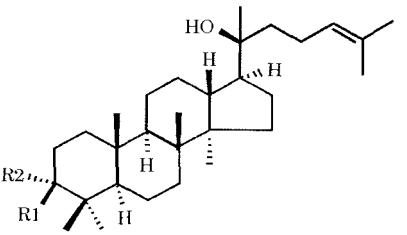
	R1	R2	R3
3-oxo-11β,16β-dihydroxyurs-12-ene	OH	H	OH
3-oxo-11α-hydroxyurs-12-ene	H	OH	H
3-oxo-11β-hydroxyurs-12-ene	OH	H	H

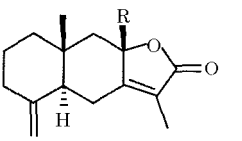


	R1	R2
3-oxo-11α-hydroxyolean-12-ene	H	OH
3-oxo-11β-hydroxyolean-12-ene	OH	H

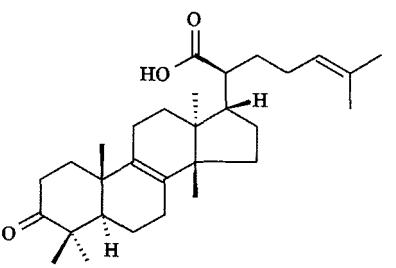
Fig 6.5. More oxygenated triterpenes from Burseraceae family

From *Trattinnickia* genus, α- & β-amyrin were identified in *T. spera* together with the sesquiterpene lactone 8β-hydroxyasterolide (Aregulin *et al.*, 2002). The non-hydroxylated form of this sesquitepene lactone, asterolide, was previously identified from *T. rhoifolia* (Diaz de Delgado *et al.*, 1995). Brein, 3β-phenylacetoxyurs-12-ene, 3β-phenylacetoxyolean-12-ene, maniladiol, 3β,16β,11α-trihydroxyurs-12-ene (Lima *et al.*, 2004), dammarenediol-II and *epi*-dammarenediol-II (3α,20(*S*)-dihydroxydammara-24-ene) were detected from *T. burserifolia* oleoresin (Lima, 2000). Tirucallane type triterpenic acids α-elemolic acid (3α-hydroxytirucalla-8,24-dien-21-oic) and 3α-hydroxytirucalla-7,24-dien-21-oic were detected from *T. burserifolia* and *T. rhoifolia* oleoresin, formerly described from *P. heptaphyllum* oleoresin, together with β-elemonic acid (3-oxo-tirucalla-8,24-dien-21-oic acid) from *T. burserifolia* (Lima, 2000; Lima *et al.*, 2004) (Fig 6.6).

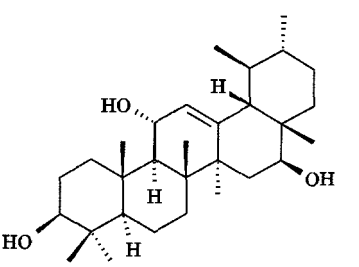




	R1	R2		R
dammarenediol-II	OH	H	8β-hydroxyasterolide	OH
3-*epi*-dammarenediol-II	H	OH	asterolide	H



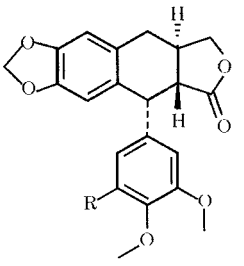
β-elemonic acid



3β, 16β, 11α-trihydroxyurs-12-ene

Fig 6.6. Triterpenes and sesquiterpene lactones from *Tratinickia*

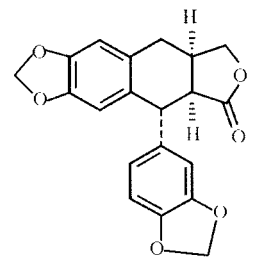
Two lignans were observed in *Bursera* oleoresin. From *B. morelensis,* deoxypodophyllotoxin and a new lignan morelensin (5'-desmethoxydeoxypodophyllotoxin) were described (Jolad *et al.,* 1977). Ariesin, a lignan isolated from *B. ariesins*, and picropolygamain (Fig 6.7), from *B. simaruba*, are two other lignans identified in this genus (Hernandez *et al.*, 1983; Perazasanchez *et al.*, 1992).



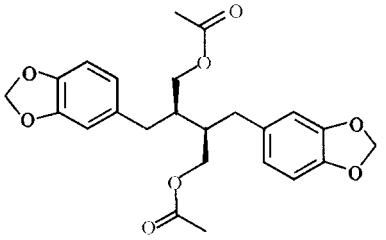
R

Deoxypodophyllotoxin MeO

Morelensin H



Picropolygamain



Ariesin

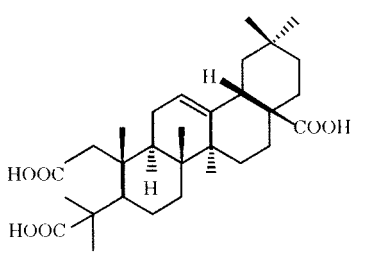
Fig 6.7. Lignans form *Bursera*

Phytochemical Studies on the Burseraceae Wood, Stems and Roots

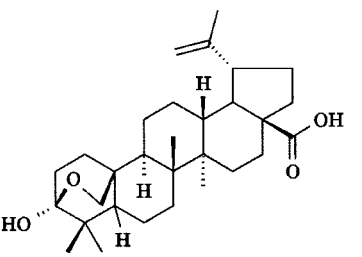
From the wood, stems and roots from Burseraceae the studies show isolation of coumarinolignoids, coumarins, flavonoids and xanthones besides the triterpenes & lignans reported earlier in oleoresin.

The oleanic acid 2,3-sec-olen-12-ene-2,3,28-trioic was detected in the wood from *Bursera graveolens* (Crowley, 1964). In a recent study, Robles *et al.* (2005) identified from the bark of the same specie the acids 3-oxo-tirucalla-8,24-dien-21-oic (β-elemonic), 3α-hydroxytirucalla-8,24-dien-21-oic (α-elemolic) and 3α-hydroxytirucalla-7,24-dien-21-oic, triterpenes that were already isolated from the *Trattinnikia burserifolia* oleoresin.

The phytochemical analysis from stems, leaves, twigs and barks from *Bursera arida* showed the pentacyclic triterpene acid 3α-hydroxy-3,25-epoxylup-20(29)-en-28-oic (Fig 6.8), known as benulin (Ionescu *et al.*, 1977).



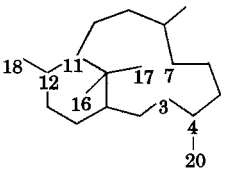
2,3-sec-olen-12-ene-2,3,28-trioic acid



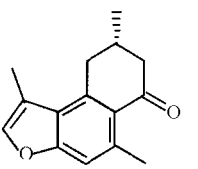
Benulin

Fig 6.8. Triterpens isolated from *Bursera* stems

Several verticillane (Fig 6.9) derivatives were observed in *Bursera suntui* and *B. kerberi* stems: verticilla-3,7,12-triene, verticilla-3,7,12(18)-triene and verticilla-4(20),7,11-triene; together with some alcohols: verticilla-3,7-dien-12-ol, verticilla-3,7-dien-12,20-diol, verticilla-3,7-dien-12,20-diol-20-acetate (*B. suntui*) and verticilla-3,7-dien-12-ol, in *B. kerberi* (Hernandez-Hernandez *et al.*, 2005). The furanosequiterpene myrrhone was isolated from the bark of the roots from *B. leptophloeos* (Fig 6.9) (Barreira *et al.*, 1996).



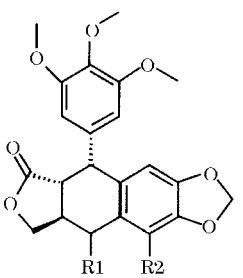
Verticillane type



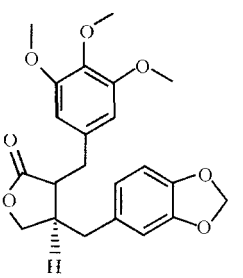
Myrrhone

Fig 6.9. Diterpeno e furanosesquiterpeno from *Bursera*

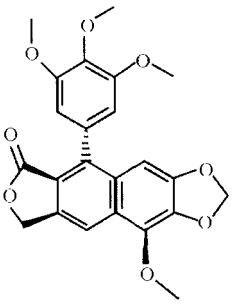
Some podophyllotoxin type lignans were also isolated from *Bursera* species. From *Bursera permollis* stems, deoxypodophyllotoxin, β-peltatin methyl ether, picro-β-peltatin methyl ether, dehydro-β-peltatin methyl ether and nemerosin were already identified (Wickramaratne *et al.*, 1995). From *Bursera tonkinensis* roots 4'-demethyldesoxypodophyllotoxin, β-D-glucoside-4-demethyldeoxypocophyllotoxin (Fig 6.10) and three others lignans (not related to podophyllotoxin) burserolignan, burseneolignan, (+)-isolariciresinol, 5-methoxy-(+)-isolariciresinol (Fig 6.11) and (+)-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxypropyl)-2,6-dimethoxyphenoxy-1,3-propanediol, dihydrodehydrodiconiferyl alcohol and (+)-5-methoxy-trans-dihydrodehydrodiconiferyl alcohol were already described (Juntiviboonsuk *et al.*, 2005).



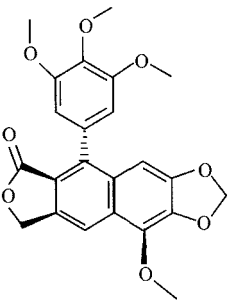
	R_1	R_2
deoxypodophyllotoxin	H	H
β-peltatin methyl ether	H	MeO



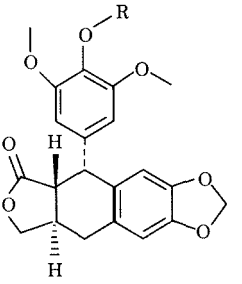
nemerosin



picro-β-peltatin methyl ether

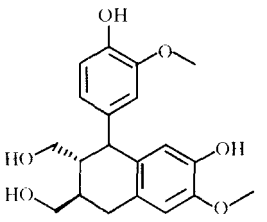


dehydro-β-peltatin methyl ether

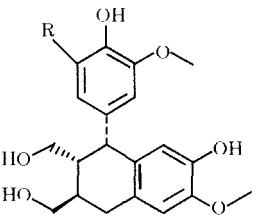


	R
4'-demethyldesoxypodophyllotoxin	H
β-D-glucoside-4-demethyldeoxy-podophyllotoxin	Glucoside

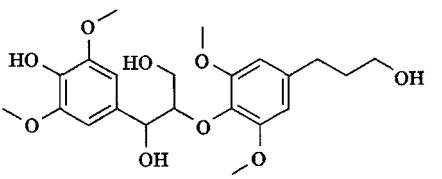
Fig 6.10. Podophyllontoxin type lignans from *Bursera*



Burselignan



	R
(+)-isolariciresinol	H
5-methoxy-(+)-isolariciresinol	MeO



Burseneolignan

Fig 6.11. Others lignans isolate of *Bursera tokinensis*

Several flavonoids were identified from *Bursera* stems. From *B. leptophloeos*, five flavonoids were isolated: 8-(3″-hydroxy-3″-methylbutyl)-5,7,4'-trihydroxydihydroflavonol, 6″,6″-dimethyldihydropyran-(2″,3″:7,8)-5,4'-dihydroxydihydroflavonol, 8-(3″-hydroxy-3″-methylbutyl)-5,7,4'-trihydroxyflavonol, 8-(γ,γ-dimethylallyl)-5,7,4'-trihydroxydihydroflavonol and 5″-isopropenyldihydrofuran-(2″,3″:7,8)-5,4'-dihydroxydihydroflavonol (Souza *et al.*, 1989).

From *Bursera tokinensis*, the phenylpropanoid bursephenylpropane and the coumarin scopoletin were isolated (Juntiviboonsuk *et al.*, 2005). This coumarin was already isolated from *P. hebetatum* and *P. heptaphyllum* (Costa, 1996; Bandeira *et al.*, 2002). The xanthone lichexanthone was another secondary metabolite isolated from this tribe, from *Trattinnickia peruviana* (Marques & Ribeiro, 1994).

In the Protieae tribe, Lima *et al.* (2001) identified the triterpenes 3β,24-dihydroxy-cycloart-25-ene and 3α,20(S)-dihydroxydammar-24-ene from the wood of *Crepidospermum rhoifolium* (Fig 6.12), together with stigmasterol, campesterol, sitosterol and its glycosilated form 3-*O*-β-D-glycopyranosil-sitosterol. In the *Tetragastris altíssima*, friedelin, taraxerol and the *sec*-isobrionoic acid were observed in the wood, the very first seco triterpene observed from *Protieae* (Lima *et al.*, 2001). In the bark of the trunk from *Protium hebetatum* only α- and β-amyrin were observed (Costa, 1996).

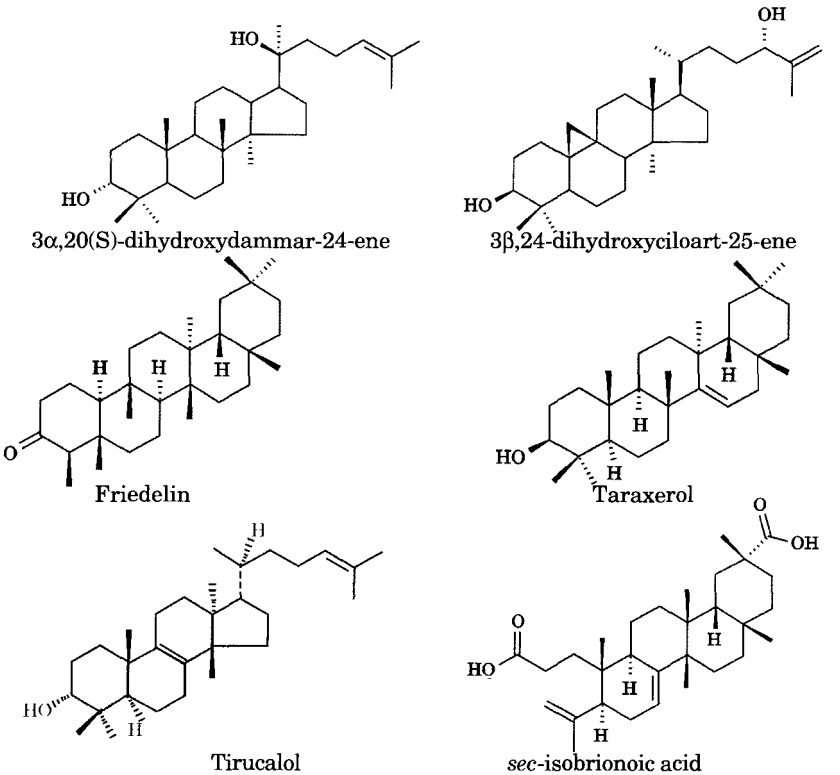
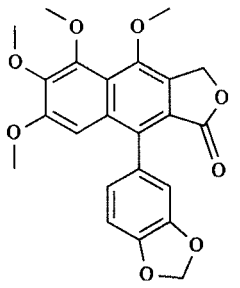
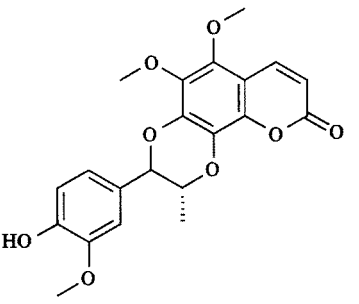


Fig 6.12. Triterpenes from species of Protieae tribe

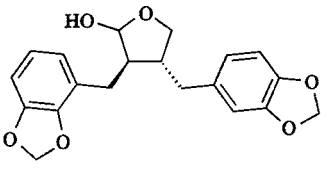
Propaicin, from *P. opacum* (Zoghbi *et al.*, 1981), and 5-methoxyjusticine A and the coumarinolignaoid 5-methoxypropaicin, from *P. unifoliolatum* (Siani *et al.*, 1998; Magalhães *et al.*, 2006) were observed in the *Protium* genus. In the *P. tenuifolium*, the lignans (-)-cubebine and (+)-(2S,3S)-2-(3″,4″-methylenedioxybenzyl)-3-(3',4'-methylenedioxyacetophenone)-butyrolactone were detected in the stems (Siqueira *et al.*, 1995). In other protieae tribe species, the lignans parabenzolactone and (-)-savinin were described from *Crepidospermum rhoifolium* and *Tetragastris altíssima*, respectively (Lima *et al.*, 2001) (Fig 6.13).



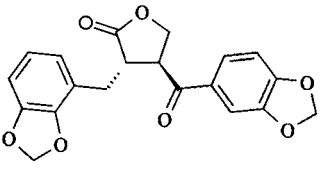
5-methoxyjusticidin A



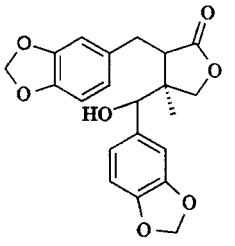
5-methoxypropacin



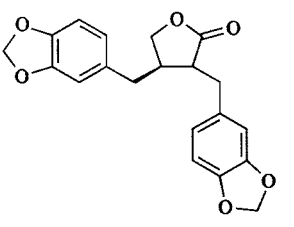
(-)-cubebin



(+)-(2S,3S)-2-(3″,4″-methylenedioxybenzyl)-3-(3',4'-methylenedioxyacetophenone)-butyrolactone



Parabenzolactone



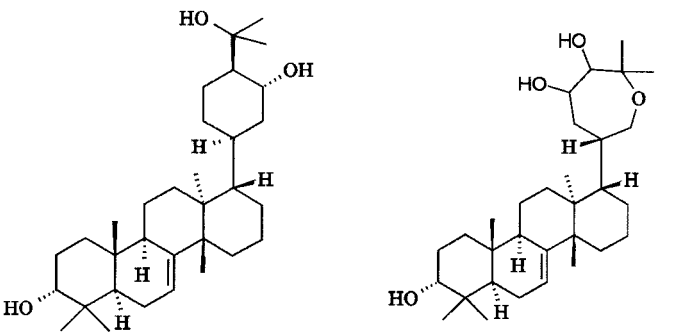
(-)-savinin

Fig 6.13. Lignans of the Protieae tribe species

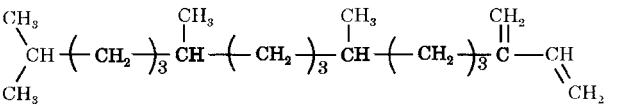
From *Dracryoides* and *Trattinnickia*, Canarieae tribe, only triterpenes and sterols were observed in the stems. Besides α- and β-amyrin (from *T. burserifolia* and *T. rhoifolia*) only lupeol, tirucalol, sitosterol and stigmasterol were detected in the *Dacryodes hopkinsii* (Lima *et al.*, 2004) and campesterol, β-sitosterol and stigmasterol from *T. peruviana* (Marques & Ribeiro, 1994).

Seus Frutos and Folhas

The fruits and leaves extracts from *Bursera* species showed the presence of triterpenes, diterpenes and steroids, as sapelin A and sapelin B (Fig 6.14), in the *B. klugii* (Jolad *et al.*, 1977); and campesterol, stigmasterol, sitosterol, α-amyrin and 3-methylene-7,11,15-trimethylhexadec-1-ene (neophytadiene), in the *B. simaruba* (Carretero *et al.*, 2008).



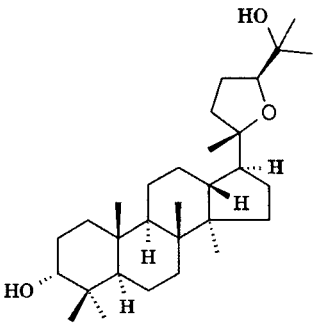
Sapelin A Sapelin B



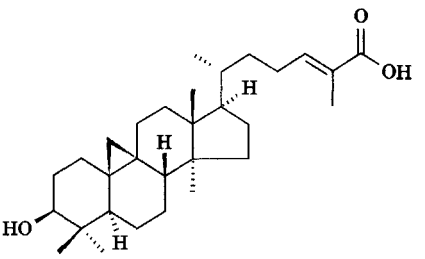
neophytadiene

Fig 6.14. Metabolites from fruits and leaves from Burseraceae

The common sitosterol was found together with lupeol and the tri-epoxy-triterpen cabraleadiol in *Protium apiculatum* leaves (Lima *et al.*, 2001). From *P. bahianum* fruits the pentacycle triterpenes, mangiferolic acid (Fig 6.15), α- and β-amyrin were identified (Oliveira *et al.*, 2006). From *P. strumossum*, Guimarães and Siani (2007) isolated 3-*epi*-fridelanol, friedeline, lupeol, lupenone, α- and β-amyrin and β-sitosterol-3-*O*-glicoside. Triterpenes were observed in the leaves extracts from *Tetragastris altissima*: friedeline, taraxerol and β-sitosterol (Lima *et al.*, 2001).



Cabraleadiol

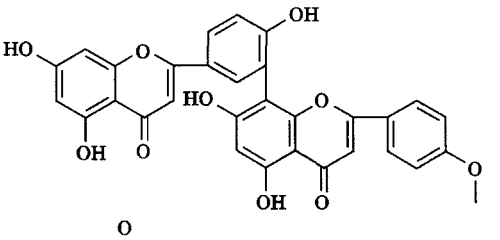


Mangiferolic acid

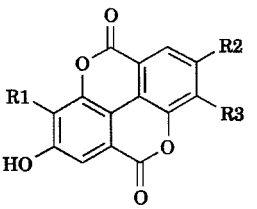
Fig 6.15. Triterpenes from Burseraceae leaves

Quercetin, quercitin-3-*O*-rhamnoside and (-)-catechin were isolated together with the coumarin scopoletin (7-Hydroxy-6-methoxycoumarin) (Bandeira *et al.*, 2002) from the *Protium heptaphyllum* leaves. Deoxypodophyllotoxin was also isolated from *B. microphylla* leaves (Bianchi *et al.*, 1968).

Ellagic, galic, 3,3',4-tri-*O*-methylellagic acid and 3,3'-di-*O*-methylellagic acids and podocarpusflavon A (Fig 6.16) were isolated and identified from *Trattinnickia glaziovii* leaves (Siani & Ribeiro, 1995).



Podocarpusflavone A



	R1	R2	R3
Ellagic acid	OH	OH	OH
3,3',4-tri-*O*-methylellagic acid	OMe	OMe	OMe
3,3'-di-*O*-methylellagic acid	OMe	OH	OMe

Fig 6.16. Aromatic compounds of *Trattinnickia glaziovii*

PHARMACOLOGY

Several studies have already been performed with oleoresins and extracts from Burseraceae family, mainly with α- and β-amyrin obtained especially from *Protium heptaphyllum* and *P. kleinii*. Anti-inflammatory was the main biological activity reported in several different models.

The dose-related edema reducing activity of α-amyrin (IC_{50}= 0.31 mg/ear) was tested in rats where 12-*O*-tetradecanoilforbol acetate (TPA) was used as inflammatory agent (Otuki *et al.*, 2005b). The α- and β-amyrin mixture showed anti-platelet and anti-inflammatory (induced by TPA) activity too (Aragão, 2004, 2007). Essential oil from the bark of the trunk of *Bursera lancifolia* showed anti-inflammatory activity induced by TPA. The oil is composed by the monoterpenes α-terpineol, terpinen-4-ol, α-thujene, linalool and limonene (Zuniga *et al.*, 2005).

Protium heptaphyllum oleoresin activity was tested in rats treated with hydrochloric acid and ethanol-HCl on gastric inflammation and ulcer models. Total acidity was observed, without gastric secretion volume reduction, when rats were orally treated with 200 and 400 mg/kg, showing cytoprotective and anti-ulcerogenic activities (Oliveira *et al.*, 2004b; Oliveira, 2005).

Hexane extract from *Bursera simaruba* leaves was tested in carrageenan-induced paw oedema test in mice. Neophytadiene, ergost-5-en-3β-ol, 24*S*-stigmast-5,22*E*-dien-3β-ol, 24*S*-stigmast-5-en-3β-ol and α-amyrin fractions showed strong activity (Carretero *et al.*, 2008).

The α- and β-amyrin mixture showed hepatoprotectivity activity in rats treated with acetaminofen. The pre-treatment of this mixture at 50 and 100 mg/kg (*i.p.*) promoted the oxidative stress and toxic metabolites formation in liver, an effect probably related to anti-inflammatory activity (Oliveira, 2005; Oliveira *et al.*, 2005a).

This same amyrin mixture at 100 and 200 mg/kg showed anti-prurigineous (Oliveira *et al.*, 2004a; Oliveira, 2005), and antinociceptive activities. The visceral pain reduction was observed with capsaicin and cistite hemorrhagic induced by ciclofosfamide and mustard oil inflammation models. The results indicate the action on opioid and vaniloid receptors (Lima Junior, 2005). In the capsaicin model the antinociceptive activity showed similar results (Oliveira, 2005; Oliveira *et al.*, 2005b). Otuki *et al.* (2005a) observed that anti-inflammatory and anti-nocipetive activities could evolve protein Kinase A and B.

Susunaga *et al.* (1996), analyzed crude extracts, neutral fraction and the tritepenes ψ-taraxastanonol and *epi*-ψ-taraxastanediol obtained from *P. heptaphyllum* oleoresin. The triterpenes showed to be 5 times more active

than the neutral fraction. Oliveira (2005), found similar analgesic activities studying the ethereal extracts and the oleoresin from *P. heptaphyllum* in formalin induced contortion tests.

Using the Ellman assay for acetylcholinesterase activity the *P. heptaphyllum* oleoresin, its essential oil and (-)-catequin obtained from the green fruits from this specie were analyzed. The hexane extract (3.3 mg/mL) and its essential oil (17 mg/mL) showed 25% and 48% inhibition, respectively (Trevissam *et al.*, 2003). In an another study, oleoresin and (-) catequin showed inhibition at 0.5 mg/mL and 1.0 μg/mL, respectively (Bandeira *et al.*, 2002).

The cytotoxicity potential from Burseraceae essential oils, extracts and oleoresin was studied using the *Artemia salina* model, showing a high toxicity to *Protium araconchini* methanolic root extract and to *P. heptaphyllum* essential oil and oleoresin (Quignard *et al.*, 2003, 2004; Cito *et al.*, 2003; Ramirez *et al.*, 2004), and at tumoral cell model, as JJ74 (monocitic rat cells), SP2/0 (rat plasmocitom cell) and Neuro-2A (rat neuroblastom cell). The effect against tumoral cells was attributed to monoterpenes from essential oils (Siani *et al.*, 1999a). From *Bursera simaruba* leaves essential oil, the cytotoxicity against A-549 (human lung carcinoma) and DLD-1 (colon adenocarcinoma) were measured indicating the activity of some of their constituents: limonene, β-caryophyllene, α-humulene and germacrene D (Sylvestre *et al.*, 2007).

Isolated lignans were tested against tumoral cells too, as β-peltatin A-methyl ether and 5'-demethoxy-β-peltatin A-methyl ether, obtained from *Bursera fagaroides*, that were tested against Walker carcinoma 256 (intramuscular) tumor system (Bianchi *et al.*, 1969). The lignans sapelin A and B, isolated from *B. klugii* leaves, showed anti-tumoral activity against 9KB (human epidermoid carcinoma) and 3PS (P-388 lymphocytic leukemia) cells (Jolad *et al.*, 1977). At KB, Col2 and LNCaP cell lines, a significant effect was observed to demethyldesoxypodophyllotoxin obtained from *B. tonkinensis* roots (Jutiviboonsuk *et al.*, 2005).

Burseraceae extracts were already tested to *T. cruzzi* (low activity), and to promastigote forms of *Leishmania sp.* (moderate activity) and *Leishmania panamensis* (low activity) (Weniger *et al.*, 2001). The antiplasmodial assay showed that *Protium glabrenses* bark extracts at 100 mg/Kg inhibited at 61% the *Plasmodium* activity (Deharo *et al.*, 2001). Using the phenylpropanoid extracts from *Protium heptaphyllum* barks at 250 mg/Kg, a reduction of 58% of *Plasmodium berghei* parasitesy was observed (Almeida *et al.*, 2001). The effect is attributed to the NO inhibition by essential oil monoterpenes (Siani *et al.*, 1999a), since NO high concentration is necessary to reproduction and development of *Plasmodium* (Deharo *et al.*, 2001).

Several studies show some anti-microorganism activity in the Burseraceae. The *Bursera simaruba* fruit essential oils and stems extracts were tested against *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcoccus aureus*, methicillin resistant *Staphylococcus aureus* (MRSA) and beta-haemolytic *Streptococcus* group A (BHSA) (Junor *et al.*, 2007). *B. lunanii* oleoresin essential oil was tested to the same microorganisms (Junor *et al.*, 2007). Extracts and fractions from *Bursera graveolens* that contain the triterpenic acids β-elemonic, α-elemolic and 3α-hydroxytirucalla-7,24-dien-21-oic showed inhibitory activity against *Bacillus subtilis* and *Staphylococcus aureus* at 250 mg/mL for extracts and 150 mg/mL for fractions (Robles *et al.*, 2005).

The antiviral activity from methanolic and water extracts from *Tetragastris panamensis* showed a viral infection reduction to HSV-1 (Herpes simplex) and VSV (vesicular stomatitid virus) (Roming *et al.*, 1992).

CONCLUSIONS

Burseraceae, a very important botanical family, distributed all over the world has several commercial and medicinal applications. From the several triterpenes already detected, the lignans and coumarins isolated and identified, almost none pharmacological activity study can correlate the chemical composition to the popular extensive use. The recently published literature show that the complete chemical composition is still to be discovered, with several highly active ligans and many other substances that are hidden behind some very common triterpenes.

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Are Well-Studied Snake Venoms Well Investigated? Strategy for Isolation of New Polypeptides from Snake Venom

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ABSTRACT

Snake venoms are complex mixtures of peptides and proteins with high biological activity. The concentrations of constituents in the venoms may differ by several orders of magnitude. The most abundant peptides and proteins in venoms of the most poisonous snakes are well characterized. The low abundant or minor polypeptides are not so well studied or not studied at all. At the same time, minor venom constituents may possess unique biological activity. To isolate these polypeptides for biological studies, the adequate separation methods should be elaborated. Moreover, although all venom proteins may be classified into only several structural types, particular components within a definite structural type may have completely different biological activity sometimes with opposite directions of effects. Such compounds with a novel, "unusual" or unique activity may be the minor constituents of the venom, that once more emphasize the necessity for the development of adequate methods for their separation from the well-studied abundant components. To achieve the target component purity, necessary for its structural and functional studies, a method including at least three different steps of liquid chromatography should be applied. Gel-filtration, which allows fractionating venom components by molecular size, is the

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method of choice for the first purification step. Use of high performance ion exchange chromatography (HPLC or FPLC) ensures further separation of components by charge, and in most cases reversed-phase HPLC is very appropriate for the final purification. We have used this scheme to study Naja kaouthia cobra venom and characterized several minor polypeptides structurally or functionally novel for cobras. Thus, from this venom we have isolated for the first time structural homologues of mamba muscarinic toxins, a dimeric long neurotoxin, functional analogue of krait kappa-bungarotoxin, thaicobrin, the first member of new protein family, and several homologues of proteins from CRISP family. Moreover, in the same venom a glycosylated cytotoxin, the first representative of glycosylated three-fingered toxins, was identified. This minor protein was found at the background of high concentration of "normal" non-glycosylated cytotoxin. The venoms of other cobras were also fractionated using this scheme that allowed isolating of minor proteins with unique biological properties. We believe that this method is the easiest way to identify and purify new minor proteins from complex protein mixtures. It allows drastic reduction of mistakes resulting from the presence of admixtures of unseparated components in the target proteins.

Key words : Chromatography, cobra, gel filtration, minor component, purification strategy, snake venom

INTRODUCTION

Snake venoms are complex mixture of polypeptide compounds demonstrating high activity and selectivity to their targets in a prey organism. This is a reason why many of these compounds are widely used as valuable biochemical tools (*e.g.* anticoagulants (Kini, 2006), neurotoxins (Tsetlin & Hucho, 2004) or biomedicines (Koh *et al.*, 2006)). However, the capacity of snake venoms as a source of valuable compounds is not fully utilized yet.

The principal manifestations of poisoning by snake venom are coagulopathy and hemorrhage caused by "coagulopathic" venoms from snakes of Viperidae family (vipers, rattlesnakes, and copperheads) or neuromuscular blockage produced by "neurotoxic" venoms from snakes of Elapidae family (cobras, mambas, kraits, sea snakes etc.). These symptoms are caused by major (prevailing) components, from 5 to 10 of which may present in venom. They are relatively well studied. However, genes of snake venom proteins undergo very high mutation rates and have tendency of

duplication (Ohno *et al.,* 2002). In contrast to the ordinary protein genes, accelerated Darwinian evolution of venom protein genes is due to rapid change in exons, but not in introns and the flanking regions, which are changed at the rates of ordinary genes. It should be noted that accelerated evolution proceeds until the isoforms have acquired their particular new function and, since then, they have evolved with less frequent mutation, possibly due to the functional conservation (Ohno *et al.,* 2003). Due to these reasons, major highly toxic venom components are almost always accompanied by less abundant (minor) homologues, that are structurally very similar to major ones and may have, as a result of a mutation, reduced toxicity or an altered spectrum of biological activity (*e.g.* see (Doley *et al.,* 2008)). Moreover, snake venoms also contain a lot of minor components belonging to structural types that are different from those of major components. A pool of minor components possesses broader spectrum of biological activity, and at the same time is much less toxic, as compared to major components.

As venoms are complex protein mixtures, the proteomic approach is a method of choice for their study. Indeed many papers were published on proteomic characterization of snake venom (see recent review by Fox & Serrano, 2008). Application of this approach to cobra venoms has allowed researchers to identify more than 60 polypeptides (Li *et al.,* 2004; Kulkeaw *et al.,* 2007). However, our observation based on the number of chromatographic peaks corresponding to toxin isoforms and homologues indicates the presence of more than 200 polypeptide components in a single cobra venom. Venoms of snakes from Viperidae family may also contain more than 100 individual compounds (Georgieva *et al.,* 2008). Nevertheless, all snake venom polypeptides, in spite of their large number and a wide variety of effects may be classified only into a few structural types (Table 7.1). Besides, the compounds listed in Table 7.1, snake venoms contain also some other enzymes (hyaluronidases, acetylcholinesterases, nucleases, etc), oligopeptides (*e.g.* bradykinin-potentiating peptides (Ferreira *et al.,* 1998), hannahpep (Gomes & De, 1999), and low molecular weight venom-nonspecific compounds (*e.g.* nucleosides; (Arid, 2005)). The data from Table 7.1 clearly show that biological effects of particular compounds belonging to one structural type (and even to one group within the type) may vary considerably. It should be noted that the content of these compounds can differ by several orders of magnitude. Venom components with unique biological activity often are present in very low amounts as minor components. Thus, their studying requires the development of adequate isolation methods.

Table 7.1. Structural types of snake venom proteins

Structural type	Group	Distribution	Biological effect(s)	Abundance
Reprolysins	Metalloproteases IIIP	All venomous snakes	Coagulopathy, platelet aggregation, or the complement system inhibition	Minor in Elapidae, major in Viperidae
	Metalloproteases IP, IIP, IVP	Viperidae	Coagulopathy	Major
	Desintegrins	Viperidae	Disrupt cell adhesion	Major
Serine proteases	Thrombin-like serine proteases	Viperidae	Coagulopathy	Major
	Kallikrein-like serine proteases	Viperidae, Australian Elapidae	Coagulopathy	
	Other	Ophiophagus	Coagulopathy	Minor
Three-fingered toxins	Short-chain α-neurotoxins	All Elapidae	Block muscle-type nicotinic acetylcholine receptors (nAChR)	Major
	Long-chain α-neurotoxins	All Elapidae	Block muscle-type and α7 nAChR	Major
	"Weak" toxins	Naja, Bungarus, Microrus	Block muscle-type and α7 nAChR, interact with mucarinic acetylcholie receptors (mAChR)	Major (minor in some venoms)
	Muscarinic toxins and muscarinic toxin-like proteins	Dendroaspis, Naja	Interact with mAChR	Minor
	Cytotoxins	Naja, Hemachatus	Cytotoxicity	Major
	Hemextins	Hemachatus	Coagulopathy	
PLA2	IA or IB	All venomous snakes	Cytotoxicity, and/or coagulopathy, and/or impair platelet aggregation, and/or induce neurite outgrouth	Major
	II	Viperidae	The same	Major

Table 7.1. *Contd.*

Structural type	Group	Distribution	Biological effect(s)	Abundance
Oxidase of L-amino acids		All venomous snakes		Major (minor in some venoms)
Trypsin inhibitors of Kunitz type	Naja, Ophiophagus Vipera	Inhibit serine proteinases		
Cysteine-rich secretory proteins		Naja, Ophiophagus, Australian Elapidae, Some Viperidae	Ionic channel blockers	Major (minor in some venoms)
Growth factors	NGF	All venomous snakes	Promote neural cell survival, induce neurite outgrouth	Minor
	VEGF-like	Viperidae		
C3b-like proteins	CVF	Naja, Austrelaps	Deplete the complement system	Minor
Cystatines		Naja, Bitis		Minor
Vesprins		Naja, Ophiophagus	Hyperalgesia	Minor
Lectins	C-type lectin-like protein	Viperidae Ophiophagus	Induce platelet aggregation	Major
Waprins		Naja	Antimicrobial	

In our laboratory, the proteomic study of the Thailand cobra *Naja kaouthia* venom has been carried out during preceding years. The main task of this study was structural and functional characterization of new minor components that can be accomplished only through isolation of an individual compound in the native form. To obtain the toxins with the purity necessary for such purpose, we have chosen a method including three different types of liquid chromatography: gel-filtration, ion-exchange and reversed phase chromatography. We believe that this scheme is necessary to purify the venom minor components which can be reliably used for structure and biological activity studies. The deviation from this scheme (*e.g.* omission of one stage) may result in obtaining of insufficiently pure samples that, in turn, can lead to misinterpretation of the results.

Three-step Liquid Chromatography for Separation of Snake Venoms

It should be noted that the choice of purification scheme is strongly dependent on the required final purity of the sample and isolation scheme for a particular compound may be adjusted to the requirements. For example, if α-cobratoxin, the main neurotoxin of *Naja kaouthia* cobra venom, should be prepared for structural studies (NMR or X-ray), in principle, a simple one-step isolation scheme elaborated about forty years ago (Karlsson *et al.*, 1971) and giving the toxin with purity of 98-99% may be used. However, if this toxin is used for toxicity studies on cell lines it should be thoroughly purified from cytotoxins and phospholipases A2 that are highly toxic to cell cultures. α-Cobratoxin is the main venom component comprising about 25% of dry venom weight and it should be purified "only" four fold to obtain the purity of 99%. This task is much more complicated for minor venom components. Thus, to gain the same 99% of purity for some minor component with content in venom of about 0.1%, it should be enriched by the factor of about 1000. At the present state of purification technique, this is practically impossible with one-step purification scheme. The only one exception may be affinity chromatography used under very strictly controlled conditions: the affinity resin should be absolutely selective to the target compound and should not bind any other venom proteins (especially homologues) structurally or functionally related to it. The affinity chromatography in relation to purification of snake venom proteins will be shortly discussed below.

Taking the above considerations into account, we have chosen for the separation of crude venom, the chromatographic scheme that allows the fractionation of proteins according to their size (gel-filtration), charge (ion-exchange chromatography) and hydrophobicity (reversed-phase chromatography).

Gel-filtration

The first and very important step of separation is gel-filtration that allows fractionating the venom proteins by size of the molecule. We consider this step necessary because low and high-molecular weight components require

different conditions for further purification. Appropriately chosen gel-filtration medium permits to solve the problem of contamination for compounds with very close charges and hydrophobic properties which may not be well separated by ion-exchange and reversed-phase chromatography. Classical low pressure gel-filtration (*e.g.* on glass column packed with Sephadex medium) gives satisfactory resolution even for large venom quantities: hundreds of milligrams or grams depending on column size. HPLC columns for gel-filtration are indispensable for analytical purposes, however according to our experience they do not exceed classical gels in efficiency of preparative separation of crude venoms.

These considerations can be illustrated by the example of phospholipase A_2 (PLA2), which is one of the main components of snake venoms. PLA2s are usually present in venom as several homologues, and their pool very often overlaps considerable range of pI and hydrophobicity. Therefore in some cases the separation of the target compound from contaminating PLA2 is not possible without gel-filtration. The only limitation is that the molecular mass of this compound should be different from that of PLA2. Interestingly, nerve growth factor (NGF) from cobra venom possessing molecular mass (about 13 kDa) similar to that of PLA2 forms non-covalent dimer under conditions of gel-filtration used for the separation of the crude venom and is separated from monomeric cobra PLA2s (Fig 71).

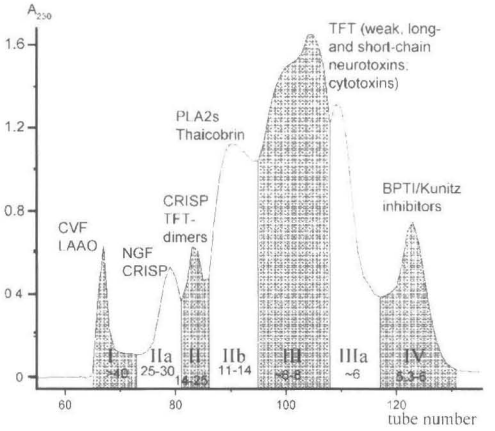

Fig 7.1. Separation of crude venom of *Naja kaouthia* by gel filtration on Sephadex G-50sf column (4, 5 x 150 cm) in 0.1 M ammonium-acetate buffer, pH 6.2, fraction volume 9 mL. Roman numbers indicate pooled fractions, and the numbers beneath – molecular masses of constituent proteins. Here and in the next figure, CLBP is cytotoxin-like basic protein, CRISP - cysteine-rich secretory protein, α-CT-α-cobratoxin, αCT-CX - dimer formed by α-cobratoxin and corresponding cytotoxin, CVF-cobra venom factor, CX-cytotoxin, DαCT-homodimer of α-cobratoxin, ISP-BPTI/Kunitz-type serine proteinase inhibitor, LAAO-L-amino acid oxidase, MTLP-muscarinic toxin-like protein, NGF-nerve growth factor, NWT-a new "weak" toxin, PLA2-phospholipase A_2, TFT-three-finger toxin, WTX Trp-containing "weak" toxin

As a buffer, we usually use 0.1 M ammonium acetate with pH 6.2. The choice of pH is based on pH value (about 6.0) of the fresh liquid cobra venom. Furthermore, the weakly acidic medium prevents the spontaneous disulfide isomerization in proteins. Ammonium acetate was chosen because of its volatility under freeze drying. Thus, after three-fold lyophilization of collected protein (dissolved each time in HPLC-grade water) it becomes practically free of ammonium acetate and could be used for electrophoresis and MALDI mass-spectrometry analysis. However, it should be noted that some high molecular weight proteins (*e.g.* cobra venom factor with molecular mass of about 145kDa) can not be freeze-dried in ammonium acetate buffer and require removal (for example by ultrafiltration) of this buffer before drying.

Ion-Exchange Chromatography

In principle ion-exchange chromatography separates proteins according their charges. If the net charge or pI of the target protein is not known the origin of the venom can be taken into account. Thus, the viper venoms contain mostly acidic proteins, which can be effectively separated by anion-exchange chromatography, while cobra venoms consist mostly of basic proteins, for separation of which cation-exchange chromatography should be used.

At present a vast array of ion-exchange resins is available in the market and sometimes several different supports should be used to isolate target protein. Thus, for the isolation of muscarinic toxin-like proteins MTLP-1 and MTLP-3 from cobra venom we used HEMA BIO 1000 CM column (Tessek), while for isolation of MTLP-2 a combination of cation-exchanger BioRex 70 (Bio Rad) and TSK CM-3SW (LKB) was applied (Kukhtina *et al.*, 2000). As to elution buffers, their choice depends on the further use of the sample. As discussed above, ammonium acetate buffer is volatile and can be easily removed by freeze drying, however the traces of this buffer can disturb coagulometric and immunological tests, therefore, Tris of phosphate buffer should be used in these cases.

Reversed-Phase Chromatography

This type of liquid chromatography is well known for high efficiency, that is why it is sometime applied as a single step for the separation of such a complex protein mixtures as animal venoms. However, presently it is not possible to obtain highly pure venom proteins using solely reversed-phase HPLC. The reason is that the hydrophobicity of venom proteins covers relatively small range: practically all venom proteins elute from C18 column in the acetonitrile concentration range from 20 to 55%. Therefore it is difficult to expect a good separation of more than one hundred proteins under these conditions.

Reversed-phase HPLC is the method of choice for the final purification step. However, the high molecular weight proteins sometimes are inactivated or denatured under conditions of this type of chromatography. Thus, according to our data, snake venom serine proteinases and cobra venom factor are inactivated after this procedure. Some alternative methods should be used for the final purification of labile proteins, *e.g.* ion-exchange re-chromatography or hydrophobic interaction chromatography.

Hydrophobic Interaction Chromatography

This method is suitable for purification of large (100kDa and more) proteins. For example, L-amino acid oxidase from *Naja oxiana* venom was purified by combination of gel filtration and cation exchange chromatography. The hydrophobic chromatography on HPS-7 (Kemotex Bio, Tallinn, Estonia) was used as a last step that allowed the removal of small impurities (Samel *et al.,* 2008). This method may be used for the final purification and seems to be ineffective as the main step due to broadness of peaks.

Affinity Chromatography

This method can be used for the isolation of known proteins or proteins with known activity. For example, some venom serine proteinases have arginine esterase and arginine amidase activities; therefore, they can specifically recognize arginine-containing substrates. To prepare the affinity medium for these enzymes, L-arginine was immobilized on Sepharose 4B via a long spacer. The affinity chromatography on the prepared column allowed to remove in one step more than 95% of venom proteins and purify the target component (Xin *et al.,* 2007). A number of amidine derivatives are able to interact specifically with serine proteases. The affinity chromatography on *p*-aminobenzamidine substituted agarose was proposed for isolation of thrombin-like proteins (TLP) from snake venom (Holleman & Weiss, 1976). However, it has been recently shown that the specificity of such an interaction is unpredictable; two different commercially available benzamidine affinity matrices bind two different TLPs from each of two snake venoms (*Bothrops jararacussu* and *Lachesis muta rhombeata*), but by the combination of both supports three other TLPs from the same venoms have been isolated (De-Simone *et al.,* 2005).

Metal ion-affinity chromatography can be used to fractionate venom metalloproteinases due to the presence of the metal ion-coordination motif in these enzymes. It has been shown that Ni(2+)-agarose is suitable for isolation of metalloproteinases from both cobra and viper venoms (Wijeyewickrema *et al.,* 2007).

Several method were suggested for affinity purification of PLA2s on the basis of different affinity supports: ethanolamine and choline phospholipids (Rock & Snyder, 1975), Affi-Gel Blue (Hazzlet & Dennis, 1985), biospecific

supports of an organo-silica type with immobilized phospholipid (Evstratova *et al.,* 1982), 2-acylamino phospholipid analogues (Dijkman *et al.,* 1997), glycyrrhizin (Ohtsuki *et al.,* 1998), and betulinic acid (Tseng & Lee, 2004). The affinity chromatography on immobilized lactose was used for the purification of lectin from *Bothrops insularis* venom (Braga *et al.,* 2006).

Several original methods were developed for isolation of minor components from snake venom. For examples, S-carboxymethylpapain-Sepharose was used to isolate a new cystatin from the Taiwan cobra venom (Brillard-Bourdet *et al.,* 1998). Thiophilic adsorption chromatography (TAC) is an efficient procedure for purification of the human component complement C3. Native CVF, its homologue, was purified by TAC in a one-step procedure from cobra venom with yield of 92% as compared to 35% by conventional approaches (Kolln *et al.,* 2007).

However, it should be noted that affinity resin can interact not only with one venom component, but with a pool of compounds with close functional (or structural) properties: serine proteinases, metalloproteinases, PLA2s etc. Affinity chromatography separates most venom components from the target protein; nevertheless to obtain the target protein of high purity, some additional purification steps are usually required. This is also true for immunoaffinity chromatography. Thus, it was reported (Stábeli *et al.,* 2005) that antibodies elicited against a fragment of *Bothrops moogeni* L-amino acid oxidase (*Bm*-LAAO) cross-reacted with snake venoms components totally unrelated to the parent molecule, confirming that drawbacks in immuno-based methods should be taken into account.

Alternatively, affinity chromatography can be used to remove "inappropriate" component(s) from the desired compound. For example, benzamidin-sepharose was applied to separate LAAO from other components of *Bothrops* venom (Izidoro *et al.,* 2006), the main constituents of which are serine proteinases capable to bind benzamidin.

It is quite possible that one will raise doubt about the necessity of such a sophisticated scheme for venom protein purification. However, to obtain a preparation of minor venom protein with high purity, even this scheme may be not be sufficient and some other steps are required. For example, for the final removal of residual PLA2 activity from the sample, modification of essential histidine residue in the active center of enzyme with affinity reagent *p*-bromophenacyl bromide can be carried out.

It should be noted that speaking about the choice of the scheme for the separation of crude snake venom we do not suggest some new method of chromatography, but just emphasize the importance of multi step purification that include classical gel-filtration.

MINOR PROTEINS WITH NEW PROPERTIES IN COBRA VENOM

As earlier discussed, the venom of *N. kaouthia* cobra may contain more than 200 polypeptides. However, only about ten proteins can be classified as major components: long chain α-neurotoxin, α-cobratoxin (accession number in UniProt Knowledgebase P01391), one or two short chain α-neurotoxins, four cytotoxins (P60305, P01445, P01446 and P60303), weak toxin WTX (P82935), and PLA2 CM2(P00596) with close homologues, which are not easily separated. Being present in the venom in high amounts, these proteins cover the whole chromatographic profile, when the crude venom is separated by ion-exchange (Fig 7.2) or reversed phase HPLC. It is obvious that some additional purification step(s) is necessary for isolation of minor venom components. Classical gel-filtration can be well applied for this purpose.

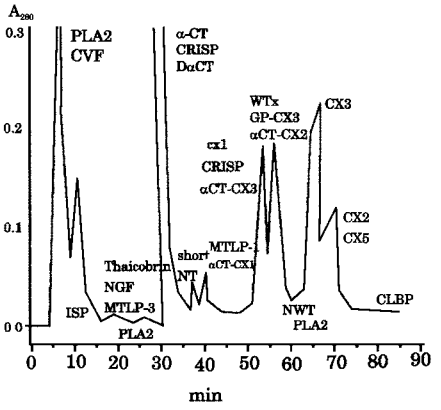

Fig 7.2. Crude *Naja kaouthia* cobra venom (7.5 mg) separated by cation-exchange HPLC on a HEMA BIO CM column (250 × 10 mm; Tessek) in gradient of ammonium-acetate buffer, pH 7.5, from 5 mm to 1 M in 100 min at flow rate 1 mL/min. The major components are signed in bold and minor - italicized

Using gel-filtration on Sephadex G-50, *N. kaouthia* venom can be separated in several fractions (Fig 7.1): fraction I containing high molecular weight (>30kDa) proteins, fraction II-proteins with masses in the range from about 9 to 30kDa, main toxic fraction III-6-8kDa and fraction IV low molecular weight compounds. To find new proteins, the main toxic fraction III was analyzed at first. Principally new for cobra venom components, the so-called muscarinic toxin-like proteins were found in this fraction (Kukhtina *et al.,* 2000). These toxins are structurally very similar to muscarinic toxins earlier found only in mamba venom. However, the noticeable affinity to muscarinic acetylcholine receptor was observed only for one toxin MTLP-1 (P82462) (Kukhtina *et al.,* 2000). Therefore, one cannot exclude that these toxins have some other biological targets in prey organism.

An abundant source of new proteins proved to be fraction II. This fraction itself can be named "minor" compared to fractions III and I (Fig 1). Analysis

of the fraction II showed that it consisted of minor and practically unstudied proteins (with the exception of nerve growth factor P61899). The main constituents of this fraction were proteins of so-called CRISP (Cysteine RIch Secretory Proteins) family (Osipov *et al.,* 2001), which were not found earlier in cobra venoms. These proteins are practically not toxic and present in venom as three groups of close homologues (Osipov *et al.,* 2005). CRISP proteins from other snake venoms (for review see Yamazaki & Morita, 2004) can also be classified into these three groups. It seems that these proteins represent a new class of toxin acting on ionic channels. Thus, some of them block cyclic nucleotide gated channels (Yamazaki *et al.,* 2002), other high-conductance calcium-activated potassium (BK(Ca) channel (Wang *et al.,* 2005) and voltage-gated calcium channels (Yamazaki *et al.,* 2003).

In addition to proteins of CRISP family, fraction II (Fig 1) contains also proteins with molecular masses of 14-16 kDa. Analysis of these proteins has revealed that they represent a new type of three-fingered toxins: disulfide-bound dimers of α-cobratoxin with cytotoxins and homodimer of α-cobratoxin (Osipov *et al.,* 2008). Interestingly, the dimerization changed the biological activity of three-fingered toxins forming dimers. Thus, the affinity of α-cobratoxin to its known biological targets (α7 and muscle-type nicotinic acetylcholine receptors as well as acetylcholine-binding protein) decreased and cytotoxins completely lost cytotoxicity. However, α-cobratoxin within dimer acquires new activity – the ability to interact with pharmacologically important α3β2 type of nicotinic acetylcholine receptor (Osipov *et al.,* 2008). It should be noted that the content of dimers in crude venom is less than 0.1%, and it was hardly possible to find theses proteins without application of gel-filtration.

Other interesting post-translational modification of three-fingered toxins is glycosylation. The first and the only one found glycosylated three-fingered toxin is cytotoxin from *N. kaouthia* venom (Osipov *et al.,* 2004). The content of this toxin in the *N. kaouthia* venom is 0.17% of dry weight that is about only 1/30 of the amount of the normal non-glycosylated cytotoxin. It should be noted that it would be very problematic to isolate this minor glycosylated toxin without gel-filtration. Cytotoxin glycosylation results in two-fold decrease of toxicity to insects. At the same time, cytotoxicity to human HL60 cells was about two orders of magnitude lower for glycosylated cytotoxin as compared to parent toxin. The reason for the presence of the modified cytotoxin in the venom is not clear. As glycosylation leads to a substantial decrease in cytotoxicity, one can suggest that this might be the way to protect the cells expressing the toxin from its toxic action. However, some other reasons are also possible.

The main components of fraction IIb (Fig 7.1) are different PLA2s. However, in this fraction a new minor protein thaicobrin (P82885) was found. It opened a new protein family called vespryns. Thaicobrin was not toxic to mice at the dose of 3 μg/g of body weight. It was later shown (Pung *et al.,*

2005) that ohanin from *Ophiophagus hannah* venom, a structural analogue of thaicobrin, induced hyperalgesia in mice.

Three-step chromatographic scheme was successfully used for the isolation of minor proteins with unique properties from venoms of different cobras. Thus, the first reprolysin oxiagin that inhibited the activation of complement classical pathway was identified in the venom of cobra *N. oxiana* (Shoibonov *et al.,* 2005).

POSSIBLE ROLES OF MINOR PROTEINS IN SNAKE VENOMS

Our studies of the venom from Thailand cobra *N. kaouthia* have shown that it contains minor components that are structural or functional analogues of proteins from venom of snakes belonging to different genera of Elapidae family. Thus, muscarinic toxin-like proteins from cobra are analogues of muscarinic toxins from mamba, dimer of α-cobratoxin is a functional analogue of κ-bungarotoxin from krait venom. Vespryn thaicobrin has a structural analogue ohanin in *Ophiophagus hannah* venom. Proteins of CRISP family are also present in venoms of snakes from different genera.

New proteins hemextins of three-fingered toxin family were identified recently in *Hemachatus haemachatus* venom (Banerjee *et al.,* 2005). Forming a 1:1 non-covalent complex, the two hemextins moderately inhibit external activation pathway of blood coagulation. It is quite possible that venoms of cobras from *Naja* genus also contain hemextin analogues. In particular we have observed a mild inhibition of external pathway by main toxic fractions of different cobra (*N. kaouthia*, *N. melanoleuca* and *N. haje*) venoms. These fractions contain mainly three-fingered toxins.

Basing on the data discussed above, one can suggest that in principle the content of minor components is similar within the venoms of snakes from Elapidae family. This probably indicates the significance of these compounds for prey poisoning. Nevertheless, there is no clear understanding why along with main highly toxic compounds which are individually capable to kill the prey, venoms contain minor compounds of low toxicity. A simple explanation is hardly possible. It is quite probable that these compounds suppress in some way the resistance of the prey organism and thus ensure the most fast and effective action of highly toxic proteins. The latter are immunogenic because of their protein nature; therefore, suppression of prey immune system may be one of the tasks of minor auxiliary venom components. Indeed in some cobra venoms the proteins affecting (depleting) blood complement system were found; for example, these are cobra venom factors (CVF). The finding of effective coagulopathic proteins in many "neurotoxic" venoms shows the importance of blood coagulation impairment in envenoming. The snake feeding way (to swallow whole) suggests the beginning of the prey digestion outside the snake stomach and it is not surprising that snake PLA2s,

analogues of digestive enzymes, constitute the main part of venoms. From this point of view venom should contain compounds destroying tissue integrity. Venom hyaluronidases in combination with PLA2s and proteolytic enzymes may perform this function. It is also well possible that minor components may modulate or increase the effects of main venom constituents as well as protect them from the activation inside the venom gland. Finally, the presence of the variety of toxin forms in snake venom extends the number of possible biological targets thus increasing the number of preys available for snake feeding. Such an extension may increase the snake adaptation to the changing living conditions and results in the better species survival.

Interestingly, cobra (and several other Elapid) venoms do not contain even in trace quantities proteins of some structural types, characteristic for Viperidae venoms (Table 7.1). Thus, there are no disintegrins and metalloproteinases of PI group in Elapidae venoms, instead they contain metalloproteinases of group PIII which contain disintegrin-like domain along with proteinase one. Elapidae venoms do not contain thrombin-like enzymes, however in these venoms metalloproteinases may perform the function of fibrinogenases. Several serine and metallo-proteinases from Viperidae venoms are capable to hydrolytically inactivate complement system. Cobra venoms practically do not contain serine proteinases (with one exception Jin *et al.,* 2007) and the content of metalloproteinases is not high, nevertheless they can disturb the complement system and this function is effectively performed by cobra venom factor (CVF).

On the contrary, Elapidae venoms contain proteins of several structural types which are absent in Viperidae. First of all these are three-fingered toxins. It seems that Elapidae acquired this dreadful and effective weapon at the later stages of evolution after divergence from Viperidae.

THE ORIGIN OF POSSIBLE ERRORS

The purification scheme discussed above is not the only one and absolutely correct, however it allows detecting some errors arising at characterization of venom proteins. In particular, traditional proteomic approach basing on the complete reduction and alkylation of cysteine residues in analyzed proteins can overlook the disulfide isomers of known toxins. Thus, in recent study (Kulkeaw *et al.,* 2007) the venom of cobra *N. kaouthia* was analyzed using this approach, but no disulfide-bound dimers of three-fingered toxins were found despite the fact that some differences in 2D electrophoresis patterns were seen under reducing and non-reducing conditions. It is quite probable that trace amounts of dimers are buried under large spots of major proteins. As discussed above the biological activity of dimers is different from that of monomeric tree-fingered toxins and this difference may lead to erroneous data at the biological tests.

Insufficient purification of some venom component from even small admixtures of nerve growth factor or PLA2 which possesses strong differentiating activity may result in detection of such an activity in analyzed component. The main venom components may not only mask the presence of the low abundant compounds, but also being not well separated may contribute to biological effects of minor components. For example, it might be possible that the described ability of cobra venom factor to induce platelet aggregation (Ding *et al.,* 2000) is explained by the not separated trace amounts of PLA2 for which this effect is well known. Otherwise, the fibrinogenolytic effect of PLA2 from *Crotalus* venom (Zhang *et al.,* 2007) may be caused by the admixture of thrombin-like fibrinogenases which are the main components of the venoms from snakes of the genus *Crotalus*. Most of similar problems can be solved by use of classical gel-filtration. Unfortunately, this time- and labor-consuming procedure is often neglected that is not always justified. The importance of gel-filtration is proved by the following example from our practice. For the isolation of new fibrinogenolytic enzyme from practically unstudied venom of viper *Vipera nikolskii* at first we decided to refuse gel-filtration to speed up the isolation procedure. After several steps of ion-exchange chromatography, we obtained the fraction with high anticoagulant activity that possessed the molecular mass of about 13.5kDa (as determined by both MALDI mass-spectrometry and SDS-electrophoresis in polyacrylamide gel) characteristic for PLA2s, which are the most abundant components in the analyzed venom. However, after other purification procedure that included three-step scheme we have found that only fraction containing proteins with molecular masses of about 30kDa possesses fibrinogenolytic activity. The last procedure is more reliable as serine proteinases that usually accomplish fibrigenolysis have molecular masses of about 30kDa or above.

CONCLUSIONS

Snake venoms contain a vast array of minor components belonging to the different structural types and possessing "unusual" or unique biological activities. Investigation of their structures and mechanisms of interaction with biological targets may give new solutions of the current problems in biology and medicine. To be studied in full details, the minor components should be thoroughly separated from any possible admixtures using as many chromatographic steps as possible, and refusal to use gel-filtration may lead to incorrect conclusions. When characterizing the minor component one should keep in mind the possibility of its contamination by some abundant compound. During the detection of "new properties" of a well studied toxin, the possibility of the presence of some minor component in analyzed sample should be taken into account. Sometimes even trace amounts of contaminating substance can greatly change the biological activity of the studied sample.

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8

Extraction, Isolation and Characterization of Solanesol from *Nicotiana tabacum* L.

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ABSTRACT

Solanesol, a naturally occurring trisesquiterpenoid (C_{45}) alcohol of tobacco is used not only as an antibiotic but also cardiac stimulant and lipid antioxidant. At present clinical trials are under progress to explore its use as an anticancer drug. It is also the starting material for many high-value biochemicals, including coenzyme Q10 and Vitamin-K analogues. As a starting material for Q10, it is used in the treatment of different cancers. Coenzyme Q10 is well known not only to reduce the number and size of tumors but also to improve cardiovascular health. Thus there is a great demand for the solanesol for production of Q10 and other uses. Therefore, the isolation of solanesol from tobacco has achieved a great importance in recent years. Several techniques were described in literature for extraction, isolation, purification of solanesol from tobacco. These include column chromatography, soxhlet, microwave assisted extraction (MAE), counter current extraction etc. This paper not only reviews the state of the art techniques used for its extraction, but also presents an integrated counter current chromatographic approach developed in our laboratory for large scale isolation, purification and characterization of solanesol from tobacco. In addition, a simple and rapid method for determination of solanesol in tobacco extracts using non-aqueous RP-HPLC in an isocratic elution mode and UV detector at 215 nm was developed.

Key words : Antioxidant, characterization, counter current extraction, isolation, NARP-HPLC, purification, solanesol

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INTRODUCTION

Recently, there is a growing popularity and faith in the use of herbal medicine worldwide. Extraction and characterization of active phyto-chemicals from medicinal plants had lead to the development to high activity profile drugs *e.g.* vincristine, vinblastine and taxol (Huie, 2002). Tobacco contains several chemicals (Leaderer, 1992) and the best known of which is nicotine (Posselt & Reimann, 1828). Various species of *Nicotiana* contain many alkaloids, fatty acids and sugars (Severson *et al.,* 1978). The medicinal uses of tobacco as antidiarrhoeal, narcotic, pain reliever, healing wounds and burns were well reported (Dickson *et al.,* 1954). In the treatment of scabies, 0.1% Nicotine salicylate was used (Silvette *et al.,* 1958).The patients of post-encephalitic Parkinsonism were treated with subcutaneous injections of nicotine for immediate improvement in muscular movement (Moll *et al.,* 1926). The tobacco leaves and juices were much used for skin disorders including basal cell cancer. The pyridine content of the tobacco smoke destroys the comma bacillus of cholera or germs responsible for diphtheria and typhus (Charlton, 2004).

Solanesol in Tobacoo

Solanesol is a naturally occurring trisesquiterpenoid (C_{45}) alcohol of tobacco. It is one of the important precursors of the tumorigenic poly nuclear aromatic hydrocarbons (PAHs) of tobacco smoke. Reduction of its levels in tobacco, leads to safe smoking products due to reduced PAH levels in cigarette smoke. It is also the starting material for many high-value biochemicals, including coenzyme Q10 and Vitamin-K analogues (Hamamura *et al.,* 2002). As a starting material for Q10, it is used in treatment of different cancers. Coenzyme Q10 is well known not only to reduce the number and size of tumors but also improve cardiovascular health (Yalcin *et al.,* 2004; Singal *et al.,* 1999). Solanesol itself could be used as an antibiotic, cardiac stimulant and lipid antioxidant. At present clinical trials are under progress to explore its use as an anticancer drug. There is a great demand for solanesol for production of Q10 and other uses. Thus its isolation not only reduces the risks of PAH from tobacco smoke but also makes use of it as a starting material in the synthesis of several value added products such as Q10 and other analogues. Therefore, isolation of solanesol from tobacco is gaining a lot of importance in recent years. Solanesol is present in the lamina of tobacco leaves while absent in stems and stalk (Severson *et al.,* 1977). The content of solanesol in tobacco depends upon a number of factors. It varies from 0.3-3.0% according to the type and variety of tobacco, duration of growth and method of curing (Zhang *et al.,* 2005; Wang *et al.,* 2005). A substantial portion of solanesol exists in the form of fatty acid esters due to which proper curing and saponification play an important role in converting them in to free solanesol (Scholtzhauer *et al.,* 1976). Tobacco also contains several other organic compounds that can be easily co-extracted with solanesol and interfere with subsequent separation and purification processes (Svob Troje *et al.,* 1977).

Extraction of Solanesol

One of the key problems in extraction of solanesol from tobacco is the selection of a suitable solvent for maximum yield and purity. As the solanesol lies in the cellular chloroplast of the tobacco leaves, not only the solubility but also penetrability of the solvent is very important for complete extraction. Further, its separation from the crude extract and purification poses several problems because of the presence of closely related fatty acids, alcohols, alkaloids, tobacco pigments, tar and other organic impurities. The food and pharmaceutical grade solanesol has to be of highest purity for quality, safety and efficacy of the finished products. Therefore, it is quite important to develop processes that can selectively separate and purify solanesol from the crude extracts of tobacco leaves. Several methods were described in the literature for extraction, isolation and purification of solanesol from tobacco (Keca *et al.,* 1997; Duan, 2000; Huang & Zheng, 2003; Zheng, 2003; Chen, 2006). Most of the methods involve multiple step procedures, which are non specific, quite tedious and time consuming. Generally, maceration, percolation, ultra sonication, soxhlet and bubble column were used for extraction of phytochemicals from the plant materials (Keca *et al.,* 1997; Doig *et al.,* 2005). The first two techniques are not only time consuming but also give low yields of the desired products. Soxhlet extraction has been the most respected among all other conventional techniques. It serves as an (i) extraction step for the isolation of phyto-constituents and (ii) used as a bench mark against which any new extraction technique is compared. The basic extraction apparatus consists of a reservoir, an extraction body, a heating source and a reflux condenser. As the extracted analyte will normally have a higher boiling point than the solvent, it is preferentially retained in the flask and fresh solvent circulates. This ensures that only fresh solvent is used to extract the analyte from the sample in the thimble. One of the major shortcomings of soxhlet extraction is the lengthy extraction time that can be 8, 16 and 24 h or more (Pastot *et al.,* 1997). It has limited analytical applications and not suitable for handling of bulk quantities of tobacco. Tang *et al.* (2007) proposed an extraction procedure with petroleum ether under reflux at 50°C followed by silica gel column chromatography for isolation and purification of solanesol from tobacco leaves. However, the heat- reflux processes involve lengthy operations, bulk amount of solvents and ultimately thermal decomposition of the target compounds. Microwave assisted extraction (MAE) coupled with saponification was reported to be effective for extraction of solanesol from tobacco leaves (Zhou & Liu, 2006). It has the advantages, such as shorter time, less solvent consumption, higher extraction rate, better products with lower cost. Saponification alleviates emulsification in this process. Zhou *et al.* (2006) compared four different extraction methods to recover solanesol from tobacco leaves and MAE found to be faster as shown in Fig 8.1. Ultrasonic extraction combined with saponification was also reported (Chen *et al.,* 2007; Chen *et al.,* 2008) for extraction of solanesol from tobacco leaves. Highest yields were obtained using 20 mg/mL KOH in

ethanol at 60°C in 4 h. However, both MAE and ultrasound sonication consume high energy and not suitable for commercial production. Supercritical fluid extraction (SFE) is one of the environmental friendly processes for separating the active ingredients from plant materials. It uses supercritical fluids (*e.g.* CO_2) as the extraction solvents. The supercritical fluids are less expensive and extract the analytes faster when compared to organic solvents. By adding modifiers to supercritical fluids, the polarity could be changed for increasing the selectivity. However, the technique is sensitive to process control and the phase transitions. SFE was used as a refining method to produce solanesol from crude cream (Qunly *et al.,* 2005). It was also employed as a method for extracting high-purity solanesol from waste tobacco leaves. However, the methods described involve several pre-treatment procedures and extraction steps (Qunly *et al.,* 2001; Xiaoling Guan, 2006). Recently, Ruiz-Rodriguez *et al.* (2008) have reported extraction of solanesol with supercritical CO_2. The average yields of solanesol were 18.8% of extract. Nicotine was also co-extracted which could be hazardous. Recently, high speed countercurrent chromatography (HSCCC) for isolation of solanesol from the crude extracts of tobacco was reported (Du & Daijie, 2006). Here, the crude extract instead of raw tobacco was used as a feedstock for purification of solanesol. The purity of solanesol thus obtained was less than 95%. A slow rotary counter current chromatography (SRCCC) involving a non-aqueous two-phase solvent system of sunflower oil-ethanol was also used to produce food grade solanesol in a commercial scale (Zhao & Du, 2007). However, the process is not cost effective and the purity of solanesol was only 26%. Continuous countercurrent extraction, featuring a continuous relative movement between solvent and feed materials offers high yields of extractable material with comparatively less solvent requirement and time. It is widely employed in the food industry and its extension to the pharmaceutical industry requires a better understanding of the extraction process.

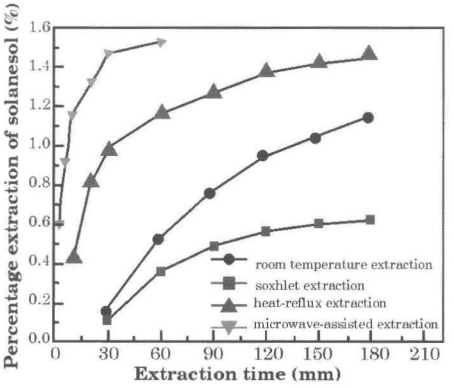

Fig 8.1. Comparison of different extraction methods on solanesol recovery from tobacco leaf sample. (Zhau, H.-Y. and Liu, C.-Z. (2006). *J. Chromatogr., B* **835:** 119-122) (Reproduced with permission from Elsevier Limited, UK)

Analysis of Solanesol

Reliable methods for determination of solanesol in tobacco are important for classification of different grades of tobacco according to their quality. A number of gas chromatographic methods for determination of solanesol in tobacco were reported (Wellburn & Hemming, 1966; Woollen & Jones, 1971; Severson, 1977; Severson *et al.,* 1978; Sheen, 1978). The low volatility and poor FID response of solanesol render the technique unsuitable (Wellburn & Hemming, 1966). The sample preparation also involves a number of time-consuming derivatization steps (Chamberlain *et al.,* 1990; Severson, 1978). Sheen *et al.* (1978) reported a packed column GC method involving lengthy extraction procedures and hydrogenation of solenesol. The other methods include gravimetry (Woollen & Jones, 1971) coulometry (Zhao, 2002) and thin layer chromatography (TLC) (Woollen & Jones, 1971). HPLC with various detectors including UV, RID, ELSD and MS was used to determine solanesol. Most of the methods reported before 2006 were in normal phase mode with UV detection (Zhao *et al.,* 1997; Zhang & Huang, 2001; Sun, 2002). Until the early 2007, not even a single reversed-phase HPLC method for determination of solanesol in tobacco was reported. Recently Zhoua *et al.* (2006) reported a RP-HPLC method using ELSD as a detector for determination of solanesol in tobacco. ELSD is not only a specialized detector but also requires a large volume of nebulizer gas of high purity. It makes the method unsuitable for routine analysis of solanesol because of the cost ineffectiveness. However, reversed phase HPLC with UV detection is often preferred not only because of its higher sensitivity but also wide availability and suitability. Quite recently Chen *et al.* (2007) reported RP-HPLC with UV and ESI-TOF/MS determination of solanesol in the crude extracts of tobacco leaves. The major drawback of this method lies in detection. The analytes were monitored at 202 nm by PDA, where acetonitrile used as one of the mobile phase solvents generally interferes. The use of such a short wavelength UV also produces baseline artifacts. The less non-polar column (C_4) was selected to elute the solanesol peak near to 10 min. Due to the increasing the demand for solanesol, a convenient and rapid method for determination as well as isolation of solanesol from tobacco is highly needed. Recently we had developed a continuous counter current extraction method for isolation of solanesol and its determination by RP-HPLC (Nageswara Rao *et al.*, 2008).

Scope of the Work

The present work i) reviews the state of art techniques used for extraction of solanesol from tobacco ii) describes the development of a simple protocol involving the use of a continuous counter current extraction followed by saponification, solvent crystallization or column chromatography. It was

compared with soxhlet extraction in terms of efficiency and recovery. Further, the development and validation of a simple non-aqueous RP-HPLC with UV method for determination of solanesol in *Nicotiana tobacum* L. was also described.

EXPERIMENTAL

Materials and Reagents

All the reagents were of analytical-grade unless stated otherwise. HPLC-grade isopropyl alcohol and methanol (Ranbaxy, SAS Nagar, India) were used. Dried tobacco received from local industries in Hyderabad, India and farmers of Nigeria, West Africa were used.

Counter Current Extraction

The continuous counter current extraction was carried out in 9 stages. Initially the dried leaves were powdered in a pulveriser and particles of 2-3 mm size were pelletizied of size 12 × 24 mm using steam. The pellets were then passed to the charge hopper of continuous extractor with the help of a bucket conveyor by means of gravity. Hexane was fed to the extractor from the storage tank. Later the solvent was recovered from the extract and recycled. The crude extract contains 15–20% solanesol. The raffinate was disposed after the recovery of solvent. Finally the solanesol enriched hexane was collected in miscilla tank. The miscilla extract was sent to multiple vacuum evaporators for solvent recovery with the help of a pump. The accumulated water in miscilla tank was separated and drained off as an effluent. In each stage the recirculation pumps were kept running at a flow rate of 10 liters/second to extract the solanesol from the pellets.

Extraction conditions

Flow rate of the feed (pellets): 2.5 tons/h, residence time: 4 h, flow rate of the circulating solvent: 6 KL/h, extractor still volume: 16 KL, ratio of solvent to solid feed: 2:4, extraction temperature: 60°C and pressure: 600 mm Hg.

Process operation parameters

Number of stages: 9, pump capacity: 10 lps, extractor dimensions (HLW): 1.5 × 14 × 1.5, total volume of extractor: 31.5 m^3, bulk density: 0.5–0.65 kg/l, bed height: 0.75 mts, vapour space: 50%, quantity in each stage except feed: 1.75 m^3, total quantity processed/hour: 2.56 t (with moisture), extract: 3.07 t/day (20% purity). extract: 0.62 t/day (100% solanesol). Extraction temperature: 60°C. The extract was dissolved in methanol, sonicated for 10 min, filtered and analysed by HPLC.

Soxhlet Extraction

Dried and powdered tobacco (25 g) was placed in 250 mL soxhlet thimble and fitted with 500 mL round bottom flask containing 250 mL hexane and refluxed for 4 h. The contents were cooled and the hexane was removed on a rotary evaporator. It was dissolved in methanol, sonicated for 10 min, filtered and analysed by HPLC. The HPLC conditions are as following: Hypersil BDS C_{18} (250 × 4.6 mm, 5 μm) column, methanol and IPA as mobile phase (40:60, v/v), flow-rate is 0.7 mL/min, detection length of UV is 215 nm, injection volume is 20 μl. The method has been applied to analyze and compare different tobacco samples. The results show that the solanesol content in samples of different raw materials varies widely from 0.43 to 1.0%. When different parts of the tobacco plant are compared, the top parts of the leaves are more abundant in solanesol content than those of lower parts.

Isolation and Purification

About 5 g of the crude extract was saponified with 120 mL of 0.5 N methanolic potassium hydroxide. The mixture was refluxed for 2 h. After cooling, the mixture was filtered through Whatmann No 42 filter paper in to a separating funnel and the flask and filter were washed with 50 mL of methanol. Hexane (150 mL), 75 mL of aqueous saturated KCl solution and 150 mL of water were added and vigorously shaken to effect solvent partitioning. The hexane layer was removed and the aqueous layer was extracted with hexane (2 × 50 mL). The hexane extracts were combined, washed with water, reduced the volume on a rotary evaporator and analyzed by HPLC (~ 45%). About 0.3 g of the sample was loaded on 30 g of 100–200-mesh silica gel and placed on a column (50 × 2 cm I.D). The column was eluted with 5% (v/v) ethyl acetate in hexane. The eluent was collected in 20 mL fractions. Fractions containing solanesol were monitored with the help of HPLC and all the similar fractions concentrated under vacuum at 40°C to obtain a pale yellow residue. The residue was crystallized by dissolving in hexane, stored at –20°C and analyzed by RP-HPLC.

Analytical apparatus

The HPLC system was composed of two LC-10 AT VP pumps, one LC-8A pump, an SPD-10AVP diode array detector an SIL-10 AD VP auto injector, a DGU-12 A degasser and SCL-10A VP system controller (all from Shimadzu, Kyoto, Japan). A reverse-phase Hypersil BDS C_{18} column (250X4.6 mm i.d., particle size 5 μm) was used for separation and determination. The chromatographic and integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system. The H^1 and C^{13} NMR spectra were recorded using Brucker 300 MHz (Varian, Palo Alto, USA) FT-NMR spectrometer containing $^1H/C^{13}$ dual probe. A 5 mm glass

tube was used to place the sample in to the magnetic field of the spectrometer under the following conditions: ^{1}H NMR; resonance frequency 300 MHz, spectral width 6188 Hz, pulse width 5.8 μs, data points 16,834, spectral resolution 0.3 Hz, probe temperature 27°C. ^{13}C NMR; resonance frequency 75.46 MHz, spectral width17985 Hz, pulse width 6.25 μs, data points17,982, spectral resolution 3 Hz, probe temperature 27°C. ^{1}H and ^{13}C chemical shifts were reported in ppm, relative to tetra methyl silane (δ 0.0; TMS). $CDCl_3$ (δ 77.0) was used as an internal standard. The IR spectra were recorded using a Fourier-Transform Infrared spectrometer (Thermo Nicolet, Nexus 670, USA) range 4000–400 cm^{-1} and resolution 4 cm^{-1}. The numbers of scans were 20. The spectra were recorded in the solid state using a 1% KBr pellet. The instrument was calibrated by using a polystyrene standard. ESI-MS spectra were recorded using a Micromass Quatro mass spectrometer (Micromass, UK) equipped with electro spray interface. Nitrogen was used as a nebulizer and dissolution gas. nebulizer gas flow 0.9 liters/min, dissolution gas flow 9.8 liters/min, nebulizer pressure 50 psi, capillary voltage 3.0 kV, cone voltage 25 V, source block temperature 20°C, ion energy 2.0 V. Mass measurements were performed in the full-scan mode over the mass range of m/z 50 to1000 with 0.21 scans/second.

Chromatographic Conditions

Analytical HPLC was performed with Hypersil BDS C_{18} (Thermo Electron corporation) column (250 × 4.6 mm *i.d.*, particle size 5 μm) using two LC-10AT VP pumps. Before delivering in to the system, the mobile-phase consisting of MeOH: IPA (60: 40 v/v) was filtered through 0.45 μm PTFE under vacuum and degassed by purging with helium. The analysis was carried out under isocratic conditions using a flow rate of 0.7 mL/min at room temperature (28°C). Chromatograms were recorded at 215 nm using SPD-10A VP photodiode array detector.

RESULTS AND DISCUSSION

Extraction of Solanesol

Extraction forms one of the basic steps in medicinal plant research as the preparation of crude extracts is the starting point for isolation and purification of chemical constituents present in plants (Romanik *et al.*, 2007). Yet the extraction step remains often neglected area, which over the years has received much less attention and research. Usually the traditional techniques require longer extraction time thus running a severe risk of thermal degradation for most of the phyto-constituents (Luque de Castro & Garcia-Ayuso, 1998). Keeping in view of the requirements, the use of new extraction techniques with shortened extraction time, reduced solvent

consumption, less pollution and care for thermolabile constituents was studied.

It is always economical to produce solanesol first in the form of a crude extract in a continuous counter current extractor. The extract was further purified to high-grade solanesol by different methods. Fig 8.2 shows the block diagram of counter current extraction of solanesol from tobacco. In the counter current extraction process steam was introduced in to the pellitiser so that, the powder absorbs around 5–8% moisture while forming the pellets. The moisture helps in the separation of water-soluble compounds and increases the content of solanesol in the extract. The water was finally collected in the miscilla tank and disposed off in the form of an effluent. The bulk density of the powder and the pellets were almost same as 0.5–0.6 kg/l. At every stage of the counter current continuous extractor, the feed solvent *i.e.* hexane was enriched with solanesol and its concentration increased till it reached the miscilla tank. In other words concentration of solanesol in pellets came down as it moved from stages 1 to 9. There by a concentration gradient was maintained to help in the mass transfer between the pellets and the solvent. During the preliminary trials, the loss of solvent was 0.5 to 1.0%. It was observed that 4 h of extraction gave a maximum yield of 5–6% of solvent. Thus the residence time was fixed at 4 h. Bed heights were fixed on the basis of amount of leaf powder/pellets to be processed. The hexane was removed from the extract by multiple evaporators and analyzed by RP-HPLC. Fig 8.3 shows the schematic representation of a continuous extractor used in the study.

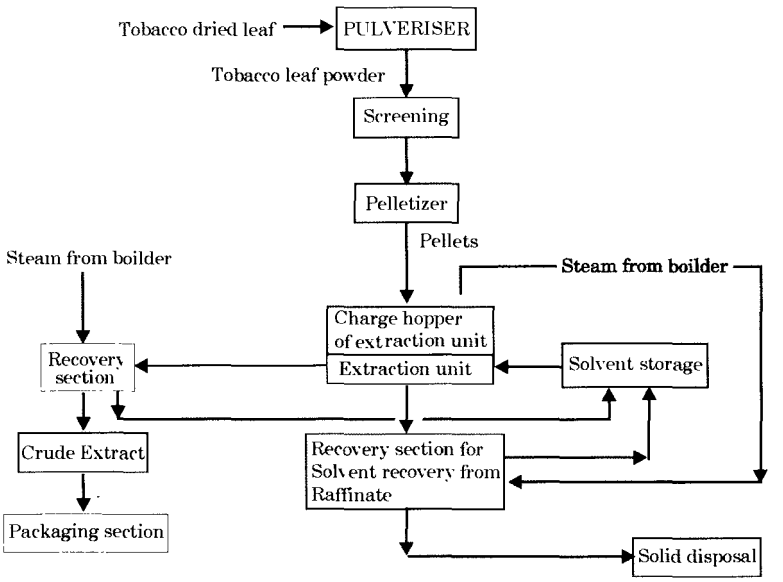

Fig 8.2. Block diagram of the counter current extraction of solanesol from tobacco

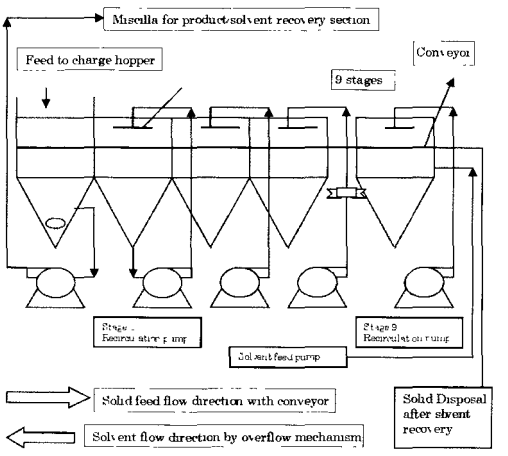

Fig 8.3. Schematic representation of the continuous extractor used in the present study

Effect of Particle Size

The effect of particle size on the extraction yield of solanesol was studied with leaf particle size varies from 2.83 to 9.51 mm. It was observed that the yield of solanesol increased when the particle size of the tobacco was small. The small size of the particles increases the surface area of tobacco dust. Therefore, it is easier to extract solanesol from tobacco having small particle size. Thereby, the optimized particle size of leaf for extraction of solanesol by continuous counter current extraction was <3 mm.

Effect of Solvent

The effect of different solvents *viz.*, hexane, trichloromethane, acetone, methanol and ethanol on the yield of solanesol was investigated. All the solvents have good solubilization ability of solanesol. Even though methanol had a good extraction capacity, keeping in view of the economics, hexane was found to be the best alternative for industrial scale, since its latent heat was three times less than that of methanol. This has not only minimized the consumption of energy but also enhanced and applicability of the process for commercial production.

Effect of Time

The optimum time for extraction was determined by analyzing the sample in different time intervals 0 to 6 h. It was observed that the extraction for 4 h gave a maximum yield of 5–6% of solanesol. The dependence of recovery of solanesol on time of extraction is shown in Fig 8.4. The counter current extraction was completed within 4 h after which there was no further improvement in the yields.

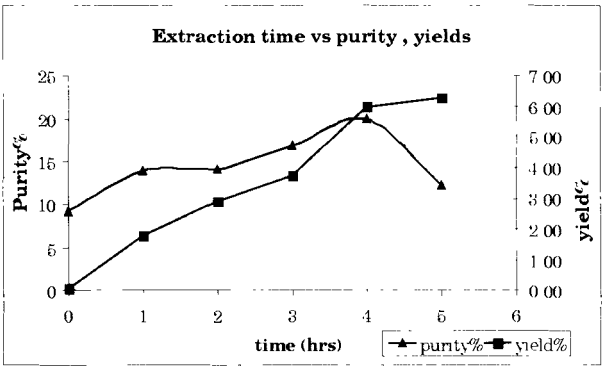

Fig 8.4. Effect of time on the yield and purity of solanesol by counter current extraction

Purification of Solanesol

The crude extract containing 15–20% of solanesol was purified by three different methods i) silica gel column chromatography ii) saponification followed by silica gel column chromatography iii) saponification followed by recrystallization with different solvents *viz.*, acetone, methanol and acetonitrile. In the first method, about 0.3 g of the sample was loaded on 30 g of 100–200-mesh silica gel packed in a column of 50 × 2 cm I.D. The column was eluted with 5% (v/v) ethyl acetate in hexane. All the similar fractions were concentrated under vacuum at 40°C to obtain a pale yellow residue. The solanesol thus obtained was 90–93% pure. It was further recrystallized with hexane to improve the purity to 95%. In the second method, the crude was initially saponified by methanolic KOH to get 45% of solanesol which was further purified by silica gel column to 95%. The product was further purified by hexane crystallization. The purity was 98% with a good yield of 2.1%. In the third method, the saponified crude of 45% solanesol was dissolved in warm acetone (10 mL/g) and kept in refrigerator for 72 h and the crystals formed were removed by filtration and analyzed, the purity of solanesol was found to be around 70%. The purity of solanesol was further improved to >90% when it was recrystallized in methanol and twice in acetonitile. Of the three methods, silica gel column purification of crude solanesol and hexane recrystallization gave about 1.8% yield of the product. In the second method, saponification followed by column purification gave good yield of 2.1% and more than 98% purity with hexane recrytallization. In the third method, the saponified fraction was subjected to recrystallization with common laboratory solvents gave the yield 0.8%. This could be due to the fact that a substantial portion of the solanesol in tobacco exists in the form of esters of fatty acids rather than free solanesol. Saponification of the crude converts the solanesyl esters to solanesol and a series of fatty acids. Therefore, the saponification of the crude extract increased the solanesol content and in turn the yield. Hence it is a best choice for isolation and purification of solanesol from tobacco. Fig 8.5 shows flow sheet of procedures

followed for purification of solanesol from the crude extracts of tobacco. The analysis results of the counter current, soxhlet extracts and the purified fractions of solanesol are given in Table 8.1.

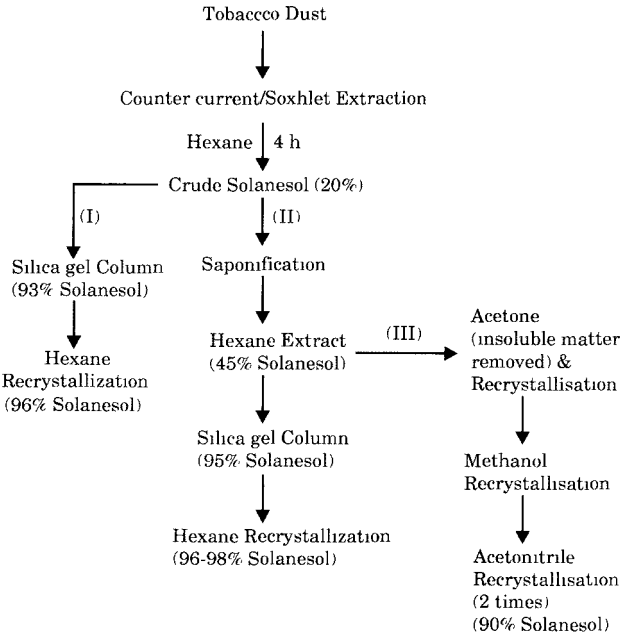

Fig 8.5. Flow sheet of procedures followed for purification of solanesol from the crude extract of tobacco

Table 8.1. Results of analysis of crude and purified extracts of solanesol by RP-HPLC

Sample	Solanesol content (%w/w)	
	Counter current	Soxhlet
Crude extract	21.01 ± 0.03	17.32 ± 0.05
Saponified	45.01 ± 0.02	43.43 ± 0.07
Acetone RC	70.02 ± 0.03	66.09 ± 0.03
Acetonitrile RC	91.45 ± 0.02	90.34 ± 0.04
Silica gel column	93.51 ± 0.01	92.53 ± 0.03
Saponified + Silca gel column	96.32 ± 0.04	95.58 ± 0.05
Silca gel column + Hexane RC	98.05 ± 0.05	96.12 ± 0.05

RC: recrystallisation

Characterization

The isolated solanesol was identified by the following spectral data. IR: 3378, 2965, 2917, 2851, 1664, 1446, 1382, 1151, 1104, 992, 875, 837, 795, 750, 599. MS (ESI) *m/z* 631 (M+H), ^{1}H NMR (200 M_Z) (solvent: $CDCl_3$) δ ppm: 1.36(3H, $1CH_3$), 1.59(21H, $7CH_3$), 1.66(s, 3H) 1.68(s, 3H), 1.90-2.12(m, 32H), 4.10(m, $2H,O\text{-}CH_2$), 5.01-5.12(t, 8H) 5.39(t, 1H) ^{13}CNMR (200M_Z) (solvent: $CDCl_3$) δ ppm: 15.97(C38-C44), 16.19(C37), 17.60(C45), 25.60(C36), 26.70(C8, C12, C16, C20, C24, C28, C32), 26.80 (C33), 29.67(C4), 39.70(C9, C13, C17, C21, C25, C29), 59.29(C1), 123.51-124.46 (C2, C34, C30, C18, C22, C26, C6, C10, C14), 131.03(C35), 134.80-135.31(C31, C19, C23, C27, C7, C11, C15), 139.49 (C3). The chemical structure of solanesol is shown in Fig 8.1.

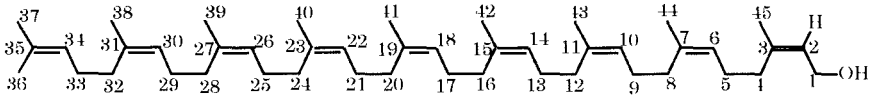

Fig 8.6. Chemical structure of solanesol

HPLC Method Development

Solanesol, the C_{45} terpenoid of the lipid soluble fraction of tobacco is soluble in polar organic solvents but insoluble in water. Thus silica was the preferred stationary phase for analytical separations. However, in normal phase mode, solvents of low polarity must be used to achieve adequate retention. But it becomes quite difficult to maintain reproducibility in such systems unless the trace amounts of water in the solvents are carefully controlled. Such problems encountered in normal phase chromatographic separation of hydrophobic compounds are generally overcome by reversed-phase chromatography. The retention of hydrophobic compounds on chemically bonded C_{18} phases is generally large and non-aqueous solvents such as methanol, acetonitrile and THF should be used to accomplish the elution in an acceptable time. For example the separation of fats, carotinoids and sterols have been generally carried out by non-aqueous reversed phase chromatography (L'isa *et al.,* 2007; Yakushina & Taranova, 1995). Under such conditions the homologues/isomers are better resolved than on silica, which is another advantage of reversed-phase separations. Thus non-aqueous reversed phase HPLC was carried out to separate solanesol effectively from other components of tobacco. Hypersil BDS C_{18} column (250 x 4.6 mm i.d., particle size 5 µm) with a mixture of isopropyl alcohol-methanol using a UV at 215 nm was used. The mobile phase composition was optimized and it was found to be IPA: MeOH (60:40 v/v) for better separation. The total run time between the injections was 15 min. Identification of solanesol was based on co-injection and comparison of retention time with that of a standard. The HPLC chromatograms of solanesol a) extracted by counter current extraction b) purified by different methods are shown in Figs 8.7 and 8.8 respectively.

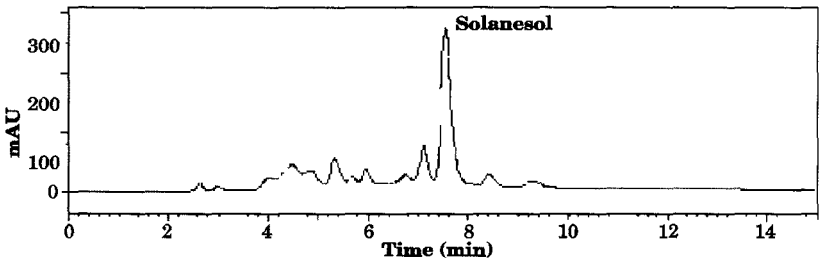

Fig 8.7. HPLC chromatogram of a continuous counter current extract of solanesol from *Nicotiana tobacum* L

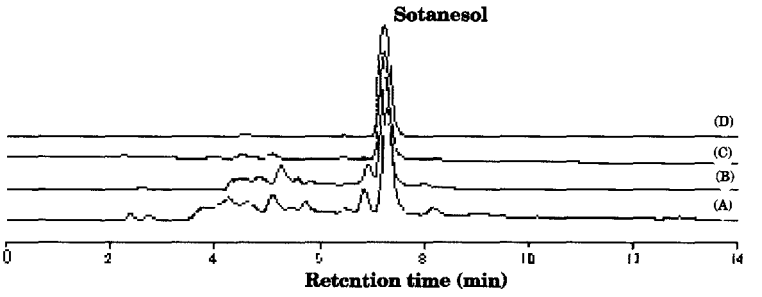

Fig 8.8. HPLC profiles of (A) crude extract; (B) saponified; (C) saponified and acetone RC; (D) silica gel column and hexane RC

Validatiosn

Linearity

Calibration was carried out in the range of 0.1–1.25 mg/mL. The mean equation of the calibration curve (n = 6) obtained from six points was y = 13473671x + 1560765 with a regression coefficient of 0.9996.

Accuracy and Precision

The precision was evaluated by repeated injections of the sample solution six times. The R.S.D. of peak area and retention time was 1.6%. Intra and inter-day variabilities were determined by analysis of standard solutions at low, medium and high concentrations of solanesol on three different days. The acceptable intra-and inter-day precisions (R.S.D.) and accuracy (relative error, RE) were <1% and ± 5% respectively. The assay precision was < 5%, and the accuracy was > 98%.

Limits of Detection (LOD) and Quantification (LOQ)

LOD was defined as the lowest concentration of solanesol at which the signal was larger than 3 times of the baseline noise S/N = 3 and LOQ as S/N =10. The measured LOD and LOQ values were 0.2 μg/mL and 0.7 μg/mL, respectively.

Applications

The developed RP-HPLC method was used for determination of solanesol in tobacco of different grades obtained from local and Nigerian farmers. The content of solanesol in different raw material of tobacco was determined and the results are given in Table 8.2. The method showed efficient separation of solanesol from different components of tobacco.

Table 8.2. Content of solanesol in different raw materials of tobacco of Nigeria as determined by RP-HPLC

Tobacco	Solanesol content (%w/w)
Fibers	0.63 ± 0.01
Mody	0.46 ± 0.01
Dust	0.44 ± 0.02
Sweeding	0.47 ± 0.02
Wet offals	0.65 ± 0.02
Top leaf	1.00 ± 0.01
Middle leaf	1.01 ± 0.02
Lower leaf	0.43 ± 0.03

CONCLUSIONS

The current state of the art techniques for isolation, purification and determination of solanesol from tobacco were reviewed. Although several techniques were reported for the isolation of solanesol from tobacco, each has its own merits and demerits. In the present chapter an economical and efficient protocol for isolation as well as purification of solanesol from tobacco using counter current extraction, followed by column chromatography, saponification and/or recrystallisation was described. The continuous counter current extraction is more suitable for isolation of solanesol on a large scale and high-purity of 95–98% solanesol was produced using common laboratory chemicals. The analysis of published data revealed that the HPLC is an effective tool for the determination of solanesol in tobacco compared to gas chromatography due to the time consuming derivatization steps involved in GC. However, most of the earlier methods reported the use of normal phase LC probable due to the hydrophobic nature of solanesol. Further the development and validation of a non-aqueous reverse phase HPLC method on a C18 column using methanol: isopropyl alcohol (40:60) as a mobile phase and UV detection at 215 nm for determination of solanesol in tobacco was described.

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Cinnamon : Molecular Evidence for the Health Benefits Through its Insulin-Like and Anti-Inflammatory Effects

CAO HEPING*, URBAN F. JOSEPH JR. AND ANDERSON A.RICHARD

ABSTRACT

Obesity is a major public health problem that leads to increased risk of developing diabetes, cardiovascular and related diseases. Obesity may be responsible for as many as 300,000 deaths and medical costs in excess of $100 billion annually in the U.S. alone. Drugs to reduce obesity and related disorders have been largely ineffective, resulting in the evaluation of complementary and alternative approaches to control disease. Cinnamon is one of a number of bioactive plant components used to alleviate the signs and symptoms of insulin resistance and type-2 diabetes due to its insulin-like activity. There is lack of knowledge at the molecular level, however, for supporting the health benefits of cinnamon. Analysis by quantitative real-time PCR compared the effects of cinnamon polyphenol extract (CPE) and insulin on the expression of 43 genes coding for the glucose transporter (GLUT) family, insulin signaling components, anti-inflammatory tristetraprolin (TTP) family, and pro-inflammatory cytokines in cultured mouse adipocytes and RAW cell line macrophages, and immunoblotting confirmed some of the PCR data. The insulin-like effects of CPE include rapid induction of TTP and reduction of vascular endothelial growth factor (VEGF) gene expression; a pro-angiogenic cytokine responsible for new blood vessel formation in adipose tissue. CPE, unlike insulin, sustained message

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expression for GLUT1, a major glucose transporter, and TTP, a protein that regulates pro-inflammatory cytokine message in autoimmune diseases, and is differentially produced in adipose tissue of obese people with metabolic syndrome. These results demonstrate that CPE regulates the expression of multiple genes that contribute to its insulin-like and anti-inflammatory properties.

Key words : Adipocytes, adipokines, cinnamon polyphenol extract, cytokines, diabetes, gene expression, glucose transporter, inflammation, insulin signaling pathway, obesity, tristetraprolin

INTRODUCTION

Reports from ancient history document the use of bioactive natural products to prevent and treat various diseases. One major class of bioactive compounds is plant polyphenols widely present in plant seeds, fruits, leaves, and bark (Yang *et al.*, 2001). Plant polyphenols are synthesized by the flavonoid biosynthetic pathway in plants such as cinnamon and tea and used for plant protection against predators (Dixon *et al.*, 2005). These compounds are commonly present in the diet (Prior & Gu, 2005) and are important for human health (Yang *et al.*, 2001).

Cinnamon and other spices including cloves, tumeric and bay leaves have insulin-like activity *in vitro* (Broadhurst *et al.*, 2000), and are proposed to be effective in the treatment of diabetes (Khan *et al.*, 2003). Several studies have demonstrated that cinnamon and cinnamon polyphenol extract (CPE) have insulin-like activity in cells, animals, and people with type 2 diabetes. Activities attributed to CPE include: 1) increased glucose metabolism in a fat cell assay (Anderson *et al.*, 2004); 2) increased insulin receptor β (INSRβ) autophosphorylation and decreased tyrosine phosphatase activity *in vitro* (Imparl-Radosevich *et al.*, 1998); 3) increased glucose uptake and glycogen biosynthesis, activation of glycogen synthase, and inhibition of glycogen synthase kinase-3β (Jarvill-Taylor *et al.*, 2001); 4) enhanced *in vivo* insulin-regulated glucose utilization in rats fed a high-fructose diet (Qin *et al.*, 2003); 5) decreased serum glucose levels, increased insulin (Verspohl *et al.*, 2005) and decreased blood pressure (Preuss *et al.*, 2006); and 6) decreased levels of glucose, triglycerides, and LDL cholesterol in people with type 2 diabetes fed cinnamon powder (Khan *et al.*, 2003; Hlebowicz *et al.*, 2007). Not all studies, however, have reported positive effects of cinnamon in patients with diabetes (Vanschoonbeek *et al.*, 2006; Baker *et al.*, 2008). This discrepancy may be due to the selection of patients, level of glucose control, oral hypoglycemic agents, and diet or type of cinnamon used.

Recent studies suggest that CPE has other health benefits. First, CPE inhibits cancer cell proliferation by altering the cell cycle pattern in myeloid cell lines (Schoene *et al.*, 2005). Second, CPE, like insulin, increases gene expression of anti-inflammatory protein tristetraprolin/zinc finger protein 36 (TTP/ZFP36) in 3T3-L1 adipocytes (Lai *et al.*, 1990; Cao *et al.*, 2007c) and RAW264.7 macrophages (Cao *et al.*, 2008b). Third, cinnamon has been shown to lower blood glucose due in part to delayed gastric emptying (Hlebowicz *et al.*, 2007). Fourth, cinnamon bark was reported to have antioxidant effects by increasing the activities of antioxidant enzymes including glutathione S-transferase, superoxide dismutase, and catalase in rat livers and hearts (Dhuley, 1999). Finally, CPE has anti-ulcerogenic activity by preventing the occurrence of stress ulcers under cold exposure or water-immersion-stress in rats (Akira *et al.*, 1986). These newest findings show the vast potential of cinnamon in animal and human health. There is, however, a lack of knowledge at the molecular level for supporting the health benefits of CPE.

We have used quantitative real-time polymerase chain reaction (PCR) to investigate the effects of CPE on the expression of 43 genes coding for glucose transporter (GLUT) family, components in the insulin signal transduction pathway, TTP family, adipokines, pro-inflammatory cytokines, and other selected targets in mouse 3T3-L1 adipocytes and RAW264.7 macrophages. Immunoblotting confirmed the PCR results by showing production of some of these proteins. In this chapter, we review the experimental approaches used, the basal level of expression of the various genes analyzed, the transcriptional regulation of genes coding for the tristetraprolin family, the glucose transporter family, and the pro-inflammatory and insulin signal transduction pathway components. Our results show that CPE has insulin-like and independent effects on the regulation of gene expression in these mouse cells.

EXPERIMENTAL APPROACHES

Cinnamon Polyphenol Extract

The cinnamon polyphenol extract (CPE) was prepared by a standard protocol (Anderson *et al.*, 2004; Cao *et al.*, 2007c). Briefly, ground cinnamon (*Cinnamomum burmannii*) suspended in 0.1 N acetic acid was then autoclaved, and the supernatant obtained after centrifugation was mixed with four volumes of absolute ethanol and refrigerated overnight. The mixture was filtered through glass wool and Whatman #1 filter paper, ethanol removed by roto-evaporation, and the remaining solution freeze-dried for

storage. CPE reconstituted at 100 mg/mL in 100% dimethylsulfoxide (DMSO) was then diluted with deionized water before addition to culture medium. The compositions of CPE mixture were analyzed by high performance liquid chromatography (HPLC) using a Symmetry Prep C_{18} column (Anderson *et al.*, 2004; Shan *et al.*, 2007).

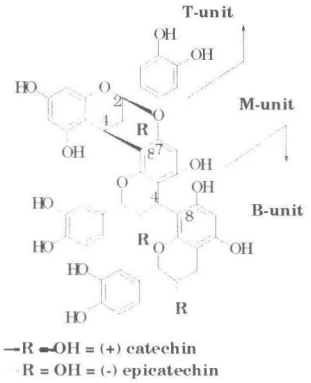
Cinnamon polyphenols (CP) were purified from CPE by HPLC using a Symmetry Prep C_{18} column using conditions similar to our earlier study (Anderson *et al.*, 2004). Cinnamon polyphenol fractions from HPLC included CP1A, CP1B, CP2, CP3, CP4, CP5, CP6 and CP7. CP2 is a procyanidin tetramer (M_r 1152 Da). CP4 and CP6 are both trimers with the same molecular mass (M_r 864 Da) (Fig 9.1A). CP7 is a mixture of monomer (M_r 288 Da) plus other oligomers as determined by mass spectrometry analyses (Anderson *et al.*, 2004). CP3 and CP5 contained mixtures of trimers and tetramers and the exact identities of CP1A and CP1B were not determined. Acetonitrile was removed by roto-evaporation and the fractions were freeze-dried for storage. The freeze-dried samples reconstituted at 10 mg/mL in 100% DMSO were used for analysis with cultured-cells.

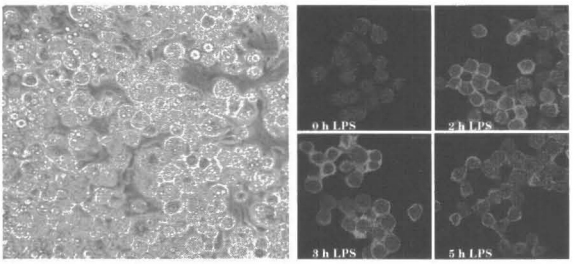
Cell Culture

Mouse 3T3-L1 preadipocytes maintained at 37°C in a humidified incubator with 5% CO_2 in DMEM containing 4500 mg/L (25 mm) glucose supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mm L-glutamine were differentiated into adipocytes (Cao *et al.*, 2007c). Briefly, mouse 3T3-L1 preadipocytes grown under the same conditions for 48–60 h had medium replaced with differentiation medium containing 1 μg/mL insulin, 0.25 μm dexamethasone, and 250 μm 1-isobutyl-3-methylxanthine. Following incubation for 48–60 h, the differentiation medium was replaced with fresh medium containing only 1 μg/mL insulin. After an additional 48–60 h incubation, the medium was replaced with fresh medium and the cells were grown for an additional 4–6 days. More than 90% of the cells accumulated lipid drops, indicating differentiation from preadipocytes to adipocytes (Fig 9.1B). The cells were then serum-starved in DMEM without any supplementation for 3–4 h before CPE (10 or 100 μg/mL) and DMSO (the vehicle control, 0.01% and 0.1%) were added to the medium for various times followed by cell extraction (Cao *et al.*, 2004).

Mouse RAW264.7 macrophages were maintained at 37°C in a humidified incubator with 5% CO_2 in the same medium as shown above for adipocytes (Cao *et al.*, 2004). RAW cells were treated with 100 μg/mL CPE (corresponding to 0.1% DMSO in the culture medium), 100 nM insulin, or 100 ng/mL lipopolysaccharide (LPS) for 0, 15, 30, 45, 60, 90, 120, 180, and 240 min.

The doses of CPE, LPS, and insulin were based on previous studies showing effective stimulation of TTP expression in adipocytes (Cao *et al.*, 2007c; Cao *et al.*, 2008c) and macrophages (Cao *et al.*, 2004) (Fig 9.1C).





A Cinnamon polyphenol **B Mouse 3T3-L1 adipocytes** **C Mouse RAW264.7 macrophages**

Fig 9.1. Chemical structure of cinnamon polyphenol and morphology of mouse 3T3-L1 adipocytes and RAW264.7 macrophages. (A) The structure of a HPLC-purified cinnamon polyphenol fraction (Cao *et al.*, 2007c). The structure of cinnamon polyphenol was determined as a doubly linked procyanidin type-A polymer by nuclear magnetic resonance (300 MHz for ^{1}H and 75 MHz for ^{13}C on a Bruker QE Plus 300 NMR spectrometer), mass spectroscopy (electrospray ionization and atmospheric pressure chemical ionization on an LCQ classic ion trap instrument) and infrared spectroscopy as described in reference (Anderson *et al.*, 2004). (B) Differentiated mouse 3T3-L1 adipocytes (Cao *et al.*, 2007c). (C) Mouse RAW264.7 macrophages after LPS induction and immunostaining with TTP antibodies (Cao *et al.*, 2004)

RNA Extraction and Real-time PCR Analysis

Total RNA was isolated from mouse adipocytes and RAW cells using TRI_{ZOL} reagent. RNA concentrations and integrity were determined using RNA 6000 Nano Assay Kit and the Bioanalyzer 2100 (Agilent Technologies) with RNA 6000 Ladder as the standards. The cDNA were synthesized from total RNA using SuperScript II reverse transcriptase (Cao *et al.*, 2007c). Shown in Table 9.1 are the gene names, GenBank accession numbers, and amplicon sizes. The sequences (5′ to 3′) of the forward primers, TaqMan probes (TET-BHQ1), and reverse primers have been described (Cao *et al.*, 2008c), along with the TaqMan reaction mixtures and thermal cycle conditions (Cao *et al.*, 2007c). PCR reactions performed in 96-well plates in an ABI Prism 7700 real time PCR instrument (Applied Biosystems) were evaluated using the $\Delta\Delta C_T$ method of relative quantification to determine the fold change in expression (Cao *et al.*,

Table 9.1. The mRNA names, GenBank accession numbers and amplicon sizes of gene targets investigated

mRNA	Accession No	Amplicon	mRNA	Accession No	Amplicon
ADIPOQ	NM_009605	180 bp	LEP	NM_008493	70 bp
AKT1/PKB	NM_033230	90 bp	LEPR	NM_146146	92 bp
APP	NM_007471	70 bp	PIK3CB	NM_053481	134 bp
CRP	NM_007768	79 bp	PIK3R1	NM_013005	118 bp
CSF2/GM-CSF	NM_009969	71 bp	PTGS2/COX2	NM_011198	106 bp
CSF3/G-CSF	NM_009971	74 bp	RPL32	NM_172086	66 bp
ELAVL1/HUR	NM_010485	69 bp	SERPINE1/PAI1	NM_008871	91 bp
GSK3B	NM_032080	106 bp	SHC1	XM_216176	85 bp
GYS1	XM_229128	119 bp	SLC2A1/GLUT1	M13979	123 bp
IFNG	NM_008337	81 bp	SLC2A2/GLUT2	NM_012879	80 bp
IGF1	NM_184052	78 bp	SLC2A3/GLUT3	NM_017102	112 bp
IGF1R	NM_010513	62 bp	SLC2A4/GLUT4	NM_012751	87 bp
IGF2	NM_010514	78 bp	SOS1	D83014	104 bp
IGF2R	NM_010515	91 bp	TAU	NM_010838	108 bp
IL1A	NM_010554	66 bp	TNF	NM_013693	74 bp
IL6	NM_031168	84 bp	VEGFA	NM_001025250	68 bp
IL12B	NM_008352	79 bp	VEGFB	NM_011697	83 bp
INS1	NM_008386	89 bp	ZFP36/TTP/TIS11	NM_011756	70 bp
INS2	NM_008387	100 bp	ZFP36L1/TIS11B	NM_007564	60 bp
INSR	NM_017071	137 bp	ZFP36L2/TIS11D	NM_001001806	77 bp
IRS1	NM_012969	68 bp	ZFP36L3	NM_001009549	70 bp
IRS2	AF050159	69 bp			

2007c). The gene expression data were analyzed by SigmaStat 3.1 software (Systat Software) using ANOVA or ANOVA on Ranks, and multiple comparisons performed with Student-Newman-Keuls Method.

SDS-PAGE and Immunoblotting

SDS-PAGE and immunoblotting used 10% SDS-PAGE and nitrocellulose membranes (Cao *et al.*, 2003). Membranes blocked with 5% nonfat dry milk and were successively incubated with buffers containing primary and secondary antibodies. Proteins on the immunoblots were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce) followed by imaging with BioChemi Image Acquisition and Analysis System (UVP BioImaging Systems). The primary antibodies were anti-MBP-TTP and anti-MBP-ZFP36L1 raised against recombinant *E. coli* maltose-binding protein (MBP) fused to the full-length mouse TTP (Cao *et al.*, 2004) or ZFP36L1/TIS11B (Cao *et al.*, 2008a). The secondary antibodies were affinity-purified goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate with human IgG absorbed (Bio-Rad).

BASAL GENE EXPRESSION LEVELS

Expression Profiles of Selected Genes in Mouse Adipocytes

The relative basal expression of several genes in untreated adipocytes was determined to provide a basis for the inductive effects of treatment with CPE (Table 9.2). GLUT 1 (glucose transporter 1) and GLUT4 mRNA represent major forms of the GLUT family expressed in adipocytes relative to low level expression of GLUT3 and undetectable levels of GLUT2 mRNA (Table 2). IGF2 expression was the most abundant message for the insulin signaling pathway followed by AKT1, IGF2R, INSR, IRS1 and IGF1R; expression levels of INS1, INS2, PIK3CB, and SHC1 mRNA were relatively low in untreated adipocytes (unpublished results). In the TTP family, TTP mRNA levels were approximately 10–15% those of ZFP36L1 and ZFP36L2 and 100-fold that of ZFP36L3 (Table 9.2) (Cao *et al.*, 2008c). Hu antigen R/embryonic lethal, abnormal vision-like 1 (HuR/ELAVL1) and vascular endothelial growth factor (VEGF) mRNA were 4.5–12 fold that of TTP. TTP mRNA levels were approximately 30-fold that of cyclooxygenase-2 (COX2), 125-fold of granulocyte-macrophage colony-stimulating factor (GM-CSF), and 100,000-fold that of tumor necrosis factor (TNF) in the adipocytes.

Table 9.2. Relative levels of GLUT and TTP family mRNAs in untreated mouse 3T3-L1 adipocytes [TTP family data are from (Cao *et al.*, 2008c)][1,2]

Class	mRNA	Cycle of threshold C_T	Expression ratio *Fold of GLUT1 or TTP*
GLUT family	GLUT1 (SLC2A1)	20.84 ± 0.11	1.00
	GLUT2 (SLC2A2)	undetectable	undetectable
	GLUT3 (SLC2A3)	32.56 ± 0.22	0.0003
	GLUT4 (SLC2A4)	21.30 ± 0.53	0.73
TTP family	TTP (ZFP36/TIS11)	24.63 ± 0.45	1.00
	ZFP36L1 (TIS11B)	21.86 ± 0.25	6.82
	ZFP36L2 (TIS11D)	21.36 ± 0.21	9.65
	ZFP36L3	31.59 ± 0.06	0.01

[1] Values are means ± SD, n = 2-4.

[2] RNA-derived cDNAs (25 ng) was used for the quantitation of mRNA levels using 50 cycles of real-time PCR program. The relative ratios of mRNA levels were calculated using the double delta C_T method normalized with RPL32 C_T value as the internal control and TTP or GLUT1 C_T value as the calibrator.

Expression Profiles of Selected Genes in Mouse RAW264.7 Macrophages

GLUT1 mRNA was the most abundant form of GLUT family in RAW264.7 macrophages, and was 8 and 1300-fold those of GLUT3 and GLUT4 mRNA, respectively (Table 9.3) (Cao *et al.*, 2008b). GLUT2 mRNA was undetectable by the PCR assay with 50 cycles (Table 9.3). INSR and GSK3B mRNA were detected in RAW macrophages, although their levels were much less than those of GLUT1 (Cao *et al.*, 2008b). In TTP family, ZFP36L2 mRNA was the most abundant molecule among those measured, and was over 4-fold that of TTP in RAW264.7 cells (Table 9.3). TTP mRNA levels were approximately 2–100-fold those of ZFP36L1 and ZFP36L3 (Table 3). HuR/ELAVL1 mRNA levels were approximately 2-fold those of TTP (Cao *et al.*, 2008b). VEGFB mRNA levels were 19% less than that of TTP (Cao *et al.*, 2008b). TTP mRNA levels were approximately 6–10,000-fold those of TNF, GM-CSF, COX2, VEGFA, interleukin (IL) 6, and IFNγ in untreated RAW cells (Cao *et al.*, 2008b).

Table 9.3. Relative levels of GLUT and TTP family mRNAs in untreated mouse RAW264.7 macrophages [modified from (Cao *et al.*, 2008b)][1,2]

Class	mRNA	Cycle of threshold C_T	Expression ratio *Fold of GLUT1 or TTP*
GLUT family	GLUT1 (SLC2A1)	22.97 ± 0.37	1.00
	GLUT2 (SLC2A2)	undetectable	undetectable
	GLUT3 (SLC2A3)	25.96 ± 0.28	0.13 ± 0.03
	GLUT4 (SLC2A4)	33.31 ± 0.28	0.0008 ± 0.0002
TTP family	TTP (ZFP36/TIS11)	23.66 ± 0.29	1.00
	ZFP36L1 (TIS11B)	24.81 ± 0.27	0.45 ± 0.10
	ZFP36L2 (TIS11D)	21.59 ± 0.33	4.18 ± 0.89
	ZFP36L3	30.13 ± 0.39	0.012 ± 0.003

[1] Values are means ± SD, n = 9–22.

[2] RT-PCR method was identical to that described in the footnotes to Table 2.

INSULIN-LIKE EFFECTS ON GENE EXPRESSION

CPE, Like Insulin, Increased TTP mRNA and Protein Levels in Adipocytes

Quantitative real-time PCR analyses showed that a 0.5 h treatment of mouse 3T3-L1 adipocytes with 10 and 100 nM insulin rapidly induced TTP mRNA by approximately 5- and 7-fold over the control, respectively (Fig 9.2A) (Cao *et al.*, 2008c). TTP mRNA levels then declined, but were still 2-fold over the control after 1 h induction (Fig 9.2A). TTP mRNA levels were similar, however, between the control and the cells treated with 10 nM insulin for 16 h or treated with 100 nM insulin for 1.5, 2, 4 or 16 h (Fig 9.2A). Immunoblotting showed that TTP protein was barely detectable in untreated cells (Fig 2B, lane 1), but significantly induced by 10 and 100 nM of insulin for 3 h (Fig 2B, lanes 2–3). In contrast, ZFP36L1 protein levels were not significantly affected by insulin under the same conditions (Fig 9.2B, lanes 4–6) (Cao *et al.*, 2008c).

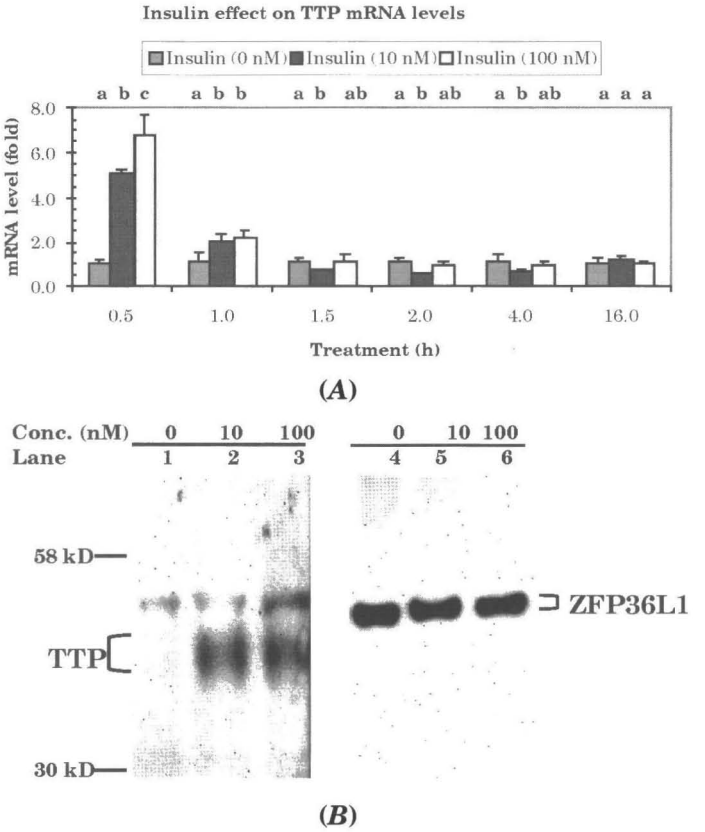


Fig 9.2. Effects of insulin on TTP mRNA, TTP protein, and ZFP36L1 protein levels in mouse 3T3-L1 adipocytes. (A) Real-time PCR assay. Total RNAs were isolated from 3T3-L1 adipocytes following treatment with the control or 10 and 100 nM insulin for 0.5–4 h after serum starvation for 4 h or treatment for 16 h after serum starvation for 8 h. The RNAs were reversely transcribed into cDNAs. RNA-derived cDNAs (25 ng) were used for quantitative real-time PCR assays. The double delta C_T method of relative quantification was used to determine the fold change in expression. Values with different lower case letters displayed above the columns of the figure are significantly different at $p<0.05$. (B) Immunoblotting. Proteins in the 10,000 *g* supernatants of 3T3-L1 adipocytes were separated by 10% SDS-PAGE. TTP was detected by immunoblotting with anti-MBP-mTTP and anti-MBP-ZFP36L1 antibodies. Each lane was loaded with 100 μg of protein. Lane 1, control; lane 2, insulin (10 nM); lane 3, insulin (100 nM) [modified from (Cao *et al.*, 2008c)]

CPE also significantly increases TTP mRNA levels in 3T3-L1 adipocytes (Cao *et al.*, 2007c). In a time course study, TTP mRNA levels increased in adipocytes treated with CPE for 30–120 min (Fig 9.3A). TTP mRNA levels in 10 μg/mL CPE-treated cells were approximately two-fold those in the controls, and those in 100 μg/mL CPE-treated cells were approximately six-fold those in the control cells after 30–60 min. CPE-induced sustained TTP mRNA in adipocytes that contrasts with the transient increase by insulin (Lai *et al.*,

1990; Cao *et al.*, 2008c). TTP mRNA levels were still 2-fold that of the control after 16 h treatment (unpublished results). Immunoblotting showed that TTP was barely detectable in untreated cells (Fig 9.3B, lanes 1 and 5), but was significantly induced by 100 μg/mL of CPE in 3T3-L1 adipocytes after 3 h (lane 3). Treatment of adipocytes with 10 and 100 μg/mL of the purified CP3 fraction of CPE increased the amount of TTP after 3 h, and higher concentrations of CP resulted in more TTP in the adipocytes (Fig 9.3B, lanes 6–8).

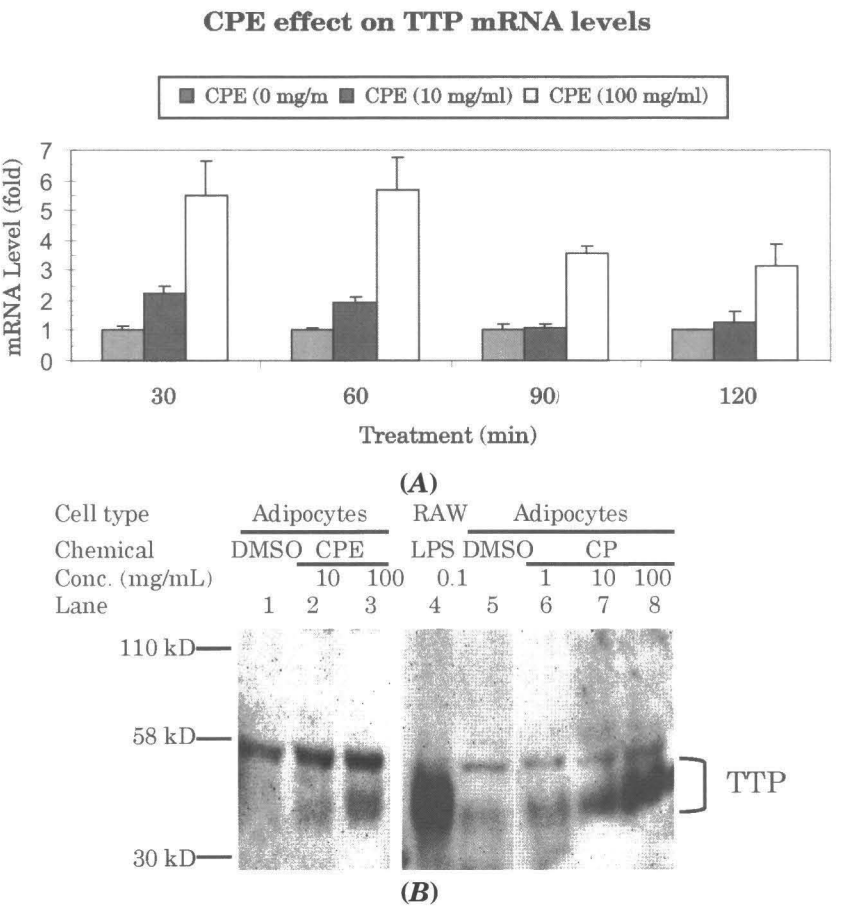


Fig 9.3. Effect of cinnamon extract and polyphenols on the expression of TTP in mouse 3T3-L1 adipocytes. (A) PCR assays. TTP mRNA levels were evaluated by RT-PCR assay with identical procedure to that in Fig 9.2 legend. (B) Immunoblotting. Proteins in the 10,000 g supernatants of 3T3-L1 adipocytes after 3 h treatment were separated by 10% SDS-PAGE, and TTP was detected by immunoblotting with anti-MBP-mTTP antibodies. Lane 1, PBS control; lane 2, CPE (10 μg/mL); lane 3, CPE (100 μg/mL); lane 4, extract from RAW264.7 cells treated with LPS (100 ng/mL) for 2 h as a positive TTP control; lane 5, DMSO control (1%); and lane 6–8, CP3 (1, 10, and 100 μg/mL, respectively). Lanes 1–3 (100 μg of protein); lane 4 (40 μg of protein); and lanes 5–8 (80 μg of protein) [modified from (Cao *et al.*, 2007c)]

CPE, Like Insulin, Decreased VEGF mRNA Levels in Adipocytes

VEGF mRNA codes for a pro-angiogenic cytokine that is a target for degradation by TTP family proteins both *in vivo* and *in vitro* (Essafi-Benkhadir *et al.*, 2007; Suswam *et al.*, 2008). Quantitative real-time PCR assays indicated that treatment of adipocytes with 10 and 100 nM insulin for 0.5–4 h after 4 h serum starvation reduced VEGFA mRNA by approximately 30–50% (Fig 9.4A). VEGFA and VEGFB mRNA levels were significantly decreased by 100 nM insulin treatment for 16 h; although VEGFA mRNA was slightly increased in adipocytes treated with 10 nM insulin under the same conditions (Fig 9.4A). CPE significantly decreased VEGF mRNA levels by more than 50% in cells treated for various times from 0.5 to 16 h (Fig 9.4B).

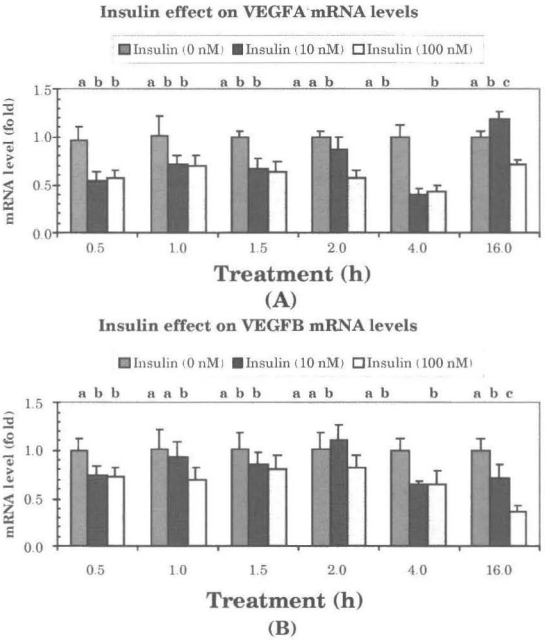


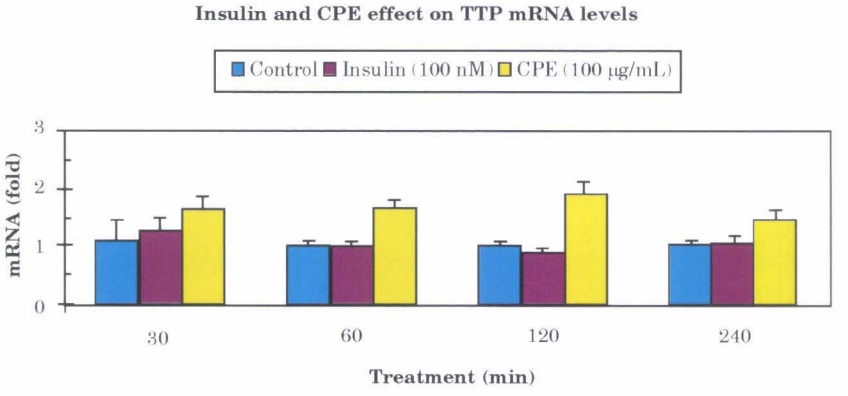
Fig 9.4. Effect of insulin and CPE on VEGF mRNA levels in mouse 3T3-L1 adipocytes. RNA isolation, cDNA synthesis, real-time PCR assays, and statistical analyses are described in Fig 9.2 legend. (A) VEGFA mRNA, (B) VEGFB mRNA. CPE results are not shown [modified from (Cao *et al.*, 2008c)]

INSULIN-INDEPENDENT EFFECTS ON GENE EXPRESSION

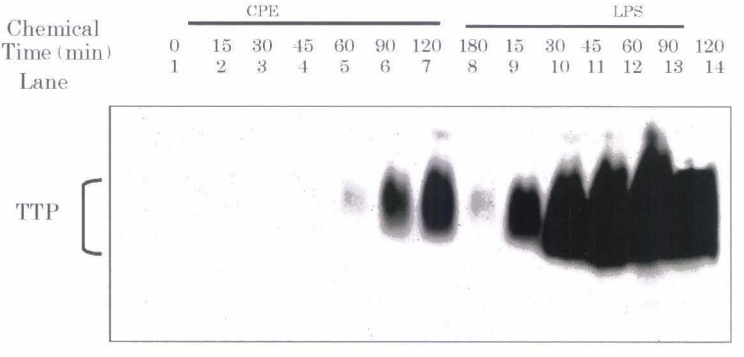
CPE but Not Insulin Increased TTP mRNA and Protein Levels in Macrophages

CPE increased TTP mRNA levels in mouse RAW264.7 cells (Cao *et al.*, 2008b). TTP mRNA levels in cells treated with 100 μg/mL CPE for 30–240 min were

approximately 50–100% greater than controls (Fig 9.5A). Insulin did not exhibit any major effect on TTP mRNA levels in RAW cells except for a slight decreased in cells treated for 120 min (Fig 9.5A). Immunoblotting showed that TTP protein increased in RAW cells treated with 100 μg/mL CPE for 90–180 min (Fig 9.5B, lanes 6–8), but was below detection in cells treated with insulin for the same length of time (data not shown).



(A)

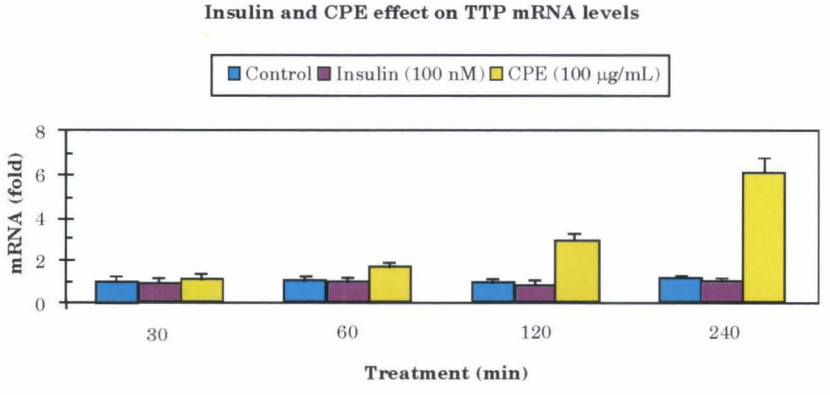


(B)

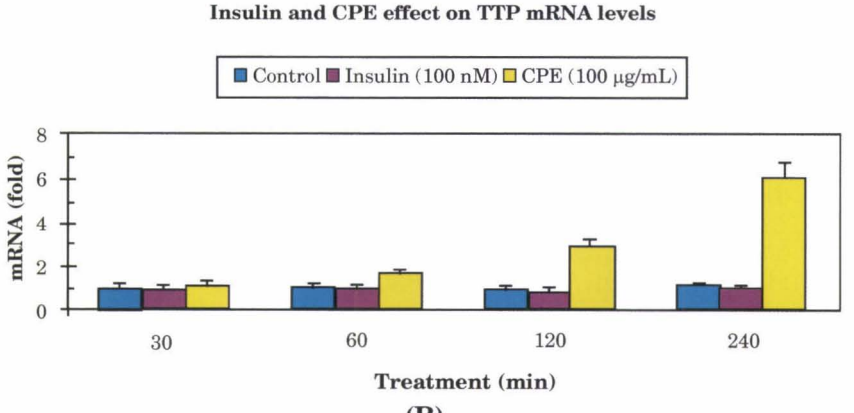
Fig 9.5. CPE but not insulin increased TTP mRNA and protein levels in RAW264.7 macrophages. (A) PCR assays. Total RNAs were isolated from RAW cells treated with DMSO control (0.1%), insulin (100 nM), or CPE (100 μg/mL). RNA-derived cDNAs (25 ng) were used for quantitative real-time PCR assays. The double delta C_T method of relative quantification was used to determine the fold change in expression. Values with different lower and upper case letters displayed above the columns of the figure are significantly different at $p<0.05$ or $p<0.01$, respectively. (B) Immunoblotting. Proteins in the 10,000 g supernatants of RAW cells were separated by 10% SDS-PAGE. TTP was detected by immunoblotting with anti-MBP-TTP serum. Each lane was loaded with 100 μg of protein. Lane 1, DMSO control; lanes 2–8, CPE treatment; lanes 9–14, LPS treatment. Insulin did not affect TTP protein levels in the same cells [modified from (Cao *et al.*, 2008b)]

CPE but Not Insulin Increased TTP-Targeted Pro-Inflammatory Cytokine mRNA Levels in Macrophages

TTP is a mRNA destabilizing factor for a number of acute response genes, such as TNF and GM-CSF genes (Blackshear, 2002). Therefore, gene expression of a selected set of pro-inflammatory cytokine was examined in RAW264.7 macrophages treated with CPE and insulin (Cao *et al.*, 2008b). TNF mRNA levels in RAW cells treated with 100 μg/mL CPE for 30-240 min were 120–620% greater than untreated controls (Fig 9.6A). Unlike CPE, insulin did not effect TNF mRNA levels in RAW cells (Fig 9.6A), and neither CPE nor insulin had a significant effect on GM-CSF mRNA levels (Fig 9.6B). Treatment of RAW cells with CPE (100 μg/mL) for 60–240 min increased COX2 and IL6 mRNA levels to approximately 200–340% and 160–350% of the controls, respectively (Figs 9.6C & 9.6D) (Cao *et al.*, 2008b). Insulin (100 nM, 0.5–4 h) did not significantly effect expression of COX2 or IL6 (Figs 9.6C & 9.6D).



(A)



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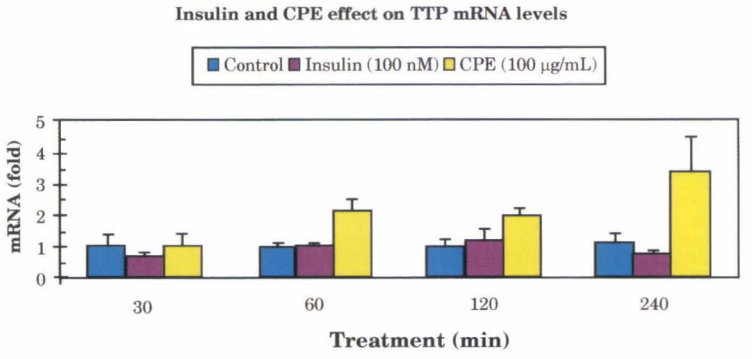

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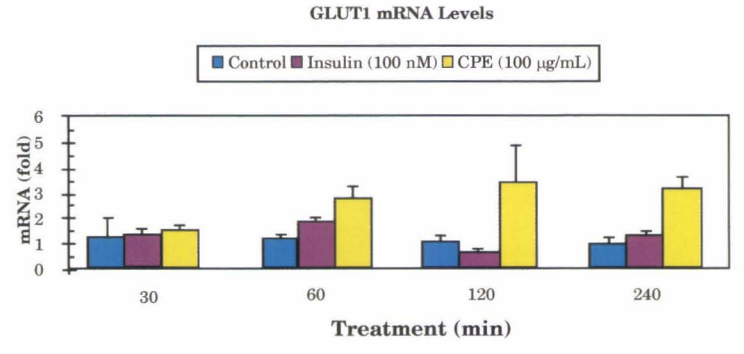
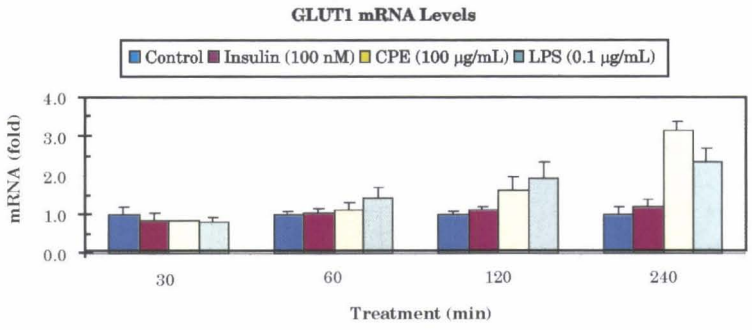


(D)

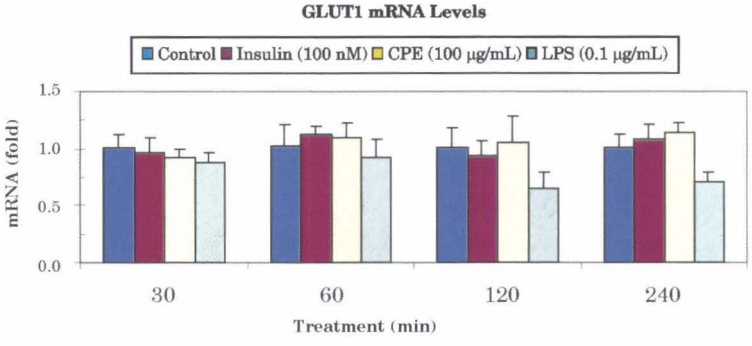
Fig 9.6. CPE but not insulin increases TNF, COX2, and IL6 mRNA levels in RAW264.7 macrophages. (A) TNF mRNA levels, (B) GM-CSF mRNA levels, (C) COX2 mRNA levels, and (D) IL6 mRNA levels. RNA isolation, cDNA synthesis, real-time PCR assays, and statistical analyses were described in Fig 9.5 legend [modified from (Cao *et al.*, 2008b)]

CPE but Not Insulin Increased GLUT1 mRNA Levels in Macrophages

Glucose is a major metabolic substrate critical for host immunity (Gamelli *et al.*, 1996; Calder *et al.*, 2007), and glucose uptake in mammalian cells is facilitated by glucose transporter (GLUT) family proteins (Fukuzumi *et al.*, 1996). CPE increased GLUT1 mRNA levels (the major GLUT mRNA in RAW cells, refer to Table 9.4) in RAW cells after 120 min treatment (Fig 9.7A) (Cao *et al.*, 2008b). After 240 min treatment, CPE increased GLUT1 mRNA levels in RAW cells by more than three-fold those of the control (Fig 9.7A). CPE did not significantly effect the mRNA levels of GLUT3 (Fig 9.7B), but increased GLUT4 mRNA levels by more than two-fold after 240 min (Fig 9.7C).



(A)



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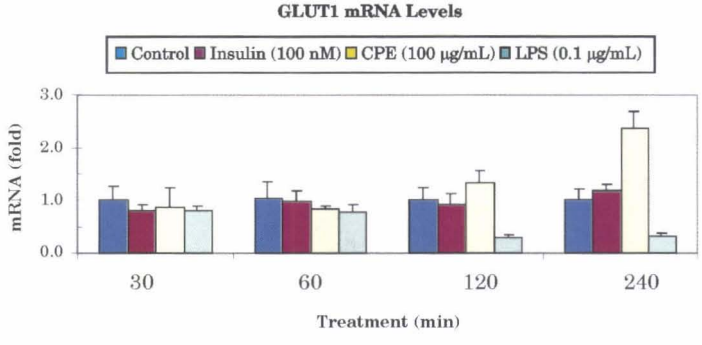

(C)

Fig 9.7 CPE but not insulin increases GLUT1 mRNA levels in RAW264.7 macrophages. (A) GLUT1 mRNA levels, (B) GLUT3 mRNA levels, and (C) GLUT4 mRNA levels. RNA isolation, cDNA synthesis, real-time PCR assays, and statistical analyses were described in Fig. 9.5 legend [modified from (Cao *et al.*, 2008b)]

DISCUSSION

Although studied extensively, the prevention of obesity, diabetes, and related disorders remains elusive. Cinnamon is one of many plant-derived products

widely used throughout history as a botanical supplement to prevent or treat various health conditions. Cinnamon and its polyphenol extract can significantly improve some diabetic conditions (Khan *et al.*, 1990; Ziegenfuss *et al.*, 2006; Hlebowicz *et al.*, 2007), however, the effectiveness for diabetes prevention and treatment vary (Vanschoonbeek *et al.*, 2006; Baker *et al.*, 2008). Detailed analyses of the biochemical and molecular effects of cinnamon on relevant cells are lacking. In this chapter, we summarize recent progress in understanding the effects of cinnamon polyphenol extracts (CPE) on selected gene expression in mouse 3T3-L1 adipocytes and RAW264.7 macrophages that represent widely used cellular models to study mechanisms of insulin action, lipid metabolism, and inflammation. Our molecular analyses demonstrate that CPE has both insulin-like and insulin-independent effects on gene expression in these cells.

One major insulin-like effect of CPE in mouse adipocytes is the induction of TTP, an anti-inflammatory and mRNA destabilizing protein (Cao *et al.*, 2007c; Cao *et al.*, 2008c). TTP is the product of the immediate-early response gene *Zfp36* in the mouse (*ZFP36* in humans) (Lai *et al.*, 1990) that binds to ARE in some mRNAs and destabilizes transcripts encoding for proteins such as TNFα(Carballo *et al.*, 1998; Lai *et al.*, 1999), GM-CSF (Carballo *et al.*, 2000), IER3 (Lai *et al.*, 2006), and VEGF (Essafi-Benkhadir *et al.*, 2007). The mRNA binding activity of TTP is zinc-dependent (Cao, 2004) and is regulated by post-translational phosphorylation (Carballo *et al.*, 2001; Cao, 2004; Cao *et al.*, 2006; Cao *et al.*, 2007a). TTP mRNA and/or TTP protein levels are increased in mammalian cells by a wide range of agents including insulin and other growth factors (Lai *et al.*, 1990; DuBois *et al.*, 1990), cytokines (TNFα, GM-CSF and IFNγ) (Carballo *et al.*, 1998; Sauer *et al.*, 2006), zinc (Cousins *et al.*, 2003), cinnamon extract and polyphenols (Cao *et al.*, 2007c; Cao *et al.*, 2008b), green tea extract (Cao *et al.*, 2007b), tumor promoters (Varnum *et al.*, 1989; Lai *et al.*, 1990), bacterial endotoxin lipopolysaccharide (Cao *et al.*, 2004; Cao *et al.*, 2008b), and viral infection (Taddeo *et al.*, 2006). Since TTP gene expression is diminished in adipose tissue of obese subjects with metabolic syndrome (Bouchard *et al.*, 2007a; Bouchard *et al.*, 2007b), the induction of TTP by CPE could improve the effects of obesity and related diseases similar to its effects in autoimmune disorders.

Another major insulin-like effect of CPE is the reduction of VEGF mRNA levels in mouse adipocytes (Cao *et al.*, 2008c). VEGF is an important mitogenic and angiogenic factor associated with tumor progression, collateral vessel formation in ischemic tissues, inflammation, and development of diabetic retinopathy (Ferrara *et al.*, 2003). VEGF is also a key regulator of adipogenesis in obesity (Nishimura *et al.*, 2007). Plasma VEGF levels in obese mice and in athymic mice implanted with 3T3-L1 adipocytes in visceral fat are significantly higher than controls (Miyazawa-Hoshimoto *et al.*, 2005). People with type 2 diabetes treated with insulin have 16% less VEGF levels in the plasma than those treated with diet alone, and VEGF mRNA levels in 3T3-L1 adipocytes are increased by troglitazone and rosiglitazone, two antidiabetic

Table 9.4. Induction levels of anti-inflammatory TTP and pro-inflammatory cytokine mRNAs relative to the base level of TTP in CPE and LPS-treated mouse RAW264.7 macrophages [modified from (Cao *et al.*, 2008b)][1,2]

mRNA	Base level	CPE stimulation			
		30 min	60 min	120 min	240 min
	Fold of TTP	*Fold of Control (Fold of Control x Base level expression)*			
TTP	1.00	1.59 ± 0.17 (1.59)	1.64 ± 0.16 (1.64)	1.88 ± 0.17 (1.88)	1.49 ± 0.15 (1.49)
TNF	0.17	1.18 ± 0.13 (0.20)	1.80 ± 0.13 (0.31)	3.07 ± 0.20 (0.52)	6.18 ± 0.64 (1.05)
GM-CSF	0.002	1.01 ± 0.20 (0.002)	1.27 ± 0.45 (0.003)	1.01 ± 0.23 (0.002)	1.11 ± 0.44 (0.002)
COX2	0.02	1.07 ± 0.25 (0.02)	2.14 ± 0.39 (0.04)	2.05 ± 0.18 (0.04)	3.44 ± 1.0 (0.07)
IL6	0.0003	1.56 ± 0.24 (0.0005)	2.75 ± 0.52 (0.0008)	3.50 ± 1.37 (0.001)	3.20 ± 0.44 (0.001)

[1] Values are means ± SD, n = 2–6. [2] RT-PCR method was identical to that described in the footnotes to Table 2

compounds shown to induce weight gain and edema (Emoto *et al.*, 2001). VEGF mRNA is destabilized by TTP family proteins in intact cells (Ciais *et al.*, 2004; Suswam *et al.*, 2008). It was reported recently that TTP might represent a novel anti-angiogenic and anti-tumor agent because it decreases RasVal12-dependent VEGF expression and the development of vascularized tumors in nude mice (Essafi-Benkhadir *et al.*, 2007). The CPE-induced increase in TTP gene expression and decrease in VEGF gene expression in adipocytes suggests a potential role of CPE in obesity prevention and care by restricting blood supply to adipose tissue (Tilg & Moschen, 2006).

The insulin-independent effect of CPE is demonstrated by increases in TTP mRNA and protein levels in mouse RAW macrophages that are not induced by insulin (Cao *et al.*, 2008b). TTP is a highly phosphorylated protein (Cao *et al.*, 2006; Cao *et al.*, 2007a) that effects the relative ratios of anti- and pro-inflammatory proteins proposed to be important in the modulation of inflammatory responses (Frasca *et al.*, 2007). CPE sustained a two-fold increase in TTP expression in RAW macrophages for 4 h after treatment; however, the expression of a number of pro-inflammatory cytokine genes gradually increased in RAW cells treated with CPE for longer times. Because TTP mRNA levels in unstimulated RAW cells are 6–3,000-fold of TNF, COX2, GM-CSF, and IL6 mRNA levels (Table 9.3), the net increases of CPE-induced TTP mRNA molecules remains larger than the level of the pro-inflammatory cytokine mRNA molecules in the same cells (Table 9.4). These results suggest that nutritional supplements containing cinnamon can modulate inflammatory responses in mammalian species.

Another major insulin-independent effect of CPE is induction of GLUT1 mRNA, the major form of GLUT family mRNAs in RAW macrophages (Cao *et al.*, 2008b). These results suggest that CPE may regulate energy metabolism of immune macrophages since glucose is the major metabolic substrate important for the host response to injury and infection (Gamelli *et al.*, 1996; Calder *et al.*, 2007). Our PCR results showed that GLUT1 mRNA levels are approximately 8-fold of GLUT3 and 1,300-fold of GLUT4 mRNA levels, and GLUT2 mRNA is undetectable in RAW cells. Previous studies reported that GLUT1 mRNA and protein is the major if not the only GLUT in murine peritoneal macrophages whose level is induced by LPS, TNFα, burn injury, and *Pseudomonas aeruginosa* infection (Gamelli *et al.*, 1996). Furthermore, increases in GLUT1 gene expression results in enhanced glucose uptake in these macrophages (Fukuzumi *et al.*, 1996; Gamelli *et al.*, 1996). The ineffectiveness of insulin on GLUT1 gene expression in RAW cells is in agreement with a previous report that showed GLUT1 protein does not respond to insulin in either resting or phorbol 12-myristate 13-acetate (PMA)/LPS activated white blood cells from healthy people (Maratou *et al.*, 2007).

The insulin-independent effects of CPE are also seen in mouse adipocytes (Cao *et al.*, 2007c; Cao *et al.*, 2008c). CPE increases TTP mRNA levels up to 9-fold during a 30–90 min treatment. TTP mRNA levels are still 2-fold those of the controls after 16 h treatment by CPE at 100 g/mL (Cao *et al.*, 2007c).

TTP gene expression is also rapidly induced by insulin, but the induction pattern is different from that of CPE (Cao *et al.*, 2008c). TTP mRNA levels are increased 7-fold that of the control with 30-min treatment of insulin, but the levels return to that of the control within 2 h treatment (Cao *et al.*, 2008c). These results suggest that the mechanism of TTP induction by CPE is different from that of insulin.

CONCLUSIONS

The results presented here support the hypothesis that cinnamon polyphenol extracts have insulin-like and unique effects on the regulation of gene expression in mouse adipocytes and RAW macrophages. The insulin-like effects of CPE on gene expression include the increased expression levels of TTP and decreased levels of those of VEGF. The effects of CPE are different from those of insulin on gene expression. CPE increases TTP and GLUT1 gene expression in RAW macrophages and the induction of TTP gene expression in adipocytes is sustained over a longer period time than the short-lived induction by insulin. Our results indicate that CPE regulates multiple genes in adipocytes and macrophages, and the health benefits of cinnamon would include effects on glucose metabolism and inflammation.

A cellular model of the beneficial action of CPE on induction of TTP and components in the insulin signal transduction pathway are shown in Fig 9.8. CPE 1) increases insulin receptor efficiency by increasing tyrosine phosphorylation and decreasing phosphatase (Imparl-Radosevich *et al.*, 1998); 2) increases the amount of INSRβ protein (Cao *et al.*, 2007c); 3) increases the amount of GLUT4 protein (Cao *et al.*, 2007c); 4) increases glycogen synthase activity and glycogen accumulation (Jarvill-Taylor *et al.*, 2001); 5) decreases GSK3β activity (Jarvill-Taylor *et al.*, 2001); and 6) increases TTP (Cao *et al.*, 2007c; Cao *et al.*, 2008b). Thus, CPE could enhance more efficient glucose transport and utilization, and CPE-induced TTP could regulate the pro-inflammatory cytokines in macrophages and adipocytes that are important in obesity related conditions.

ABBREVIATIONS

ADIPOQ, adiponectin; AKT1 (PKB), thymoma viral proto-oncogene 1 (protein kinase b); APP, amyloid beta precursor protein; ARE, AU-rich element; CP, cinnamon polyphenols; CPE, cinnamon polyphenol extract; COX2 (PTGS2), cyclooxgenase-2 (prostaglandin-endoperoxide synthase 2); CRP, C-reactive protein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; G-CSF (CSF3), granulocyte colony-stimulating factor (colony-stimulating factor 3); GM-CSF (CSF2), granulocyte-macrophage colony-stimulating factor; GLUT (SLC2A), glucose transporter (solute carrier

family 2); HPLC, high performance liquid chromatography; HuR (ELAVL1), Hu antigen R (embryonic lethal, abnormal vision-like 1); GSK3B, glycogen synthase kinase 3 β; GYS1, glycogen synthase 1; IER3, immediate early response 3; IFNγ, interferon-gamma; IGF, insulin-like growth factor; IGFR, insulin-like growth factor receptor; IL, interleukin; INS, insulin; INSR, insulin receptor; IRS, insulin receptor substrate; LEP, leptin; LEPR, leptin receptor; LPS, lipopolysaccharide; PAI1 (SERPINE1), plasminogen activator inhibitor I (serine/cysteine peptidase inhibitor 1); PCR, polymerase chain reaction; PIK3CB, phosphatidylinositol 3-kinase, catalytic, β; PIK3R1, phosphatidylinositol 3-kinase, regulatory subunit 1; RPL32, ribosomal protein L32; SHC1, Src homology 2 domain-containing transforming protein 1; SOS1, Son of sevenless 1; TAU, microtubule-associated protein tau; TNF, tumor necrosis factor; TTP, tristetraprolin. VEGF, vascular endothelial growth factor; ZFP36, zinc finger protein 36; ZFP36L, ZFP36-like.

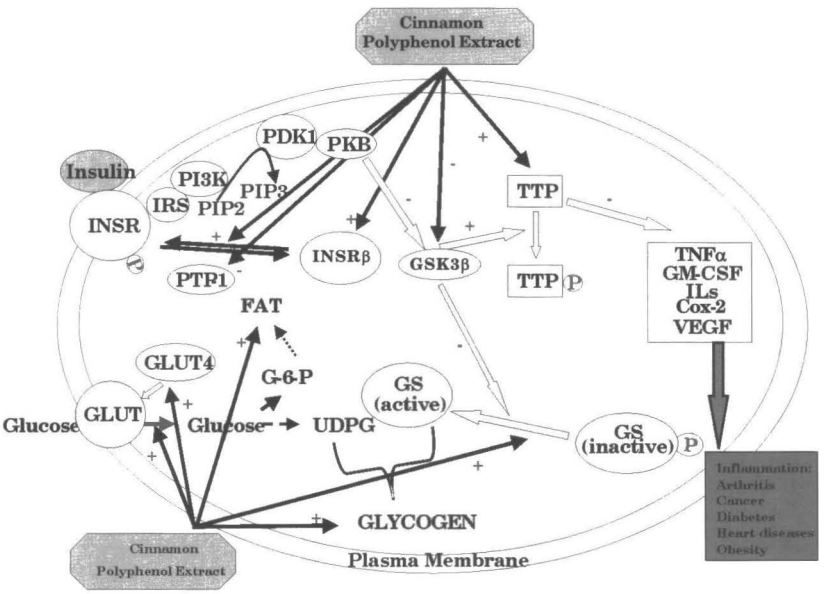

Fig 9.8. A model of actions by CPE and TTP in the insulin signal transudation pathway leading to the beneficial effects in people with type 2 diabetes: 1) CPE activates IR by increasing their tyrosine phosphorylation activity and by decreasing phosphatase activity that inactivates the receptor (Imparl-Radosevich *et al.*, 1998); 2) CPE increases the amount of INSRβ protein (Cao *et al.*, 2007c); 3) CPE increase the amount of GLUT4 protein (Cao *et al.*, 2007c) and GLUT1 mRNA levels (Cao *et al.*, 2008b); 4) CPE increases glycogen synthase activity and glycogen accumulation (Jarvill-Taylor *et al.*, 2001); 5) CPE decreases GSK3β activity (Jarvill-Taylor *et al.*, 2001); and 6) CPE increases TTP gene expression (Cao *et al.*, 2007c; Cao *et al.*, 2008b). Refer to the text for more details ("+" represents positive effect and "-" represents negative effect) [modified from (Cao *et al.*, 2007c)]

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10

Flax Cyanogenic Glycosides

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ABSTRACT

Flaxseed (Linum usitatissimum L.) is an oilseed crop of growing international interest due to its high α-linolenic acid (an ω-3 fatty acid) content. Flaxseed oil is used industrially in the production of paints, stains, surface coatings and various ω-linolenic acid-derived oleo chemicals. Flaxseed is also one of the richest sources of ω-3 fatty acid, making both the seed and its oil of significant neutraceutical importance. Flaxseed and flaxseed meal (a byproduct of the oil extraction process), is being used increasingly as a feed supplement for economic cost recovery in the case of meal and in order to incorporate α-linolenic acid into livestock and poultry products in the case of whole seed. Both flaxseed and flaxseed meal contain cyanogenic glycosides; linustatin (2-[6-β-D-glucosyl-β-D-glucopyranosyloxy]-2-methylpropionitrile) and neolinustatin ((R)-2-[6-β-D-glucosyl-β-D-glucopyranosyloxy]-2-methylbutyronitrile), gentiobiosides of acetone cyanohydrin and 2-butanone cyanohydrin, respectively. The corresponding cyanogenic monoglycosides linamarin (2-β-D-glucopyranosyloxy-2-methylpropionitrile) and lotaustralin (R)-2-β-D-glucopyranosyloxy-2-methylbutyronitrile) have been found in seedlings, developing plants, flowers at anthesis and immature seed, but are present only at trace levels in mature seed. Cyanogenic glycosides liberate cyanide upon hydrolysis either by endogenous seed enzymes or by acid-catalyzed hydrolysis (which can occur in the gut, especially in ruminants). The presence of these anti-nutritives in flax seed or meal restricts their use in animal feed mixtures. Many methods exist in the literature for the detection of cyanogenic compounds. Historically, wetted macerated plant material was incubated in a sealed flask, with hydrolysis of cyanogenic species occurring either by endogenous hydrolytic enzymes or chemically by added dilute

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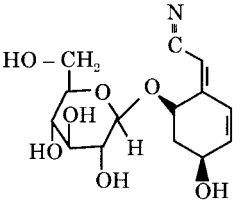
sulfuric acid and heat. Liberated cyanide was detected by filter paper strips suspended above the solution that were coated with either sodium picrate (brick red response) or guaiacum resin treated with dilute copper sulfate and allowed to dry (blue response). Plants with high cyanogenic potential were measured quantitatively by chemical hydrolysis of macerated plant material with tartaric acid, steam distillation of the liberated cyanide and titration of the distillate with standardized silver nitrate. The main drawbacks of this technique are low sensitivity, lack of specificity (the identities of the specific cyanogenic molecules are not revealed), and the fact that it is extremely labor intensive. Methods in the literature specific to the detection of cyanogenic glycosides in flax include chemical and enzyme-linked colorimetric tests, TLC, HPLC and GC of trimethylsilyl (TMS) derivatives. Chemical hydrolyses function by employing strong acids at elevated temperatures to hydrolyze the cyanogenic glycosides and then quantify the liberated cyanide gas by a colorimetric method. Although amenable for rapid analysis of large sample sets, it was found to be much less accurate than enzyme-linked assays. Enzyme-linked assays function by employing endogenous hydrolytic enzymes (either as is or spiked with a crude enzyme preparation) to hydrolyze the cyanogenic glycosides. Quantification is achieved by measuring either the liberated cyanide or glucose using a colorimetric test. Although these methods have been demonstrated to be as accurate as HPLC, they do not provide information on levels of individual cyanogenic glycosides. Additionally, the use of endogenous or crude enzyme preparations adds a variable that could severely impair repeatability and reproducibility from a validation perspective. Although existing TLC methods are capable of resolving linustatin and neolinustatin, they are not capable of full resolution of diglucosides from their respective monoglycosides; moreover, TLC is generally not considered a quantitative test. GC or HPLC methods is typically the preferred method of analysis due to its high resolving power, capability of direct quantification of analytes and automation. This work presents current knowledge on cyanogenic glycosides in flaxseeds and discusses the analytical methods used to measure them.

Key words: Flax, cyanogenic glycosides, linustatin, neolinustatin, linamarin, lautostralin

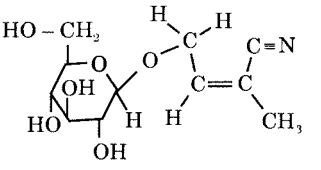
INTRODUCTION

There are two types of cyanide-containing compounds, the cyanogenes or cyanogenic glycosides and the non-cyanogenic cyanoglycosides. Non-cyanogenic cyanoglycosides compounds have a nitrile group not adjacent to the glycosidic linkage (Fig 10.1); *e.g.* menisdaurin (Yamasaki, 1997) found in *Menispermaceae* or mulfidin present in latex from *Jatropha mulfidita* (van den Berg *et al.*, 1995). Cyanogenic glycosides are glycosides issued

from an amino acid-derived cyanohydrin; the nitrile group *alpha* to the glycosidic linkage (Fig 10.1) can be released as cyanide by acidic or enzymatic hydrolysis. There are about 75 known cyanogenic glycosides in more than 2500 plant species including grains such as wheat, barley and flax (Conn, 1994; Jones, 1998). Cyanogenic glycosides have an uneven distribution in the plants. They could be present in the whole plant (cassava), in the seeds but not in the flesh of the fruits (apple), or in the roots and the leaves of the plant but not in the fruits or the seeds (mango, papaya or cereals) (Jones, 1998).

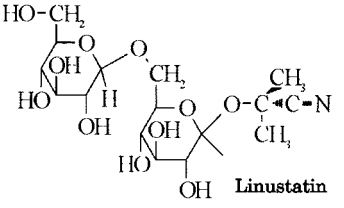


Menisdaurin

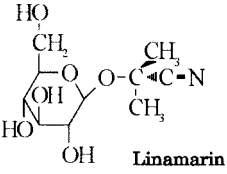


Multifidin

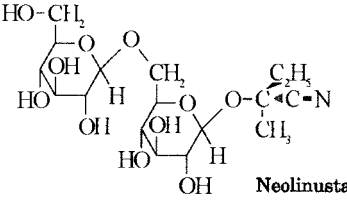
(*a*) Non-Cyanogenic cyanoglycosides



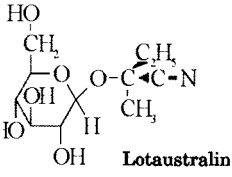
Linustatin



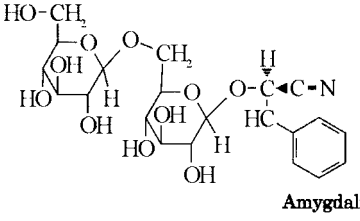
Linamarin



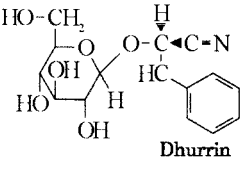
Neolinustatin



Lotaustralin



Amygdalin



Dhurrin

(*b*) Cyanogenic cyanoglycosides

Fig 10.1. Scheme of the known non-cyanogenic and cyanogenic cyanoglycosides

In his review on the distribution of cyanogenic glycosides in higher plants, Jones (1998) suggested that human might have preferably domesticated cyanogenic plants as food sources because these plants were healthier and offered higher yield. The cyanogenic glycoside provided the plants resistance to predators (herbivores, nematodes or mold).

Amygdalin, also known as laetrile, is derived from L-tyrosine and is likely the most notorious cyanogenic glycoside (Fig 10.1). It can be found in seeds of the *Rosaceae*, *e.g.* bitter almonds (Conn, 1969), apple (Lu & Foo, 1998), apricot (Femenia *et al.*, 1995) and prunes (Santamour, 1998). It became infamous because of its questionable use in cancer treatment in Mexico (Herbert, 1986; CA, 1991a,b; National Cancer Institute, 1996). The two crops that over the years gave the most concern regarding their cyanogenic glycoside contents are cassava (*Manihot esculenta* C.) and lima bean (*Phaseolus lunatus* L.) for human and white clovers for animals. Cassava contains two cyanoglycosides: linamarin and lotaustralin (Fig 10.1) (Cooke, 1985). The cyanogene content of cassava could vary from 15 to 400 mg/kg of fresh weight; consumption problems arise from the high amount of cassava ingested and the poor nutritional state of the consumers (Abuye *et al.*, 1998). In processed cassava food, variable amounts of residual cyanide are found. Several breakdown products such as acetone cyanohydrin and free cyanide are also present (Aletor, 1993; Jackson, 1994; Egan *et al.*, 1998; Ojo & Deane, 2002).

Currently, flaxseed or linseed (*Linum usitatissimum L.*) is receiving a lot of attention from researchers as well as from the general public because of its nutritional qualities. Flaxseed and flaxseed oil contain the highest level of α-linolenic acid (ALA), a ω-3 fatty acid, when compared to other oilseeds. This fatty acid is an essential fatty acid and has several beneficial effects on heart disease, inflammatory disease to name a few (Cleland & James, 2003; Dupasquier *et al.*, 2006). Flaxseed also contains lignans (secoisolariciresinol diglucoside or SDG), which have antioxidant properties and may help protect against certain cancers (Thompson, 2003). The increased use of flaxseed in food and feed because of their nutritional effects brought all the anti-nutritional components of flaxseed under the spotlight and cyanogenic glycosides are the most reported anti-nutrients found in flaxseeds. The presence of cyanogenic glycosides in flaxseed was first reported by Halverson *et al.* (1955) who showed that flax meal contained some compounds, unidentified at the time, soluble in 50% ethanol having a protective effect in rats against chronic selenium poisoning. Later, these compounds (Fig 10.1) were identified as cyanogenic glycosides and were named linustatin and neolinustatin (Palmer *et al.*, 1980; Smith *et al.*, 1980). The main cyanogenic glycosides of flaxseeds are linustatin (2-[6-β-D-glucosyl-

β-D-glucopyranosyloxy]-2-methyl-propionitrile) and neolinustatin ((*R*)-2-[6-β-D-glucosyl-β-D-glucopyranosyloxy]-2-methyl-butyronitrile), two diglucosides (gentobiose) with different hydroxynitrile substituents (Fig 10.1). Linamarin (2-β-D-glucopyrano-syloxy-2-methyl-propionitrile) and lotaustralin ((*R*)-2-β-D-glucopyrano-syloxy-2-methyl-butyronitrile), two monoglucosides (glucose) with the same two hydroxynitrile substituents, were also found in the cotyledon leaves and the seeds (Bulter & Conn, 1964; Oomah *et al.*, 1992; Niedźwiedź-Siegeñ, 1998). Conn (1969) reported that flax cotyledon leaves contained a mixture of 50-50 linamarin and lotaustralin.

It was reported that in flax, the mature seeds contain the cyanogenic diglucosides (linustatin and neolinustatin) but only the cyanogenic monoglucosides (linamarin and lotaustralin) are found in the seedlings and in the leaves, stem and roots of the plant (Niedźwiedź-Siegeñ, 1998; Krech & Fieldes, 2003).

It seems the simultaneous occurrence of the four cyanogenic glycosides (two mono- and two diglucosides) in flaxseed might be questionable for some authors. Typically only one or two cyanogenic glycosides, characteristic of the specie, are found in a given plant (Vetter, 2000). It was suggested that the quantified cyanogenic monoglucosides were in fact the products of hydrolysis of the cyanogenic diglucosides by β-glucosidase and only cyanogenic diglucosides were present in flaxseed (Smith *et al.*, 1980). We believe that in sound flaxseed, the four cyanogenic glycosides are present but at different levels; linustatin and neolinustatin are the main cyanogene compounds whereas linamarin and lotaustralin should be at the trace levels. The monoglucosides are precursors of the diglucosides (Fig 10.2); it is likely that they are still present in the seed at very low levels. Incomplete extraction, poor detection limit (refractive index) of the analytical method and lack of good standards would not allow the detection of the low concentration (traces) of these cyanogenic mono-glycosides. At the same time, reporting significant levels of linamarin and lotaustralin in mature seed is likely the results of the hydrolysis of linustatin and neolinustatin suggesting a poor extraction method.

As in other cyanophoric plants, cultivar, age, development, environment, nutritional and genetic factors affect the cyanogenic contents of flax seeds and flax oil (Oomah *et al.*, 1992; Mazza & Oomah, 1995). In mature flaxseed the amount of cyanogenic glycosides was found around 0.1% of the dry-weight of the seeds whereas in young seedlings this level could reach up to 5% of the dry weight of the plant (Bulter & Conn, 1964). The leaves of the flax plant contain higher levels of cyanogenic glycosides when compare to the stems or the roots of the plant (Bulter & Conn, 1964).

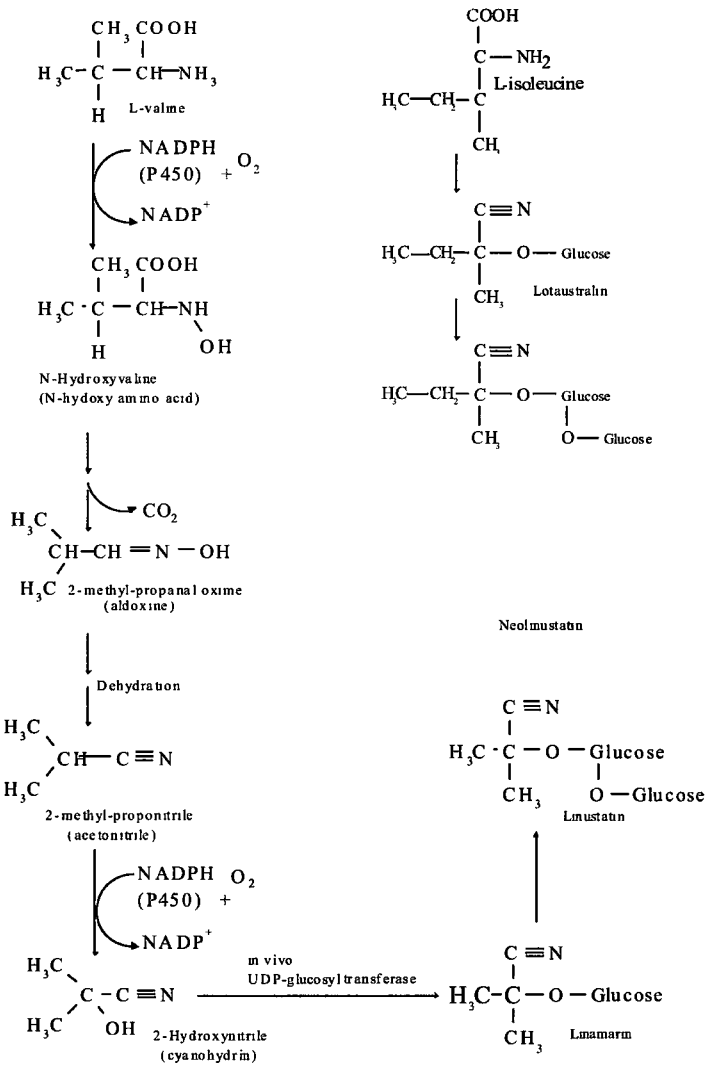


Fig 10.2. Scheme of the synthetic pathway of flax cyanogenic glycosides (Smith *et al.*, 1980)

BIOSYNTHESIS

The biosynthesis of the cyanogenic glycosides in plants has been studied for over 40 years. Conn (1994) reviewed it and presented his perspective on this work.

Amino acids are the starting block of cyanogenic glycoside synthesis (Fig 10.2). L-valine is the precursor for linamarin and linustatin and L-isoleucine is the precursor of lotaustralin and neolinustatin. The synthesis of linamarin takes place in the cotyledons with a high specific activity (Cutler

& Conn, 1981; Cutler *et al.,* 1985). The specificity towards the amino acids in the first step of the pathway is responsible for the specificity of the biosynthesis of the cyanogenic glucosides. In a model system with microsomal enzyme system obtained from 3 day seedlings, only valine, isoleucine and L-allo-isoleucine were able to promote the production of HCN whereas following steps of the biosynthetic pathway of the cyanogenic glycosides was not specific since from a large variety of aldoximes was able to produce cyanogenic compounds (Cutler *et al.,* 1985). The scheme of the synthetic pathway (Fig 10.2) of linustatin from L-valin in flax dipicted some of the research done on the biosynthesis of various cyanogenic glycosides (linamarin, lotaustralin and dhurrin) (Hahlbrock & Conn, 1970; Zilg *et al.,* 1972; McFarlane *et al.*, 1975; Smith *et al.*, 1980; Culter & Conn, 1981; Cutler *et al.,* 1985; Halkier *et al.,* 1990; Kock *et al.,* 1992; Vetter, 2000).

The enzymes responsible for the biosynthesis of the cyanogenic glycosides are located in different parts of the cell. The enzymatic system responsible of the production of acetone cyanohydrin from L-valine with NADPH of cytochrome P-450 as co-factor is located in the tonoplast of the cell whereas the UDPG-glucosyl transferase in located in the vacuole. The same UDPG-glucosyl transferase is responsible for the synthesis of linamarin and lotaustralin from the corresponding two cyanohydrins in presence of UDPG-glucose with only UDPG-glucose accepted as a donor for this reaction (Hahlbrock & Conn, 1970). The cyanogenic glycosides are also transported, often as diglucosides. In cassava, linamarin is synthesized in the cotyledons and then transported to the roots, in mature plant the synthesis in located in the leaves (Vetter, 2000).

The metabolism of each amino acid into its corresponding cyanogenic glycoside is influenced by the content of the other amino acids. Carbon 14 valine was able to inhibit the production of HCN from isoleucine by 40% while 14C-isoleucine inhibited the metabolism of valine by 16% (Cutler *et al.,* 1985).

DEGRADATION

Cyanogenic glycosides upon hydrolysis generate HCN which is lethal for the plant. To ensure the non spontaneous/uncontrolled hydrolysis of the cyanogenic glycosides in sound tissues and therefore its survival, it is suggested that a mechanism has been develop by the plant to ensure that cyanogenic glycosides are not hydrolyzed. Linamarase, the β-glucosidase responsible of the hydrolysis of linamarin with high specificity, is present in the apoplastic space between the endosperm storage tissue and the cotyledon (Selmar *et al.*, 1988) whereas the cyanogenic glycosides are located in the vacuoles (Gruhnert *et al.,* 1994).

Uncontrolled degradation of cyanogenic glycosides with the liberation of HCN occurs in damaged seeds with disrupted cellular structures since degradation enzymes and cyanogenic substrates are in different cellular

structures (Conn, 1994). Flax seed contains two distinct β-glucosidases that are performing stepwise removal of the glucose residues (Fig 10.3) from the cyanogenic diglucosides liberating α-hydroxynitrile. The first enzyme, called linustatinase, is responsible for the hydrolysis of the diglucosides (linustatin and neolinustatin) into cyanogenic monoglucosides; this enzyme showed little activity towards the monoglucosides, linamarin and lotaustralin. The cyanogenic monoglucosides are then hydrolyzed by another β-glucosidase, linamarase, which is inactive towards β-diglucosides (Fan & Conn, 1985; Mazza & Oomah, 1995). The common characteristics of these enzymes are that they are specific towards their native substrates, have mannose and/or glucose in their structure, have a protonated amino acid (aspartate or glutamate) in their active site and need the substrate to be hydrolyzed to have a β-configuration (Fan & Conn, 1985; Conn, 1994). It has been found that against all odds, ground flaxseeds were able to retain more β-glucosidases activity than whole flaxseeds during heating (Chadha *et al.*, 1995).

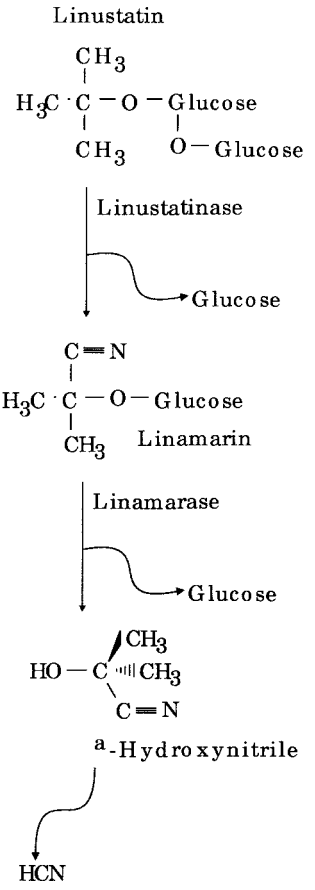


Fig 10.3. Scheme of the enzymatic hydrolysis of linustatin in flaxseeds (Mazza & Oomah, 1995)

ROLE

Cyanogenic glycosides are secondary compounds of the plants metabolism, the literature showed that they might be involved as nitrogen storage compound for the synthesis of amino acids and/or as plant deterrent against herbivores, mold and/or insects.

In *Hevea*, linamarin could be a nitrogen storing compound involved in the biosynthesis of asparagine (Lieberei *et al.*, 1986; Selmar *et al.*, 1988). The seed of *Hevea* opposite to flax does not contain significant amount of linustatin, only the mono-glucoside forms are found; linamarin mainly (Selmar *et al.*, 1987) with traces of lotaustralin in some *Hevea* species (Lieberei *et al.*, 1986). During germination and plant development, linamarin is hydrolyzed without release of HCN. Linamarin is translocated from the endosperm where it is stored, to the young leaves where it is used via the apoplastic space. To avoid the hydrolysis of linamarin in the apoplastic space by linamarase, linamarin is glycosylated to form linustatin that could not be hydrolyzed by the *Hevea* linamarase. In the leaves, linustatin is then hydrolyzed by a different glucosidase to give gentobiose (no stepwise hydrolysis) without release of any HCN. In the leaves, there is β-cyanoalanine synthase that is able to use the acetone-cyanohydrin from the hydrolysis of linustatin to form β-cyanoalanine which then gives asparagine. This enzyme system (glucosidase plus synthase) is responsible of the non-release of HCN and its use to produce amino acid (Selmar *et al.*, 1988).

In flax, the quality and the quantity of cyanogenic glycosides change depending on the tissue analyzed (seed, seedling, leaf, root or stem) and the time of analysis. There is an increase in cyanogenic glucoside content in the seedling during the first five days after germination when compared to content of the mature sound seed (Bulter & Conn, 1964; Niedźwiedź-Siegeñ, 1998; Fieldes & Gerhardt, 2001; Krech & Fieldes, 2003). Then, the cyanogenic glycoside content of the developing flax plant decreases to increase again just before flowering, without reaching the levels observed in the first 5 days after sowing (Neidzweidz-Siegien, 1998). Initially, during germination, the cyanogenic diglucosides of the seed are degraded during the first 3–4 days this is followed by a *de novo* synthesis (Niedźwiedź-Siegeñ, 1998; Fieldes & Gerhardt, 2001; Krech & Fieldes, 2003). The highest concentration of the cyanogenic glycosides (monoglucosides) is found in the leaves and the cotyledons of the seedling. The stem contains about 4 times less cyanogenic glycosides than leaves and cotyledons of the seedling; this level becomes similar to the leaves concentrations in the mature plant. The cyanogenic contents of the roots of the seedlings are at the trace levels at the beginning to increase marginally in the mature plant (Niedźwiedź-Siegeñ, 1998, Fieldes & Gerhardt, 2001; Krech & Fieldes, 2003). In immature seeds, both

cyanogenic mono-glucoside and diglucosides are found but in mature seeds only the cyanogenic diglucosides are found (Niedźwiedź-Siegeñ, 1998; Krech & Fieldes, 2003). The leaves and the stem of the flax are the part of the plants that are more susceptible to herbivores and other predators; these results suggested that in this case cyanogenic compounds could have a role in the defense of the plants against predators. This would agree with Jones (1998) who suggested that cyanogenic glycoside were involved as a defense mechanism by plants against herbivores.

In flax, no cyanogenic diglucoside has been found in any of the tissues of the plant except the mature sound seeds. This suggested that cyanogenic diglucosides are likely not used for translocation of the cyanogenic monoglucosides. Opposite to *Hevea*, flax seed possesses two distinct β-glucosidases with different specificity towards the cyanogenic mono or diglucosides (Fan & Conn, 1985). In flax seed, the activity of linamarase, responsible of the hydrolysis of the cyanogenic monoglucosides, changes during the early stages of the seed germination (before 13 days) and it seems that the intensity of the activity could affect the development of the cotyledons. After 13 days, there is no more effect of linamarase activity on the cotyledon development (Fieldes & Gerhardt, 2001). Both flaxseed cyanogenic diglucosides are hydrolyzed in the first 84 h of the germination process without generating any HCN. This hydrolysis is followed by an important *de novo* synthesis of cyanogenic monoglucosides (Krech & Fieldes, 2003). This suggested that cyanogenic glycoside hydrolysis has other purposes than nitrogen storage/production in the flax plant. It also seems that purpose might depend on the development stage of the plant (Fieldes & Gerhardt, 2001). In young seedlings, cyanogenic glycosides could be used to influence the germination and the intensity of the plant development. It was suggested that at the early stages of the development cyanogenic glycosides could be used in the regulation/synthesis of ethylene implicated in the breaking of seed dormancy and the promotion of embryonic growth. This hypothesis was favored by Krech and Fieldes (2003) after observing the sharp momentary decrease in cyanogenic glycosides linked to the strength of the seedling development only at the beginning of germination.

TOXICOLOGY

Animals, including humans, react differently to cyanogenic glycosides, depending on the anatomical structure of their digestive system, their capacity to detoxify HCN and the amount of cyanogenic glycosides ingested. Cyanogenic glycosides have the potential to be lethal because of the HCN produced upon their enzymatic or chemical hydrolysis. Hydrogen cyanide, a well know poison with a faint bitter almond-like odor, interferes with the cytochrome oxidase system of the electron transport chain of the

mitochondria making oxygen unavailable to the cell or the organism leading to death of the organism. Exposure to HCN may be acute (not very frequent) and then fatal or chronic (more frequent) – small amount on a regular basis- leading to neurological damages and/or growth depression. Several pathways help to metabolise HCN and eliminate it. The main detoxification route is the excretion of thiocyanate in the urine via transformation of HCN by rhodanese, a sulfur transferease from the liver mitochondria (Bodansky, 1929). The formation of 2-imino-4-thiazolinecarboxylic acids by reaction with free cystine is a minor detoxification pathway, about 5 times less important than the thiocyanate formation (Wood & Cooley, 1956). In France, hydryxocobalamin (vitamin B12a) has been used as an effective antidote to cyanide poisoning leading to the formation of cyanocobalamin (vitamin B12) (Borron *et al.*, 2007).

Numerous publications deal with the toxicology of ingestion of cassava or linamarin alone in humans and animals but little to no literature has been reported on the metabolism of flaxseed cyanogenic glycosides in human or animal. It could be assumed that the metabolism of the cyanogenic diglucosides from flax would be very similar to the metabolism of the other cyanogenic glycosides, mainly cyanogenic monoglucosides. However, cassava diets are usually very poor in protein whereas flaxseed contains on average over 23% protein.

For the cyanogenic glycosides to be a problem, they have to be hydrolyzed to produce HCN. This implies that the cell structure of the cyanogenic plant/seed has to be damaged to allow the degradation enzymes (β-glucosidases) from the seeds or gut microflora to be in contact with the cyanogenic compounds to degrade them in HCN and sugar.

Bovines and sheep are ruminants, they are more susceptible to cyanogenic glycoside poisoning than human, rats or pigs. Ruminant animals have a stomach with several compartments containing a large varied microflora and an important quantity of enzymes. The pH of their stomach varies from 5.8 to 7.0 promoting the hydrolysis of the cyanogenic glycosides by the enzymes and/or microflora and resulting in the production of HCN which is then rapidly adsorbed into the blood stream. There were several reports of "sudden death syndrome" in animals grazing in pasture, all related to cyanide poisoning due to cyanogenic plants (Vetter, 2000). At low doses, cyanogenic glycosides from flax seed demonstrated a goitrogenic effect in sheep that could be reversed by addition of potassium iodide or di-sodium L-thyroxine (Care, 1954).

Animal model studies (rats) with cyanogenic glycosides showed that part of the ingested linamarin was excreted intact in the urine and another part was metabolized by the liver to for thiocyanate (Philbrick *et al.*, 1977; Maduagwu, 1989). In presence of an excess of vitamin B2, larger amounts of intact urinary linamarin was excreted when compared to control animals,

while it took an extra 24 h for protein deficient rats to eliminate HCN as thiocyanate (Umoh *et al.*, 1986). These results suggest that the dietary deficiencies (vitamin or protein) will exacerbate the toxic effects of cyanogenic glycosides.

In healthy human subject fed cassava porridge (250–750 g of cassava product/day, one day diet), a large portion (1/4) of the linamarin ingested was eliminated following an unknown pathway. Some linamarin was rapidly excreted in the urine of the subjects while the rest of the linamarin was hydrolyzed, probably in the gut to produce HCN. In the liver, the HCN was also transformed into thiocyanate by an enzymatic reaction (rhodanese) (Carlson *et al.*, 1999). In the blood stream, HCN binds with methemoglobin to form cyanomethemoglobin, which is not able to fix oxygen. HCN is known to inhibit respiration by interrupting the electron transport down the cytochrome chain by inhibiting the cytochrome a to cytochrome a (3) step. Animals with a very acidic stomach, such as swine, horses and humans, the acid could react with the HCN to form less toxic substances. The consequence of the metabolic effect of cyanogenic glycosides depends on (1) the amount of cyanogenic glycosides ingested, (2) the presence of other components interacting with them, (3) the nutritional and health status of the consumer (protein deficiency) and (4) the frequency of consumption period (Jackson, 1994). The no-observed-adverse affect level was 4.5 mg/kg of body weight in rats, at 12.5 mg/kg of body weight small effects were observed in the reproductive system of the male rats without affecting the fertility (WHO, 2004). High cassava consumption, therefore high cyanogenic glycoside ingestion, combined with low protein diet (low sulfur availability and low vitamin B) makes cyanogenic glycosides detoxification ability greatly reduced (Umoh *et al.*, 1986). A nutritional study performed on volunteers ingesting 30 to 100 g of flaxseed for several weeks showed no sign HCN intoxication; long term intake resulted in an increase in plasma and urinary thiocyanate levels (Schulz *et al.*, 1983).

Toxicology studies suggested that it is important to measure cyanogenic glycosides as such and report the results as cyanogenic glycoside content and not as HCN equivalent only. The metabolism of HCN in human and animals is well known and it is different from the metabolism of the cyanogenic glycosides. Moreover, plants contain combination of cyanogenic glycosides with various nitrile groups and various glycoside radicals, reporting only HCN equivalent gives no indication on the identity of the measured compounds. Once ingested, various cyanogenic glycosides might be different metabolism in the organism and their toxicological potential might have different. It is well known that the gut microflora varies with the nutrition so the metabolism of linustatin and neolinustatin might be (or not) different when the usual diet contains only linamarin and/or lotaustralin. Moreover, when studying the metabolism pathway (biosynthesis or

degradation) of the cyanogenic glycosides, all the glycosides and their degradation products should be identified. This will allow us to have a complete picture of the role of these important secondary compounds in the plant kingdom.

METHODS OF ANALYSIS

Many methods have been reported in the literature for the analysis of cyanogenic glycosides in flax and other plant species. Although cyanogenic glycosides may differ between plant species, many analytical methods are universally applicable with only small changes necessary to accommodate differing sample matrix or cyanogenic glycoside identity (for chromatographic methods). As there are a multitude of methods including several official methods for the analysis of cyanide in almost every conceivable matrix, it is not surprising that many investigators sought methods to liberate and isolate cyanide from cyanogenic glycosides and then apply a known method for analysis. Nearly three decades ago, Cooke (1978) and Harris *et al.* (1980) acknowledged that most methods for measuring cyanogenic glycosides have three parts; (1) hydrolysis of the glycosides, (2) isolation of liberated cyanide and (3) measurement of cyanide. Both groups also acknowledged that measurement was not a challenge due to the presence of many highly characterized methods for cyanide analysis; it was the hydrolysis and isolation that were the most challenging. Challenges for the development of methods for the analysis of cyanogenic glycosides have been the lack of certified or standard reference material (CRM or SRM), poor purity of cyanogenic glycoside standards or seed samples, complete unavailability of commercial lotaustralin, unavailability of purified β-glycosidases specific for cyanogenic glycosides in flax and lack of published data on comparison among various methods.

Methods may be broadly based on (see Fig 10.4) whether the cyanogenic glycosides are hydrolyzed or extracted intact followed by quantification (directly or indirectly by liberated sugar or cyanide). Hydrolytic methods are capable of detecting all forms of hydrolyzable cyanide in a sample and therefore are preferable where degradation is suspected or the identity of the cyanophore is unknown. Instrumental methods such as high performance liquid chromatography (HPLC) or gas chromatography (GC) are amongst the most costly methods but are preferable when the identity and quantity of each cyanogenic glycoside are required. Spectrophotometric methods benefit from being the least expensive, not even requiring a spectrophotometer if accuracy may be sacrificed and a series of standards are run for comparison. Each method has advantages and disadvantages that must be weighed against the requirements of the analysis.

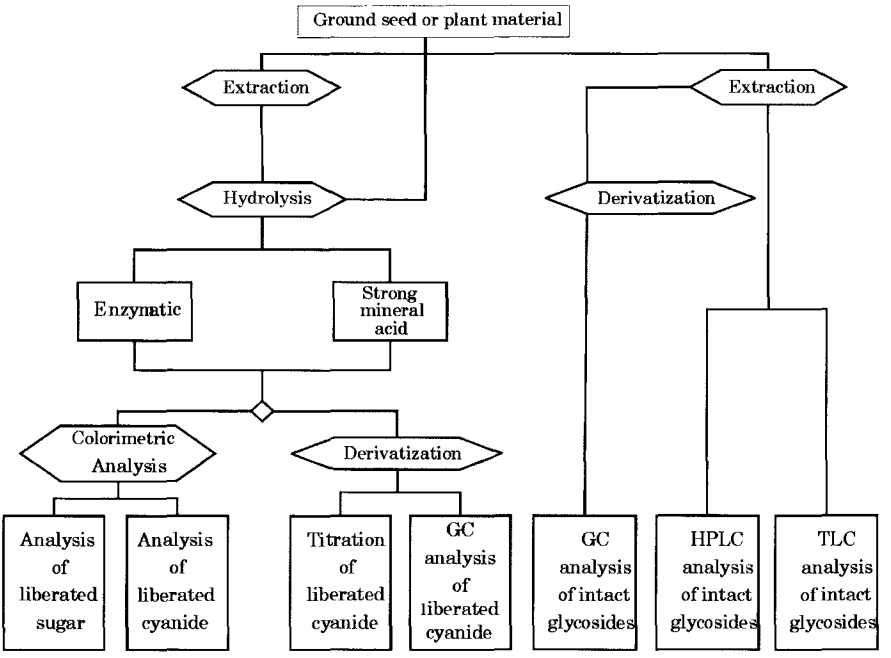


Fig 10.4. Summary of analytical methods for cyanogenic glycosides

Extraction of Cyanogenic Glycosides from Flax

The accuracy of any analytical method can be no better than the extraction method used to prepare the sample. If the extraction method does not have a high, well-defined and repeatable extraction efficiency, it cannot compensate for normal matrix differences (*e.g.* oil content, moisture, protein content, etc.) or has not been evaluated for extract stability (between extraction and analysis), then the best analytical method can not provide meaningful results. Extraction efficiency is controlled by four main factors; sample grinding method, extraction solvent choice, extraction method and stability of extracted analyte. Optimization of each of these parameters is critical to ensure high extraction efficiency and repeatability. Published methods for the extraction of cyanogenic glycosides from flax vary in the literature with no clear consensus on a preferred method. The most widely used extraction method involves extraction of ground seed with 70% methanol in a 30°C sonicating water bath for 1 h (Schilcher & Wilkens-Sauter, 1986; Cunnane *et al.*, 1993). Variations on this method involve shortening the extraction time to 30 min (Oomah *et al.*, 1992) and using 70% ethanol (Kobaisy *et al.*, 1996), 80% methanol (Krech & Fieldes, 2003; Park *et al.*, 2005), or boiling water (Kobaisy *et al.*, 1996) instead of 70% methanol. Other methods include overnight room temperature

extraction of ungrounded seeds in methanol on a shaker (Frehner *et al.*, 1990), grinding in liquid nitrogen followed by extraction with boiling 80% ethanol (Niedźwiedź-Siegień, 1998), grinding to pass through a 1 mm sieve and shaking in 0.1 M orthophosphoric acid for one h (Harris *et al.*, 1980) and electric homogenization in water followed by autohydrolysis (Chadha *et al.*, 1995). Wanasundara *et al.* (1993) extracted glycosides with 70% ethanol (7°C, 1 h), took the extract to dryness, resuspended it in methanol then chloroform (1:2 ratio), clarified it by centrifugation, evaporated the supernatant and redissolved the dried material in aqueous methanol (15%). Recent work (Bacala & Barthet, 2007) has shown that the extraction efficiency varied up to 12% between three grinding methods (impact mill, coffee grinder and impact + sieving mill), up to 18% between four extraction methods (single extraction in sonicating water bath, single extraction using a vortex mixer, single and triple extraction using a Polytron style homogenizer) and by 97% between four concentrations of aqueous ethanol (50, 80, 90 and 100% ethanol). The highest recovery combination involved grinding in a Retsch ZM200 mill (18000 rpm, 1.0 mm sieve) followed by triple extraction with 70-80% methanol in a 40°C sonicating water bath. Aside from this work, there is a total lack of characterization regarding the efficiency and reliability of any one extraction method and no comparison between extraction methods. These results show a clear need to develop a standardized method with a well-defined extraction efficiency and repeatability.

HYDROLYSIS METHODS AND DISTILLATION

Enzymatic Hydrolysis Methods

The most critical factor to consider for enzymatic hydrolysis methods is the choice of enzyme. As discussed previously, there are two β-glycosidases present in flax that are required for the sequential hydrolysis of linustatin and neolinustatin to their corresponding cyanohydrin linamarase and linustatinase and then hydroxynitrile. Linustatinase will convert linustatin to linamarin and neolinustatin to lotaustralin (at 59% of the rate of hydrolysis of linustatin) and possesses only marginal activity on linamarin (Conn, 1994). Linamarase actively converts linamarin and presumably lotaustralin to their cyanohydrin aglycones and possesses weak activity on linustatin and neolinustatin (1.3 and 4.5% of the conversion rate of linustatin to linanmarin, respectively) (Fan & Conn, 1985). Hydroxynitrile lyase is then responsible for the hydrolysis of the cyanohydrin into ketone and cyanide. β-Glycosidases from other sources must be used with caution, as they exhibit high substrate specificity in general (Hösel & Conn, 1982). For example, Harris *et al.* (1980)

was able to observe cyanide production from flax meal with sweet almond suspension, but not from a commercially available purified β-glycosidase. It has long been known that linamarase from flax has low activity against amygdalin, the main cyanogenic glycoside of almonds, and that the β-glycosidases from almond emulsin have low activity on linamarin and lotaustralin (Coop, 1940).

Most modern enzymatic methods use endogenous flax enzymes, either within the sample itself or prepared separately from flax and used in the assay. Both linamarase and linustatinase have been purified to homogeneity (Fan & Conn, 1985), however it would be tedious and unnecessary to purify both enzymes separately in sufficient quantity to use for a large number of assays as for sound seed, the enzyme present in the sample is sufficient (Harris *et al.*, 1980; Bhatty, 1993; Varga & Diosady, 1994; Feng *et al.*, 2003; Chadha *et al.*, 1995; Haque & Bradbury, 2002). Other investigators have used crude enzyme preparations prepared from flaxseed (Kobaisy *et al.*, 1996; Niedźwiedź-Siegień, 1998). In the case of flaxseed meals and processed samples where the endogenous enzyme maybe destroyed, raw ground flaxseed can be added to samples to provide enzyme, providing the results are corrected for the cyanide content of the ground flax used as enzyme source (Varga & Diosady, 1994; Yamashita *et al.*, 2007).

The conditions of the hydrolysis reaction (time, temperature and buffer composition) vary greatly (Table 10.1). Chadha *et al.* (1995) investigated autohydrolysis by homogenizing seed or ground seed in water and allowing the mixture to stand at room temperature. In this assay, cyanide release peaked after 2-3 h for 10 cultivars analyzed. Optimal hydrolysis was observed after 1.5 h at pH 6.3 (unbuffered homogenate) or after 4.5 h at pH 4.0. Cyanide formation decreased dramatically when the pH was adjusted above or below this range. It should be noted that pH adjustments were made using sodium hydroxide or phosphoric acid and no formal buffers were used to control pH; pH change during the reaction time was also not investigated. Whole and ground seed were also evaluated as is and after heating at 37°C or 177°C for 1 h. There was no difference between ground or whole seed as is or after heating at 37°C, however heating at 177°C decreased cyanide production greatly. Essentially no cyanide was produced when the seed or homogenate was boiled prior to assay, presumably due to denaturation of the enzymes required for hydrolysis. Results for one cultivar (Linott) were significantly lower than those reported for the same cultivar by Oomah *et al.* (1992) from solvent extraction and HLPC quantitation of intact cyanogenic glycosides; although the significance of this is questionable as there is no indication that the samples were procured from the same growing location or crop year and a different analytical method was used.

Table 10.1. Survey of published hydrolytic methods for the analysis of cyanogenic glycosides in flax

References	Flax tissue analyzed	Hydrolysis method and enzyme source (if applicable)	Hydrolysis medium	Reaction temperature	Aeration/ Steam distillation conditions	Quantification method
Harris *et al.* 1980	Meal	Enzymatic hydrolysis (sweet almond suspension)	Orthophosphoric acid (0.1 M)	18 h (hydrolysis and aeration), 30°C	Simultaneous with hydrolysis	König (bromine-water/pyridine-phenylenediamine) OR GC as CNBr
Bhatty, 1993	Seed	Enzymatic hydrolysis (endogenous enzyme)	Na citrate (0.1 M, pH 5.5)	1 h, 45°C	400-450 mL	König (chloramine-T/pyridine-barbituric acid)
Varga & Dios-ady, 1994	Meal	Enzymatic hydrolysis (raw ground flaxseed)	Citrate (pH 5.5 – concentration and cation not reported)	16-24 h, 50°C	450 mL	König (chloramine-T/ pyridine-barbituric acid)
Chadha *et al.* 1995	Seed	Enzymati chydrolysis (endogenous enzyme)	Water (no pH adjustment)	2-3 h, room temperature	Not used	Ion chromatography
Kobaisy *et al.*, 1996	Seed	Enzymatic hydrolysis (crude flaxseed enzyme preparation)	Na phosphate (0.1 M, pH 6)	1 h, 30°C	Not used	Konig (chloramine-T/pyridine-pyrazolone)

Table 10.1. *Contd.*

References	Flax tissue analyzed	Hydrolysis method and enzyme source (if applicable)	Hydrolysis medium	Reaction temperature	Aeration/ Steam distillation conditions	Quantification method
	Seed	Enzymatic hydrolysis (crude flaxseed enzyme preparation)	Na acetate (0.1 M, pH 6)	1 h, 30°C	Not used	König (chloramine-T/pyridine – barbituric acid)
Nied Ÿwied Ÿ-Siegieñ, 1998	Extracted TLC zones	Enzymatic hydrolysis (crude flaxseed enzyme preparation)	Na acetate (0.1 M, pH 5.6)	3 h, 37°C	Not used	Liberated glucose, enzymatic
Haque & Bradbury, 2002	Meal	Enzymatic hydrolysis (endogenous enzyme)	Phosphate buffer (0.1 M, pH 5-6)	16 h, 30°C	Not used	Alkaline picrate (spectrophotometric)
	Meal	Acid hydrolysis	Sulfuric acid, 2 M	75 min (flaxseed), 100°C	Not used	König (chloramine-T/ isonicotinic acid-barbituric acid)
Feng *et al.*, 2003	Seed	Enzymatic hydrolysis (endogenous enzyme)	Water (no pH adjustment)	2 h, room temperature	Used, volume /time not specified	Titration
Yamashita *et al.*, 2007	Meal	Enzymatic hydrolysis (raw ground flaxseed)	Na citrate (0.1 M, pH 5.9)	18 h, 30°C	120 mL	König (chloramine-T/ pyridine-pyrazolone)

There are two major drawbacks of enzymatic hydrolysis methods. The first is the lack of commercial purified linamarase and linustatinase, which would be necessary to truly characterize the optimal hydrolysis conditions for an *in vitro* assay. Varietal, geographic, developmental and seed soundness differences in titres of each enzyme can theoretically affect the reliability, precision and accuracy of the method in a way that may not be detectable within a sample without more exhaustive analysis. Such effects can only be identified and controlled using enzyme sources of known activity, whether formulated from purified enzymes or produced from a highly characterized seed sample using a robust protocol. The second drawback is the lack of published data establishing the completeness of hydrolysis. The only published recovery data (Harris *et al.*, 1980) assessed recovery of linamarin and amygdalin in the absence of plant matrix and then from four animal feed formulations, but not from flaxseed or flaxseed meal. This data is likely quite reliable due to the fact that samples were analyzed by two different cyanide assay methods (GC as cyanogen bromide and König-based spectrophotometric assays) and that recoveries were greater than 97% with less than 1% variation between the two assays. Unfortunately, this data does not describe the efficiency of the extraction method or the aeration method used to trap the liberated cyanide and suffers from the primary limitation of spiked recovery; the spiked standard is extraneous to the sample matrix (feed products containing flax meal in this case) and does not assess the ability of the enzyme to access and hydrolyze the analyte contained within the sample matrix. Unfortunately there is no published recovery data from flaxseed or flaxseed meal.

Acid Hydrolysis Methods

Acid hydrolysis utilizes mineral acid to hydrolyze cyanogenic glycosides directly to hydrocyanic acid and ketone or aldehyde. Acid hydrolysis is non-specific, unlike β-glycosidases, so the amount and identity of cyanogenic glycoside is much less relevant to the performance of the assay. Acid may also be added in known volumes and concentrations, unlike enzyme preparations where enzyme activity may vary significantly due agronomic extraction and, storage conditions besides, cultivar differences. Despite these advantages, adoption of acid hydrolysis to flax analysis has been slow. Haque and Bradbury (2002) performed acid hydrolysis by combining flaxseed meal (suspended in 0.1 M phosphoric acid) and adding sulfuric acid (final concentration 2 M) in a glass stoppered test tube and incubating in a boiling water bath. Seventy five min was determined to be the optimal reaction time for flaxseed meal. Cyanide production peaked (75 min for flax) and then slowly decreased at a rate that was kinetically zero-order. This loss of cyanide was attributed to escape of HCN through the glass stopper and could be corrected for by extrapolating the equation for the rate of loss to zero time.

Chadha *et al.* (1995) also investigated acid hydrolysis during steam distillation in the presence or absence of a small volume of concentrated sulfuric acid. The cyanide recovery in the presence of sulfuric acid peaked at 1.5 h and was only 3.5% of that obtained from flaxseed alone, which peaked after 13 h of distillation. The authors proposed that the low recovery in the presence of sulfuric acid was caused by the hydrolysis of cyanide to ammonia, as it had been previously reported for the acid hydrolysis of lima beans (Montgomery, 1969). The loss reported by Chadha *et al.* (1995) was more severe than that reported by Haque and Bradbury (2002) but the difference in severity is likely attributable to the fact that the latter authors used 2 M sulfuric acid instead of concentrated (18 M) acid. It is important to remember that cyanide will be oxidized to isocyanate or hydrolyzed to formamide then to formic acid and ammonia at any acidic pH. It is simply a matter of whether or not the rate of hydrolysis (governed by pH and temperature) is significant within the time frame of the analysis. Although it is possible that the zero order cyanide loss that Haque and Bradbury (2002) reported is due at least in part to loss through the glass stopper in the assay tube but hydrolysis and oxidation are more likely explanations. No investigation was made by either group to substantiate the mechanism of loss of cyanide from the reaction mixture.

Acid hydrolysis (analyzed by a König reaction method) has been compared to enzymatic hydrolysis using endogenous enzyme (analyzed by an alkaline picrate method) and was found to give comparable results, however the enzymatic hydrolysis/alkaline picrate assay combination was found to be more accurate and reproducible (Haque & Bradbury, 2002). No data was reported to establish as to what extent the observed differences arose from differences in the hydrolysis or analytical method.

Steam Distillation of Cyanide

Many methods utilize steam distillation or aeration to purify liberated cyanide from the reaction mixture prior to analysis. Typically, steam distillation is carried out on hydrolysis mixtures with or without a carrier (*e.g.* nitrogen gas) and the distillate is trapped in a sodium hydroxide solution. Variables include time and volume of distillate required to complete cyanide recovery. Aeration is conducting by drawing air through a sodium hydroxide solution (optional, removes trace amounts of sulfur dioxide) and bubbled through a reaction mixture containing ground seed or cyanogenic glycoside extract and enzyme. Hydrocyanic acid volatilizes in the carrier stream and is then trapped by bubbling sequentially through a sodium hydroxide solution. For higher recovery, a second sodium hydroxide solution can be used sequentially after the first; these two solutions would then be pooled prior to analysis.

Both purification methods have severe drawbacks. Steam distillation requires specialized equipment that not all labs possess and both methods are time- and space- intensive. Incorporation of steam distillation or aeration would likely render any method unsuitable for the analysis of large numbers of samples (evaluating breeder's lines or in a regulatory lab, for example). The critical factors for both methods are the completeness of removal of cyanide from the hydrolysis mixture, trapping efficiency in the sodium hydroxide and loss of cyanide by oxidation or hydrolysis (refer to the Acid Hydrolysis Methods section for discussion on the latter). There is a much lower probability of loss of cyanide by hydrolysis in an aeration method due to the lower temperatures, however there also is a greater chance that not all cyanide will be recovered from the hydrolysis mixture as hydrocyanic acid is in equilibrium with both its vapor and its anion. The use of sodium hydroxide solutions (usually 0.5 M) for both methods is another possible source of cyanide loss by hydrolysis. The replacement of either method by solid phase extraction (SPE), which could be performed directly on the hydrolysis mixture or to trap volatile cyanide during aeration has so far not been reported but may provide an elegant solution to the hydrolysis problem.

MEASUREMENT OF CYANIDE

The König reaction

All published methods for the spectrophotometric determination of cyanide from cyanogenic glycosides utilize the König reaction (König, 1904) or the picrate method. The König reaction involves the oxidation of cyanide (CN^-) to a cyanogen halide (CN^+) using an oxidizing agent. The cyanogen (CN^+) then reacts with pyridine to form an intermediate that hydrolyzes to form glutaconic acid, a conjugated dialdehyde. This dialdehyde is then reacted with a chromophoric primary amine (also called a coupling or color reagent) to produce a light absorbing Schiff base. Lambert *et al.* (1975) described that with of N-chlorosuccinimide in the presence of excess succinimide as the oxidizing agent, pyridine-barbituric acid was more sensitive than 2,4-quinolinediol (but both were suitable for color reagents) while 2,5-piperazinedione and hydantoin as color reagents showed much poorer sensitivity. Asmus and Garshagen (1953) used chloramine-T as oxidizer and barbituric acid as color reagent. Epstein (1947) reported a method that later became the basis for an official method for cyanide in waste water by the American Public Health Association (APHA, 1971) using chloramine-T as oxidizer and a mixture of water saturated 1-phenyl-3-methyl-5-pyrazolone and 0.1% bis-pyrazolone in pyridine (pyridine-pyrazolone method) as color reagent. While Epstein stated that the color

reagent should be prepared fresh every three days, the official method (APHA, 1971) stated that both oxidizing agent and color reagent solutions should be prepared fresh. These methods are the basis of all published spectrophotometric methods for cyanide from cyanogenic glycosides in flaxseed.

Applications of these methods to the analysis of flaxseed primarily differ by the choice of oxidizing and color reagents, assay conditions and whether or not the cyanide was purified from the hydrolysis mixture prior to analysis (Table 10.1). Harris *et al.* (1980) used bromine water as oxidant and *p*-phenylenediamine as color reagent, based on work by Bark *et al.* (1964). The mean recovery for the method (linamarin and amygdalin) was 97.9% ± 1.24% SD for linamarin and amygdalin added at 10–0 µg cyanide/g sample. Haque and Bradbury (2002) and Bhatty (1993) analyzed acid hydrolyzed extracts using chloramine-T as oxidizer and isonicotinic acid/ barbituric acid as color reagent. Varga and Diosady (1994) isolated cyanide from hydrolyzed flax meal by steam distillation into sodium hydroxide spectrophotometrically using a modified König reaction published by Michels and Siegfried (1986). Cyanide release was quantified against a KCN standard curve and the results corrected for the cyanide release from the ground flax used as the enzyme source. Yamashita *et al.* (2007) used a pyridine-pyrazolone based König method and further developing by establishing that 10% (by weight) freshly ground flax produced optimal hydrolysis (0–40% evaluated) with an 18 h reaction time at 30°C (0–24 h evaluated).

The use of König-based spectrophotometric assays for the analysis of cyanide has been well established and accurate. Therefore, it is the hydrolysis, cyanide isolation and matrix effect of the assay sample that are the critical elements of such methods; the limitations of these factors have been addressed in their respective sections. From a throughput and robustness perspectives, methods that do not incorporate steam distillation or aeration are preferable, provided they are accurate and precise. The unfortunate problem is that due to the turbidity of enzyme incubations, most investigators have elected to use steam distillation or aeration prior to assay. Those that were able to eliminate steam distillation or aeration did so by extracting cyanogenic glycosides from the seed prior to hydrolysis (Kobaisy *et al.*, 1996). Extraction has its own critical elements (discussed in the respective section) and although extraction prior to hydrolysis may make the assay faster than steam distillation after hydrolysis, the overall method will not necessarily be as or more accurate or precise.

The Picrate Method

The use of picrate paper (as a semi qualitative method) or guaiacum resin-copper sulphate paper (qualitative) are among the earliest methods of detecting cyanide from hydrolyzed plant matter (Vetter, 2000; Evans, 2001) and remains an official method of the Association of Analytical Chemistry (AOAC, 2000a). The picrate method was developed by Guignard (1906) and exploits the ability of cyanide to reduce picric acid to isopurpuric acid, which is orange and has an absorbance maximum between 510 and 520 nm. In a quantitative adaptation of the assay (Bradbury, 1994; Haque & Bradbury, 2002) macerated plant tissue or extracted cyanogenic glycosides are hydrolyzed (using endogenous enzyme) in a vial with phosphate buffer and a plastic-backed picrate coated strip of paper. After 30 h at 30°C, the picrate papers were extracted in water for 30 min and the absorbance of the resulting solution measured at 510 nm. The assay was calibrated using standardized potassium cyanide solutions and the recovery (amygdalin) was reported to be 101.9% ± 0.64% (SD).

Alkaline picrate methods have been described as being insensitive relative to the König reaction based methods (Epstein, 1947). Additionally, the picrate method must be carried out at alkaline pH, while König reaction based methods may be conducted at slightly acidic, neutral or slightly alkaline pH. Lastly and perhaps most importantly, the use of picric acid (2,4,6-trinitrophenol, TNP) is hazardous due to the fact that improperly stored or old picric acid can be explosive.

Titrimetric Methods

Two titrimetric methods have been published for the measurement of cyanide from hydrolyzed cyanogenic glycosides, both involving steam distillation of cyanide from the hydrolysis mixture. Cyanide may be distilled into dilute acidified silver nitrate (acidic method) and titrated with standardized potassium thiocyanate, or distilled into sodium hydroxide (alkaline method) and titrated with standardized silver nitrate. Official methods have been published for both titrations (AOAC, 2000b) and the alkaline method has been used for flax by at least one investigator (Feng *et al.*, 2003). Both methods are tedious and can lead to erroneous results due to losses of HCN (Cooke, 1987; Harris *et al.*, 1980).

Other Methods

Ion chromatography has been employed for the measurement of cyanide from flaxseed under autohydrolysis conditions (Chadha *et al.*, 1995). The reaction mixture was filtered through a 30 KDa centrifugal filter and injected immediately on an HPLC system equipped with an ion exchange column

and a silver/silver chloride electrochemical detector. The reported repeatability between replicate samples was 3.9% (n = 4) and the limit of detection was 1 µg cyanide/g seed at a signal-to-noise ratio of 10.

Gas chromatography has been used for the analysis of cyanide as cyanogen bromide (Harris *et al.*, 1980). The mean recovery for the method (linamarin and amygdalin) was 98.2% ± 0.98% SD for linamarin and amygdalin added at 10-20 µg cyanide/g sample. The limit of detection was 1 µg cyanide/g seed (method for determination not reported).

MEASUREMENT OF INTACT GLYCOSIDES

High Pressure Liquid Chromatography (HPLC) and Gas Chromatography (GC)

The first reported HPLC method for separation of flax cyanogenic glycosides (Schilcher & Wilkens-Sauter, 1986) employed a C18 reverse phase column with refractive index detection. Linamarin, linustatin and neolinustatin were resolved (lotaustralin was not evaluated) using a mobile phase of 94.5% water/ 0.05% phosphoric acid/ 5% methanol as the mobile phase. This basic method has been employed by several other investigators (Kolodziejczyk & Fedec, 1995; Kobaisy *et al.*, 1996; Oomah *et al.*, 1992; Cunnane *et al.*, 1993). Krech and Fieldes (2003) reported resolution of all four glycosides using 93.5% water/ 0.05% phosphoric acid/ 6.5% methanol. Some investigators employed a mobile phase consisting of 15% methanol and 85% water (Amarowicz *et al.*, 1993; Wanasundara *et al.*, 1993). It should be noted, however, that the water content must exceed 90% on standard C18 column in order for the monoglycosides to be resolved from the diglycosides (unpublished data). It would be expected that under these conditions, that linamarin would co-elute with linustatin and lotaustralin would co-elute with neolinustatin. Although it has been established that the monoglycosides (lotaustralin and linamarin) are absent or present at trace levels in mature sound seed (Niedźwiedź-Siegieñ, 1998; Oomah *et al.*, 1992), they are significant in immature seed, in plant tissues (Frehner *et al.*, 1990, Niedźwiedź-Siegieñ, 1998) and possibly in damaged seed. Mobile phases with less than 95% water should only be used with C18 columns when the analyst can be sure that the monoglycosides are not present. Frehner *et al.* (1990) analyzed the diglycosides on a C18 column using 4% acetonitrile/ 96% water; it is not clear whether or not the monoglycosides would be resolved from their respective diglycosides under these conditions, as acetonitrile is a preferred organic solvent than methanol. The main restriction of HPLC methods remains detection limit. Since flax cyanogenic glycosides are non-chromophoric and do not fluoresce, detection is limited to refractive index, evaporative light scattering (ELSD) or mass spectrometry. Although mass

spectrometry holds the promise of a lower detection limit, the ionization yield of many neutral species is notoriously low in LC-MS. Amperometric detection is also a promising alternative, however, there have been no published applications to cyanogenic glycosides in flax so far.

Although gas chromatography has been used for the measurement of cyanogenic glycosides in other plants, its adoption for analysis of flax has been slow. As cyanogenic glycosides are not volatile, derivatization is necessary for analysis. Zilg *et al.* (1972) reported the analysis of linamarin and lotaustralin from plant tissue as their trimethylsilyl ether (TMS) derivatives using a packed OV-1/OV-17 column. Recently, our lab has developed a gas chromatography method for the analysis of TMS derivatives of cyanogenic glycosides in flax (Bacala & Barthet, 2007). The limit of detection for the assay was 308 ± 37 and 731 ± 79 ng/mL sample for linustatin and neolinustatin, respectively. This corresponded to 620 pg and 1.50 ng on column for each standard, respectively. The instrumental repeatability, assessed at the lower and upper limits of quantitation over two days, was lower that 1%. The main strength of GC methods is its superior detection limit compared to LC methods. The GC method has been further modified in our lab to allow to quantify linustatin and neolinustatin in single seeds (unpublished results).

Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) and paper chromatography have been used by several investigators for the separation of cyanogenic glycosides from flax. Butler and Conn (1964) successfully separated linamarin and lotaustralin from flax plant tissue using paper chromatography and a mobile phase consisting of 30:10:6 methyl ethyl ketone/acetone/water. Niedźwiedź-Siegień (1998) was able to resolve linamarin, lotaustralin, linustatin and neolinustatin using previously reported methodology (Nahrstedt, 1970) consisting of cellulose MN300 as the stationary phase and water saturated *n*-butanol as the mobile phase. Amarowicz *et al.* (1993) investigated the ability of six solvent systems to resolve linamarin, linustatin and neolinustatin on silica gel TLC plates and high performance TLC (HPTLC) plates. Two solvent systems were capable of resolving all three glycosides; 2:2:1 chloroform/methanol/17% ammonium hydroxide (first reported by Smith *et al.*, 1980; Palmer *et al.*, 1980) and 65:35:10 chloroform/methanol/water. Another solvent system, previously reported by Brimer *et al.* (1983) consisting of 40:30:12:10:8 ethyl acetate/ acetone/chloroform/methanol/water was able to resolve linamarin from linustatin, but not linustatin and neolinustatin. The retention factors of these methods are summarized in Table 10.2.

Table 10.2. Summary of published thin layer chromatography (TLC), high performance TLC (HPTLC) and paper chromatography (PC) methods

Author	Method	Stationary phase	Mobile phase	Retention factor (R_f)			
				Linamarin	Lotaustralin	Linustatin	Neolinustatin
Butler and Conn (1964)	PC	Not specified	30:10:6 methyl ethyl ketone/acetone/water	0.59	0.72	ND	ND
Niedźwiedź-Siegeñ (1998)	PC	Cellulose MN300	Water saturated butanol	0.59	0.64	0.12	0.21
Amarowicz *et al.* (1993)	TLC	Silica gel	2:2:1 chloroform/ methanol /17% NH_4OH	0.68	ND	0.48	0.54
	HPTLC	Silica gel	2:2:1 chloroform/ methanol/17% NH_4OH	0.74	ND	0.56	0.63
	TLC	Silica gel	65:35:10 chloroform /methanol/water	0.59	ND	0.39	0.45
	HPTLC	Silica gel	65:35:10 chloroform /methanol/water	0.73	ND	0.50	0.58

Several methods have been developed for the detection of cyanogenic glycosides on TLC plates. The most basic method is the non-specific charring of plates by spraying with sulfuric and heating briefly in an oven. While this method is sensitive, it is non-specific and will cause the visualization of all spots. The picric acid method has also been applied to the detection of cyanogenic glycosides on TLC plates. The first report of such a method (Butler & Butler, 1960) used aqueous solutions of commercially available β-glucosidase or linamarase isolated from flax meal was sprayed on developed TLC plates. The plate was then pressed against a paper that had been impregnated with an alkaline picric acid solution and allowed to dry. Liberated cyanide reacted with the alkaline picrate yielding orange spots where cyanogenic glycosides were present. This method was later refined into a densitometric method (Brimer *et al.*, 1983) by using sheets of ion exchange membrane that were sequentially pre-coated in saturated aqueous picric acid, sodium carbonate and cetyl alcohol solutions (with drying after each step). Reagent sheets prepared according to this method were reported to be stable for several months at room temperature or under refrigeration at 0% or 100% relative humidity, as long as the sheets were protected from light and corrosive vapors. The developed sheets were reported to be stable for 28 days.

An alternative to the picrate method is the Fiegl-Anger spot test (Fiegl & Anger, 1966). This test uses Fiegl-Anger test paper, which is prepared by soaking filter paper in a solution of copper (II)-ethylacetoacetate and di-(4-dimethylaminophenyl)-methane and allowing to dry (Tantiwesie *et al.*, 1969). This method was adapted to a microtiter plate based assay that reported a detection limit of 40 nmol HCN from raw plant material (Kakes, 1991) and later used as a detection method for TLC by spraying developed plates with a buffered β-glucosidase solution and sandwiching against a Fiegl-Anger test plate (Niedźwiedź-Siegeñ, 1998). The detection limit for the latter application was 4 times lower (10 nmol cyanide), presumably due to direct liquid contact in the TLC sandwich assay compared to the microtiter plate assay where the Fiegl-Anger plate was affixed to the top of the microtiter plate and liberated cyanide had volatilized in order to reach the plate. The Fiegl-Anger method is reported to be superior to the picrate method in that the color reaction is faster (almost instantaneous), the detection limit is lower and the use of picrate (potentially explosive) and toluene (toxic) is avoided (Kakes, 1991).

The picrate and Fiegl-Anger detection methods can be described as semi-quantitative at best. Quantitative results from TLC analyses may be obtained by scraping zones from plates corresponding to individual cyanogenic glycosides, extracting with buffer, enzymatic hydrolysis and detection of liberated sugar (Niedźwiedź-Siegeñ, 1998). Although liberated

cyanide could also be quantified by any of the methods already discussed, analysis of freed glucose would be preferable as enzymatic glucose assays are much more sensitive and glucose is not prone to loss by volatilization like cyanide could be. As a final note, assay for liberated sugar is only possible in purified cyanogenic glycoside samples. Such assays have been attempted in our lab but were not successful due the high background from free sugars and other glycosides that could also be hydrolyzed in the assay (unpublished results).

Comparing Methods

Published values for the cyanogenic glycoside content of flax may be used as a means of comparing the methods used to obtain them. Table 10.3A is a summary of published results where the contents of individual cyanogenic glycosides were assayed. Table 10.3B is a summary of results where only total cyanogenic glycosides or cyanide equivalent were measured. Total cyanogenic glycosides were expressed as μmoles per gram, as this is the only accurate method for summing quantities with different molecular masses. Where reported, the HCN equivalent was reported. Where it was not, it was calculated from the total reported cyanogenic glycosides. Differences between published values are a result of differences between methods, analyst skill and the samples analyzed; both the content and composition of cyanogenic glycosides vary with cultivar and growing conditions. While it is clear that some results are markedly lower than the range reported by most analysts (Cunnane *et al.*, 1993; Bhatty, 1993), the inability to resolve differences in sample composition from analyst and method bias makes any further discrimination impossible. We have recently demonstrated the choice of grinding method and extraction solvent alone can affect the extraction efficiency by as much as 18% (Bacala & Barthet, 2007). The only true comparison would be a "round robin" style study where a set of common standards including a certified reference material were analyzed in a large number of labs. No data on such a study have been published, nor does a certified reference flaxseed or meal sample exist to date.

There have been several comparisons of methods within individual laboratories. Schilcher and Wilkens-Sauter (1986) compared extraction and HPLC with a König-based spectrophotometric assay and concluded that the HPLC method yielded results 2% lower than the spectrophotometric assay. By viewing the individual results of 43 seed lines evaluated by both methods, it is apparent that the percent difference between methods for the same seed sample ranges from -35% to +32% and as little as 0.2% (as a percentage of the spectrophotometric method). Additionally, 25 of 43 samples

had a percent difference between methods below 10% and 18 samples had a value above this. This wide range of difference for the same sample implies serious uncontrolled bias between the methods that need to be resolved before any real comparison may be made. Kobaisy *et al.* (1996) compared the same HPLC method to two König-based spectrophotometric assays (pyridine-barbituric acid and pyridine-pyrazolone). The bispyrazolone and HPLC methods showed correlation coefficients of 0.9649 and 0.9170, respectively, to the pyridine-barbituric acid method. The biases between the three methods are low and although the HPLC results tended to be higher than the other assays, there were no statistically significant differences between the assays. Haque and Bradbury (2002) compared acid hydrolysis coupled with a König-based spectrophotometric assay (isonicotinic acid-barbituric acid) to an enzymatic hydrolysis coupled with an alkaline picrate assay. Values for 10 seed and meal samples differed by less than 5%, despite observations by the authors that the acid hydrolysis method was problematic and HCN loss from the system during the assay had to be corrected for. These comparisons demonstrate that methods are likely quite comparable, as long as they are performed within the same laboratory on the same samples. The real challenge for any method is its reproducibility with other analysts in the same or other laboratories.

There are several other biases that are present in the methods published to date. There is a lack of a certified reference material for standardization within or between labs. In order for the development of such a standard, more work will be necessary to properly and completely validate the assays that would be used to certify it. There is also a lack of commercially available standards. Linamarin is available from a number of North American vendors, however lotaustralin is not. The fact that it has not been assayed in many studies compared to linamarin mainly due to its unavailability (Table 10.3A-B). There are a very small number of sources worldwide for linustatin and neolinustatin, and none provide a suitable primary reference standard of highly characterized purity, let alone a standard reference material. The omission of linamarin and lotaustralin from total cyanogenic glycoside calculations by many investigators also presents a small bias between hydrolytic methods, which estimate cyanide from all possible sources. While these species have been identified in seed by some investigators and not by others, it is not clear whether this is due to differences in seed samples, shortcomings in some methodologies or both. The most robust methods would have to quantify all four glycosides or would at least incorporate a limit test for linamarin and lotaustralin to qualify that their omission from final results was valid for each sample tested. All of these biases will have to be addressed before methods can truly be compared and candidates selected for standardization.

Table 10.3A. Summary of published analytical methods and cyanogenic glycoside levels in flax and flax products where individual glycosides were quantified.

Sample	Linamarin (mg/100 g)[a]	Lotaustralin (mg/100 g)[a]	Linustatin (mg/100 g)[a]	Neolinustatin (mg/100 g)[a]	Method	Reference(s)
Flaxseed	0	NT[b]	218-538	73-454	HPLC	Schilcher & Wilkens-Sauter, 1986
Flaxseed	0-32	NT	213-352	91-203	HPLC	Oomah *et al.*, 1992
Flaxseed	NT	NT	0.26	0.35	HPLC	Cunnane *et al.*, 1993
Flax oil			0	0		
Flax muffin			0	0		
Flaxseed	NT	NT	442	190	HPLC	Wanasundara *et al.*, 1993
Immature seed	140	190	470	250	TLC, enzymatic extraction, colorimetric assay	Niedźwiedź-Siegień, 1998

[a] Where multiple seed samples were evaluated, the ranges stated here are the minimum and maximum value for each glycoside irrespective of sample.
[b] NT = Not tested

Table 10.3B. Summary of published analytical methods and cyanogenic glycoside levels in flax and flax products where only total glycosides or cyanide were quantified

Sample	Total cyanogenic glycoside content (μmol/g)	CN or CN equivalent (ppm)	Analytical method	Reference(s)
Flaxseed meal		390[a]	Spectrophotometric	Harris *et al.*, 1980
		378[a]	GC of CNBr	
Flaxseed	0.80-2.00	217-541	HPLC	Schilcher & Wilkens-Sauter, 1986
	0.88-1.98	237-534	Spectrophotometric	
Flaxseed		0.072	$AgNO_3$ titration	Bhatty, 1993
Flaxseed meal	15.8-19.8[b]	428-535	Spectrophotometric	Varga & Diosady, 1994
Flaxseed	8.8-13.8	238-373	HPLC	Oomah *et al.*, 1992
Flaxseed	15.3	413	HPLC	Wanasundara *et al.*, 1993
Flaxseed		124-196	Enzymatic hydrolysis, ion chromatography	Chadha *et al.*, 1995
Flaxseed		626	Spectrophotometric A	Kobaisy *et al.*, 1996
		594	Spectrophotometric B	
		709	HPLC	
Immature flaxseeds	35.7	964	TLC	Niedźwiedź-Siegień, 1998
Flaxseed	5.79	367	HPLC	Wanasundra *et al.*, 1999
Flaxseed		140-360	Acid hydrolysis	Haque & Bradburry, 2002
		140-370	Spectrophotometric	
Flaxseed meal		390		
		360	Acid hydrolysis Spectrophotometric	Haque & Bradburry, 2002
Flaxseed		377	$AgNO_3$ titration	Feng *et al.*, 2003
Flaxseed		400	Spectrophotometric	Yamashita *et al.*, 2007

[a] As linamarin, moisture- and oil- free basis
[b] Mean of four results.

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11

Isolation and Preliminary Characterization of Antimicrobial Proteins and Peptides from *Ctenophores* and *Cnidaria*

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ABSTRACT

Like vertebrates, invertebrates are susceptible to infection by bacteria, fungi and viruses. Antimicrobial proteins and antimicrobial peptides are important animal host defense molecules that play a significant role in the survival against invading bacteria and fungi. Molecules exhibiting antimicrobial activity have been identified from every animal. The focus of this project was to isolate and characterize antimicrobial proteins and antimicrobial peptides in the body fluids and mucus secretions of Ctenophores (Leidy's Comb Jellyfish; Mnemiopsis leidyi) and Cnidaria (Moon Jellyfish; Aurelia aurita). Jellyfish collected from offshore waters were tested for the presence of bacteria. DAPI staining studies showed that no bacteria were present. Bacterial lysis assays were conducted to detect antibacterial activity against both gram-positive and gram-negative bacteria. Antibacterial assays showed that there was a <500 dalton protein fraction isolated from both species that exhibited bacterial growth inhibition. The peptides were characterized using column chromatography and SDS-PAGE. We have also isolated a large molecular weight protein (~40,000 daltons) exhibiting antibacterial activity (i.e. lysozyme) from the fluids as well. The isolation and study of these endogenous natural host defense products from invertebrates should have implications in understanding the evolution of immunity as well as in developing new drugs to help in the fight against human diseases.

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INTRODUCTION

Immune Reactions

Immunology is the study of the body's defenses against infection. One of the first artificial manipulations of the human immune system was Edward Jenner's discovery of vaccination to treat smallpox in 1796 (Abbas & Janeway, 2000). He knew the vaccine worked but was uncertain of how and why it was successful at treating his patients. Later discoveries revealed that host defense systems fall into two major categories. These include cellular immunity (first described by Ilya Metchnikoff in the early 1880's) and humoral immunity, (which was proposed by Paul Ehrlich in 1897) (Beck & Habicht, 1996). Metchnikoff discovered cellular immunity when he stuck thorns into a starfish larva and upon later observation noticed that mobile cells, thought to be engulfing bacteria, surrounded the thorns. This cell mediated response was therefore characterized as the host's defenses by various non specific cells such as phagocytes which protect against intracellular bacteria and viruses. Further studies in mammals revealed that cellular responses are also mediated by antigen specific T lymphocytes which are capable of protecting the host against microbial and viral infections as well as cancers in vertebrates. Contrary to the cell mediated response, the humoral response involves host defenses that are mediated by antibodies and other secreted molecules (*i.e.* complement) present in the plasma, lymph and body fluids following exposure to a pathogen (Abbas & Janeway, 2000). These secreted antibodies bind to antigens on the surface of invading microbes such as bacteria and viruses which are tagged for destruction. The humoral response is also involved with the production and release of cytokines, memory cell generation and the general neutralization of toxins and pathogens.

Vertebrates have highly developed immune systems, which are characterized by an adaptive and an innate immune response. An adaptive immune response is distinguished by its specificity for identifying the site on the antigen that is recognized and bound by an antibody or a T cell receptor. Adaptive immune responses also allow the body to reduce the delay in response by recognizing antigen that has previously come into contact with the body. Specificity is produced by antigenic receptors of B and T lymphocytes and is encoded by genes that are produced by somatic recombination of gene segments during cell maturation (Abbas & Janeway, 2000). This process is referred to as immunological memory where, the immune system is able to respond more strongly to successive exposures to the same antigen. The other arm of vertebrate immunity involves an innate response which is the first mode of defense against microorganisms. It is

important for sending signals to activate co-stimulatory molecules such as cytokines and chemokines involved with an adaptive response (Ausubel, 2005; Semple *et al.*, 2003). Innate immunity is therefore described as the non-specific host defenses that exist prior to exposure to an antigen. This involves anatomic barriers such as mucus membranes which entrap foreign microbes. Physiologic barriers include chemical agents such as lysozyme which cleave bacterial cell walls whilst phagocytic and inflammatory mechanisms involve the breakdown of toxins via macrophages and monocytes often resulting in tissue damage (Beck & Habicht, 1996). Other cellular components of the innate response include phagocytes and natural killer cells, circulating proteins such as complement which are serum proteins containing cytolytic activity, and many antimicrobial proteins and peptides which also destroy and eliminate foreign bodies (Medzhitov & Janeway, 2000b; Uzzell *et al.*, 2003; Zasloff, 2002b).

While vertebrates' immune responses employ both the adaptive and innate systems, invertebrates are only capable of eliciting an innate response (Beck & Habicht, 1996; Hoffmann & Reichhart, 2002). Innate responses in invertebrates and plants are activated when microbes succeed in entering the body cavity. Molecules called pathogen recognition receptors (PRRs) are associated with the specific recognition of microbes. The PRRs make excellent identifiers of pathogens because they are able to detect pathogens at any stage of their life cycle (Akira *et al.*, 2006; Zipfel & Felix, 2005). Molecules that are evolutionarily conserved have been found to exhibit microbe associated patterns known as pathogen associated molecular patterns or PAMPs. The PAMPs have been derived from pathogens that can distinguish between the host and pathogens. A few examples of PAMPs include lipopolysaccharide, lipoproteins, peptidoglycan, chitin and bacterial flagellin (Akira *et al.*, 2006; Ausubel, 2005; Zipfel & Felix, 2005). The specificity of these receptor molecules associated with invertebrates (and vertebrates) allow them to efficiently eliminate pathogens.

Other modes of defense such as cellular defense mechanisms of invertebrates are involved primarily with phagocytes and macrophage-like cells. For example, *Drosophila* defense occurs via plasmatocytes (Hoffmann & Reichhart, 2002) whilst insects and arthropods such as shrimp and crab use hemocytes that are found in hemolymph (Hikima *et al.*, 2003; Hoffmann & Reichhart, 2002; Leclerc & Reichhart, 2004; Medzhitov & Janeway, 2000a; Medzhitov & Janeway, 2000b). Toll-like receptors (TLRs) play an important role in the elimination of pathogens by recognizing receptors to antigen in multi-cellular organisms including plants (Ausubel, 2005; Bang *et al.*, 1997; Ganz, 2003; Hoffmann & Reichhart, 2002; Maher & McClean, 2006; Means *et al.*, 2000a; Means *et al.*, 2000b). In *Drosophila*, the Toll pathway is involved with the removal of Gram positive bacteria and fungi whilst in plants, microbes of all origin (Gram positive, Gram negative and fungi) are recognized by TLRs (Leclerc & Reichhart, 2004; Lien *et al.*, 2000). In response to microbial

infections invertebrates can also carry out humoral responses that involve destruction by lytic peptides commonly found in nature. Humoral defense of invertebrates is characterized by several contributing components such as TLRs, prophenoloxidase and antimicrobial proteins and peptides (AMPs) amongst other chemical agents. Prophenoloxidase is found in invertebrates, plants and fungi (Nagai & Kawabata, 2000). Prophenoloxidase is an enzyme that exhibits activity that is important in the defense against infection as well as for the healing of wounds. This enzyme produces quinones which have been found to aid in the killing and or segregation of pathogens within the host (Beck *et al.*, 1996; Jiang *et al.*, 1998).

Finally, the innate response in invertebrates also involves hemolytic and hemagglutinating activities seen in hemocytes of organisms such as sea hares and tunicates (Cavalcante *et al.*, 2000; Melo *et al.*, 1998). Lytic processes occur when hemocytes containing AMPs are released. The antimicrobial agents in invertebrate innate responses include pre-formed as well as inducible AMPs (Bang *et al.*, 1997; Beck & Habicht, 1996; Benkendorff *et al.*, 2001; Dimarcq *et al.*, 1998; Hancock *et al.*, 2006).

Innate responses have been proven to be vital to both vertebrates and invertebrates. Studies have suggested that, in some ways, the adaptive immune response relies on signals from the innate system in order to elicit an appropriate line of defense (Medzhitov & Janeway, 2000b; Semple *et al.*, 2003). For example, the receptors involved with innate immunity are specific for PAMPs so a major function is to signal microbial infection. The signals that are induced from an innate response in turn, signal an adaptive response. An adaptive response to a pathogen can be elicited only after the innate system has recognized and contained that pathogen (Medzhitov & Janeway, 2000b). It is important to study invertebrate innate responses so that information gained from these studies can be used to make comparisons between both vertebrates and invertebrates with regards to the evolution of molecules and their functions in adaptive and innate immunity. Other differences between cells such as hemocytes and phagocytes, chemical agents (such as those mentioned above) and pathways can also be compared.

Antimicrobial Proteins

Antimicrobial peptides are a class of small cationic molecules [molecular weight (Mr), ~10 kDa] (Boman, 2003). These molecules have been found to possess anti-viral, anti-bacterial and anti-cancer activities as well as contributing to innate immune response (Powers & Hancock, 2003). There are currently over 900 known AMPs which have been isolated from plants, animals and bacteria (a complete list of these AMPs can be found at http://aps.unmc.edu/AP/main.php). The AMPs work primarily by targeting negatively charged lipopolysaccharide in the bacterial membranes. This is

where killing begins. In order for an AMP to be successful at eliminating a pathogen, its chemical effects must react faster than the bacteria can grow. The first AMP isolated from insects was cecropin (Boman, 2003). Defensins, another AMP, are found in various organisms including mammals, insects and plants (Ganz, 2003; Gueguen *et al.*, 2006; Zhao *et al.*, 2007a; Zou *et al.*, 2007). Other AMPs have been found in almost every organism including bacteria, amphibians, mollusks, arthropods and fish (Bang *et al.*, 1997; Benkendorff *et al.*, 2001; Toke, 2005). Some examples of AMPs include magainin, dermaseptin, melittin and sapecins. The AMPs work because all organisms including microbes contain them. Microbes often do not recognize these proteins and peptides since AMPs are capable of binding to and destroying lipopolysaccharide and or peptidoglycan. Bacteria therefore have very limited defense against AMPs and proteins. Over 800 AMPs have been isolated from varying organisms and are classified by their structure: alpha helical, beta sheet, loop and extended peptides (Powers & Hancock, 2003; Zasloff, 2002b). Alpha helical and beta sheet AMPs are most commonly found in nature, however all four types (alpha helical, beta sheet, loop and extended) of AMPs may be synthetically produced.

Alpha helical peptides are characterized by their alpha-helical shape and often contain a bend in the center of the molecule. In some cases such as magainin, found in *Xenopus laevis*, it has been shown that the bend is critical for selective suppression of hemolytic activity (Powers & Hancock, 2003). Magainin is the Hebrew word for shield and was discovered by Michael Zasloff in 1987 (Zasloff, 2002b). The discovery of magainin was made when he noticed that frogs required no antibiotics in order to recover from non-sterile surgery (Beck & Habicht, 1996; Zasloff, 2002b). Alpha-helical peptides are linear, amphipathic and lack cysteines (Boman, 2003).

A second class of peptide known as beta sheet antimicrobial peptides are characterized by the presence of beta sheets that are stabilized by three disulfide bonds (Boman, 2003). The AMP tachyplesin was identified and isolated from *Tachypleus tridentatus*, the Japanese horseshoe crab (Powers & Hancock, 2003). Beta-defensins are small AMPs with a high density of cationic charge and contains a six cysteine residue motif. These beta-defensins work by penetrating the cell membrane of microbes via electrical attraction. This attraction allows the AMP to form pores in the bacterial membrane which leads to lysis of the cell (Boman, 2003; Ganz, 2003).

A third class of peptide lacks traditional secondary structure and is therefore characterized by its proline and glycine rich content. Extended peptides are formed by hydrogen bonds as well as Van Der Waal interactions. Indolicidin, found in cytoplasmic residues of bovine neutrophils is a 13-residue AMP displaying this conformation. Finally, loop peptides contain a loop in their structure aided by a single bond that may be disulfide, amide or isopeptide. This type of structure was found in *Podisus maculiventris*, the spined soldier bug (Powers & Hancock, 2003).

Not only are AMPs characterized by their chemical structure but they are also classified by their location and site of activity within an organism. They can be found in internal fluids such as lymph, phagocytic cells and mucosal surfaces. Antimicrobial peptides have gained much attention because of the value they possess, in that, they may be used as therapeutic agents such as defense against drug resistant infections as well as for the treatment of cancer (Buckling & Brockhurst, 2005; Hancock & Chapple, 1999; Nikaido, 1994b; Oh *et al.*, 2006; Spratt, 1994; Tennessen, 2005; Toke, 2005; Zasloff, 2002a). Pexiganan is a synthetically produced ribosomally encoded AMP which has been derived from magainin (Buckling & Brockhurst, 2005). It has been modified so that it can be used as a chemotherapeutic agent. This cationic AMP triggers apoptosis by disrupting the mitochondrial membrane of cancer cells resulting in lysis of the affected cells (Mader & Hoskin, 2006). Antimicrobial peptides exist in all multi-cellular organisms and therefore pose an exciting venture into the little researched world of these molecules which provide innate immunity.

Although AMPs could be very powerful agents in the fight against microbes, some researchers in the field believe that bacteria will inevitably gain resistance to these drugs within a few years. It has been suggested that microbes will modify their outer cell layers by simple changes in amino acid structure (Bell & Gouyon, 2003). Other modes of resistance would be by proteolysis of PhoP which breaks down helical peptides or by efflux mechanisms. PhoP is a part of the two component regulatory system (along with PhoQ) which is found in many species of bacteria and activity is driven mainly by a change in the Mg^{2+} concentration (Oyston *et al.*, 2000; Tu *et al.*, 2006). In efflux systems drugs are removed from the cytoplasm faster than they can be pumped in because of changes in the porin channels (Bell & Gouyon, 2003; Nikaido, 1994a; Nikaido, 1994b). It is also a concern that resistance to naturally occurring AMPs may be promoted if synthetic AMPs are over used for the treatment of microbial infections or as chemotherapeutic agents. This resistance would occur in all bacteria including those occurring naturally in organisms thus making it more difficult to interact with bacteria that are currently "harmless" to us (Buckling & Brockhurst, 2005). The bacterial resistance could lead to AMPs being less effective at treating microbes because of uncontrollable populations colonizing an organism (Buckling & Brockhurst, 2005; Tomasz, 2006). Antimicrobial peptides are favorable candidates for drug development because they provide us with new ways of combating microbes. These concerns are valid, however, the conserved structures and mode of action (present for millions of years) will make it extremely difficult for microbes to evolve and become resistant to AMPs.

Lysozyme

In 1921 Alexander Fleming cultured nasal mucus on an agar plate and after observing it weeks later, noticed that the bacteria that were covered in mucus had been destroyed while the untreated bacteria remained unaffected.

The agent responsible for eliminating the bacteria was an enzyme called lysozyme. Lysozyme, also referred to as muramidase, is an antimicrobial protein found in biological fluids and tissues, avian eggs, animal secretions, human milk, tears, saliva, airway secretions and polymorphonuclear leukocytes (Bachali *et al.*, 2004; Ibrahim *et al.*, 2005; Xue *et al.*, 2007; Xue *et al.*, 2004). Lysozyme is biologically important for self defense against microbial infections and is more effective at eliminating infections caused by gram positive bacteria. Some other functions of lysozyme include killing of viruses, tumors and immune modulatory and anti-inflammatory activities (Ibrahim *et al.*, 2005).

Lysozymes typically have a high isoelectric point, are heat stable, and have low molecular mass ranging from 11-22 kDa (Ito *et al.*, 1999; Xue *et al.*, 2007). Muramidase activity is characterized by its ability to split the 1,4 linkage between N-acetylmuramic acid and N-acetylglucosamine of the bacterial peptidoglygan (Bachali *et al.*, 2004; Bachali *et al.*, 2002; Hikima *et al.*, 2003; Xue *et al.*, 2004). When this linkage is broken the bacterial cell wall lyses causing cell death. It has been found that two amino acid residues, glutamate and aspartate, are important for the proper functioning of lysozyme. Glutamate works in conjunction with serine while aspartate is involved with covalent binding to the sugar substrate of the molecule (Bachali *et al.*, 2004; Bachali *et al.*, 2002). Lysozymes have been found in numerous organisms ranging from microbes to plants and animals. As a result, lysozyme has been categorized into several classes which include chicken, goose, insect, phage, plant and bacteria type lysozymes (Bachali *et al.*, 2002; Hikima *et al.*, 2003; Ito *et al.*, 1999; Xue *et al.*, 2007; Xue *et al.*, 2004). Each class of muramidase differs by its amino acid sequence, molecular weight and enzymatic properties.

Muramidase is an important component of the humoral immune system of invertebrates (Bachali *et al.*, 2002; Zhao *et al.*, 2007b). It is involved with bacteriolytic events as well as digestive functions within invertebrates. As a digestive enzyme lysozyme works to break down bacteria in high acid environments in ruminants, leaf eating monkeys, *Drosophila* and birds (Xue *et al.*, 2007). The eventual break down of the bacteria is what provides the nutrients for the organism. It has also been found that all lysozymes involved in digestion are of the chicken type (Ibrahim *et al.*, 2005; Ito *et al.*, 1999; Xue *et al.*, 2004).

Many studies have been conducted on invertebrates in order to isolate and characterize lysozyme. Lysozyme has been found in oysters, shrimp, *Drosophila*, starfish, bivalves, conch and earthworms to name a few (Bachali *et al.*, 2004; Bachali *et al.*, 2002; Hikima *et al.*, 2001; Hikima *et al.*, 2003; Ito *et al.*, 1999; Xue *et al.*, 2007). These lysozymes have been found to be of either the chicken (*c*), goose (*g*) or invertebrate type (*i*). It has been estimated that both the *c* and *i* type lysozymes have been in existence for over 600 million years and alignment of both types of lysozymes show that specific amino acid residues are preserved (Bachali *et al.*, 2002). To date, *Drosophila* is the only metazoan which has been found to have both the *c* and *i* type

lysozymes. The *i*-type lysozyme was first described by Jolles and Jolles in 1975 (Jolles & Jolles, 1975). Later studies by Hikima (Hikima *et al.*, 2003) revealed that *i*-type lysozyme is more closely related to *g*-type lysozyme whilst *c*-type lysozyme seems to be more ancestral to both *g* and *i*-type lysozymes. The *i*-type lysozyme has been found in nematodes, echinoderms, annelids and mollusks.

MATERIALS AND METHODS

Materials

Media and tissue culture reagents were obtained from Flow Laboratories (McLean, VA). Pyrogen-free water and pyrogen-free saline were obtained from Travenol (Deerfield, IL). Bacteriological reagents were obtained from Difco (Becton Dickinson, Sparks, MD). Plastic ware was obtained from Falcon (Oxnard, CA). All other reagents were of analytical grade or better and were obtained from Sigma (St. Louis, MO) or from Fisher Scientific (Fairlawn, NJ).

Collection of Specimen

Comb (Leidy's Comb Jellyfish; *Mnemiopsis leidyi,*) and Moon jellyfish (*Aurelia aurita*) were collected during the summer months from Nantucket Harbor. A sample from the inside and outside of each Comb jellyfish was obtained for 1) sterile streak onto nutrient broth agar and 2) fixation in formalin for 4', 6-diamidino-2-phenylindole (DAPI) staining. The remaining jellyfish were stored at -20°C (Nantucket Field Station) until transported to a -80°C freezer.

DAPI Staining

Comb jellyfish samples were fixed in 1.5 mL of 10% formalin. Fixed cells were centrifuged in a microcentrifuge for 30 seconds. Media was aspirated off and the pellets were re-suspended in 300 µl of PBS. Cells were centrifuged and washed once more in 1 mL PBS. The wash was decanted and the cells were re-suspended in 500 µl PBS. The DAPI was added at 1 µg/mL according to the manufacturer's instructions (Sigma). The cells were stained for 5 minutes with gentle agitation then washed twice with 500 µl of PBS. Cells were re-suspended in 200 µl of PBS and viewed at 60x and 100x under an epifluorescent microscope.

Preparation of Jellyfish Fluids

Lyophilization

Jellyfish were frozen at -80°C in conical plastic bottles. Frozen samples were then placed into a lyophilizer and left for reduction. Lyophilized jellyfish were re-suspended in saline, acidified with acetic acid to a final concentration of 5% and centrifuged at 12,000 rpm. The supernatant and pellet were collected.

Ultrafiltration

Jellyfish fluids were subjected to ultrafiltration at 4°C as described in detail previously (Raftos *et al.*, 1991). A 10,000 molecular weight cut off (m.w.c.o.) filter (Millipore Corp., Bedford, MA) was used for supernatant separation and the retentate and flow thru were saved. A 500 m.w.c.o.filter was used for flow thru separation. The retentate (>500) and flow thru (<500) were saved. Assays for bactericidal activity were conducted on pellet, retentate and 500 m.w. fractions. SDS-PAGE analysis was also conducted on the whole lyophilized jellyfish sample, the pellet and the 500 m.w. fractions.

SDS-PAGE

SDS-PAGE analysis was conducted for both the Moon and Comb jellyfish as described previously by (Hetru & Bulet, 1997). The samples used were the starting material, 500 Da isolated regions and the pellet along with a molecular weight standard (BioRad, Hercules, CA) as a guide. Before loading the 4-20% gradient acrylamide gels, the samples were treated with β-mercaptoethanol and then heated at 95°C for 5 min. The gels were run at 100 volts for ≈ 1h or until sample reached bottom of gel. They were stained in silver nitrate solution and analyzed for protein bands and their molecular weights.

Solid-Phase Extraction

Two grams of lyophilized whole jellyfish were re-suspended in 10 mL of super pure distilled water. Two mL 0.1 % TFA was added to 2 mL of jellyfish suspension. The mixture was incubated for 30 min in an ice-cold water bath with gentle agitation and then centrifuged at 15, 000 g for an additional 30 min. The supernatant was collected and the pH adjusted to ≤ 4. A Sep-Pak C18 cartridge (Waters Corporation, Taunton, MA) was used for the extraction of the acidified jellyfish fluids (Hetru & Bulet, 1997). The Sep-Pak C18 cartridge was washed with 5 mL methanol then equilibrated with 5 mL of 0.05% TFA. A two mL jellyfish sample was then loaded onto the column where samples were eluted in a stepwise fashion with increasing concentrations of acetonitrile (10, 40 and 80% in 0.05% TFA). Each fraction was collected in 1 mL aliquots in microfuge tubes. The samples were lyophilized in a speed-vac and saved for later analysis. A total of thirty-one fractions were collected from starting material through to the 80% acetonitrile fraction (Hetru & Bulet, 1997). Additionally, samples of just 10%, 40% and 80% acetonitrile were collected, lyophilized and saved for later use as controls in bactericidal assays.

Preparation of Bacteria

Several strains of Gram negative and Gram positive bacteria (obtained from Tom Durant, UMass Boston Biology Department) were grown in liquid media [8 g Nutrient Broth (Difco) in 1 liter distilled water, autoclaved at 121°C for 15 min] to mid-log phase at 37°C in a shaking water bath. The concentration of bacteria was between 0.1 and 0.4 at an absorbance of 600 nm. The final concentration of cells used in bactericidal assays was 10^8 cells/mL.

Antibacterial Assays

Assays were conducted in 96 well plates and were set up in triplicates as described (Beauregard *et al.*, 2001). Each experiment contained jellyfish extract (whole jellyfish extract, pellet or 500 Da extracts), nutrient broth and bacteria. Positive controls comprised nutrient broth and bacteria. Negative controls consisted solely of nutrient broth. Plates were incubated at 37°C with absorbance readings (OD_{640}) taken at varying time points between 0 to 24 h. All data was collected, saved and analyzed in MS Excel.

Lysozyme Assays

Lysozyme assays were conducted on samples (10, 40, 80% Sep-Pak fractions) collected from solid phase extraction. These assays were also conducted in 96 well plates and were prepared in triplicates (Hetru & Bulet, 1997; Xue *et al.*, 2004). Each experiment contained either 10, 40 or 80% fractions in addition to nutrient broth and *Micrococcus luteus*. Lysozyme has been shown to have specific activity against *M. luteus* (Ito *et al.*, 1999). Positive controls included *M. luteus* and nutrient broth while negative controls were nutrient broth only. Plates were incubated at 37°C with absorbance readings (OD_{640}) taken at varying time points between 0 to 24 h. All data was collected, saved and analyzed in MS Excel.

RESULTS

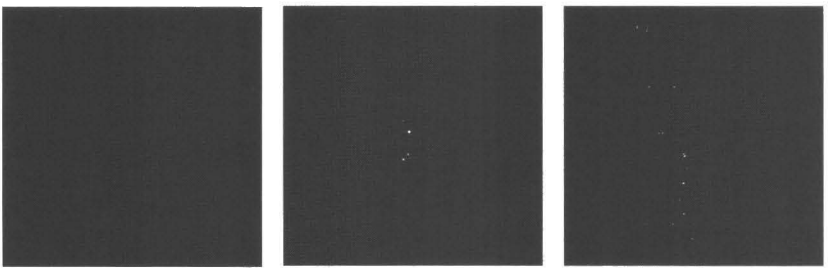
Sterile Streak and DAPI Stain

This experiment was conducted in order to determine whether bacteria were present on the outer surfaces or inner orifices of jellyfish. Upon completion of sterile streaks of Comb jellyfish, results showed no evidence of bacteria on either inner or outer surfaces. This suggests that jellyfish have very strong defenses against microbes in the ocean. Sterile streaked plates revealed no countable colonies on the plates (Fig 11.1A). Scoring of colonies was via an arbitrary scale where 0 was least bacterial growth and 5 was the most bacterial growth per streak. A score of 0 indicated that no colonies were formed on the plates while a score of 5 indicated >10 colonies on a plate. Only one jellyfish had a high rate of bacterial colonization from an internal streak with a score of 5. This result could have occurred because the jellyfish could have been severely compromised and was no longer able to fend off infection. The DAPI stains (Fig 11.1D) revealed similar results for bacterial colonization of jellyfish. The mean field count obtained for the total internal streaks was 1.2 cells while total external streaks had mean field counts of 1.6. Controls taken from seawater, air and media tubes had mean values between 0.2 and 0.4 cells, (Fig 11.1E). Each slide was viewed under an epifluorescent microscope and ten fields were counted per slide. The average of all the fields counted was then taken for both the internal and external jellyfish samples as well as the seawater and air controls. This data is shown in Fig 11.1E where the highest bacterial counts came from samples from external jellyfish streaks. This was not surprising as it was expected that microbes would be found sticking to the outside of the jellyfish since they are in constant contact with a microbe filled environment. The

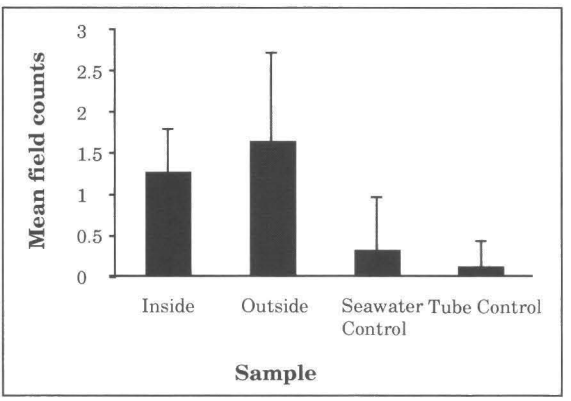
lower bacterial counts for the internal streaks was also what was expected as, it would be likely that in order to keep the jellyfish healthy it would need to have a means by which it can eliminate microbes from its internal cavity.

	Plate Number and Score								
Jellyfish Surface	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Exterior	1	0	2	1	3	2	1	1	1
Interior	0	0	2	1	3	2	1	5	1
Seawater	0	0	0	0	0	0	0	0	0
Seawater	0	0	0	0	0	0	0	0	0
Air	0	0	0	0	0	0	0	0	0

(A)



(B) **(C)** **(D)**



(E)

Fig 11.1. DAPI Stain and Sterile Streaks of Comb Jellyfish. Fig 1A shows that sterile streaked plates revealed no countable colonies. Nine Comb jellyfish were used in this experiment. A score of 0 = no bacteria present, 5 = many bacteria present. Fig 1 B,C,D show DAPI stains of jellyfish number 2 and 5 (external and internal streaks respectively) and *E. coli* used as a positive control at a magnification of 100x. Sterile streaks were taken, treated with 1 μg/mL DAPI, and viewed at 100x. Fig 1E shows the mean field counts of Comb Jellyfish with DAPI stain

SDS PAGE Analysis

From the SDS-PAGE studies conducted, two similar bands in both the Comb and Moon jellyfish were detected (Fig 11.2). These bands are suspected to be of a lysozyme-like molecule (≈40kDa) since a molecular weight similar to that of lysozyme (≈30kDa) (Bachali *et al.*, 2004) was detected.

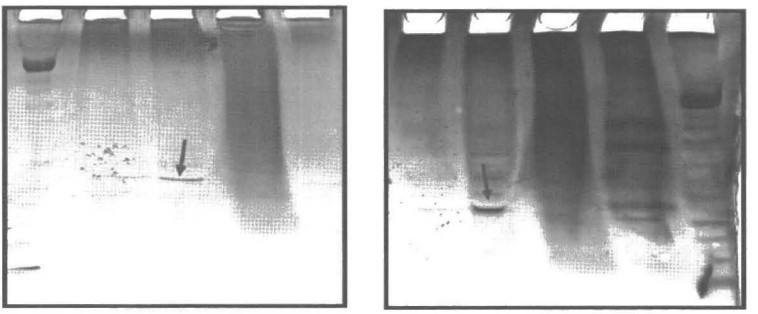


Fig 11.2. SDS-PAGE for Comb (left) and Moon (right) Jellyfish. Left: wells from left to right are the molecular weight standard, the >500 fraction, the <500 fraction, the pellet and a whole jellyfish fraction. Right: wells from left to right the >500 fraction, the <500 fraction, the pellet, a whole jellyfish fraction and the molecular weight standard. A predominant band is seen at the <500 fraction (arrow head) in both gels

This high molecular weight fraction detected in the 500 Da regions may have been due to poor filtration methods or other unforeseen circumstances. This 40 kDa band was also seen in all the other fractions in the gel (*i.e.* 500 Da fractions). The identity of this band has not yet been confirmed.

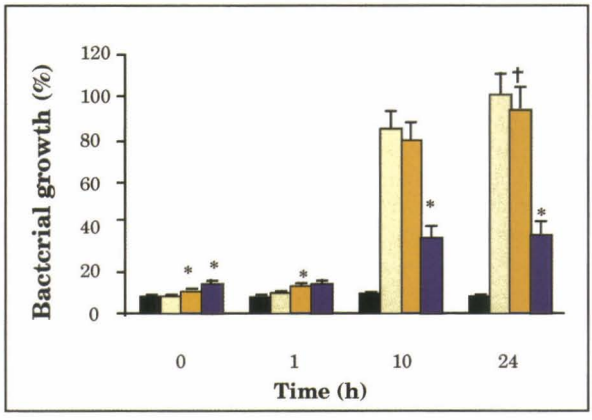
Antibacterial Assays

Fractions obtained via ultrafiltration from Comb and Moon jellyfish were used in antibacterial assays. Bactericidal assays were conducted on fourteen strains of bacteria from either Gram positive or Gram negative origin. *Alcaligenes faecalis, Enterobactor aerogenes* and *Escherichia coli* (all Gram negatives) had cell deaths of 36, 23 and 25% respectively when treated with Comb jellyfish fluids of Mr <500 Da. Gram positive bacteria (*i.e. Staphylococcus auresus, Staphylococcus epidermis* and *Bacillus subtilis*) had deaths of 29, 16 and 35% respectively when treated with Moon jellyfish fractions of <500 Da (Table 11.1). Other bacteria such as *Proteus mirabilis*, *Proteus vulgaris* and *Chromobacterium violaceusm* showed no cell death when treated with either Comb or Moon jellyfish fractions. The data represented in Fig 11.3 A-D shows a graphical interpretation of data obtained in Table 11.1. The graphs in Fig 11.3 show results obtained from bacteria lysis assays against *P. aeruginosa* and *S. aureus* with Comb and Moon jellyfish

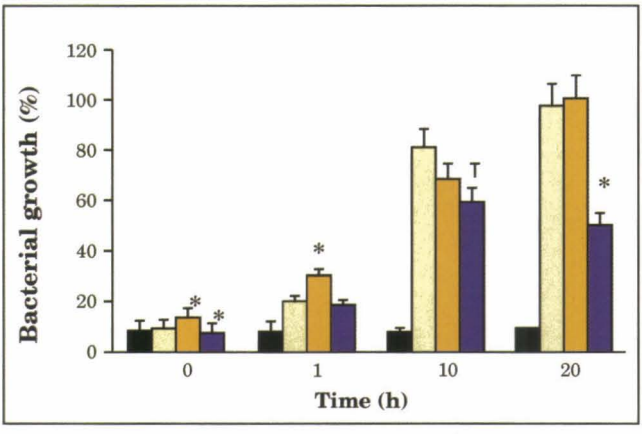
fractions. The greatest bacterial growth inhibition was observed in the <500 Da fractions of both jellyfish. Similar graphs were constructed for all bacteria assayed against jellyfish fractions (data not shown).

Table 11.1. Percent Bacterial Cell Death Induced by Comb or Moon Jellyfish Samples (10 hr. time point). The table shows data obtained from bacterial lysis assays. The jellyfish samples were assayed against Gram-negative and/or Gram-positive bacteria. Indicated above is the percent cell death of bacteria after 10 hours of exposure to the samples. Several Gram-negative as well as Gram-positive strains were killed by the jellyfish samples

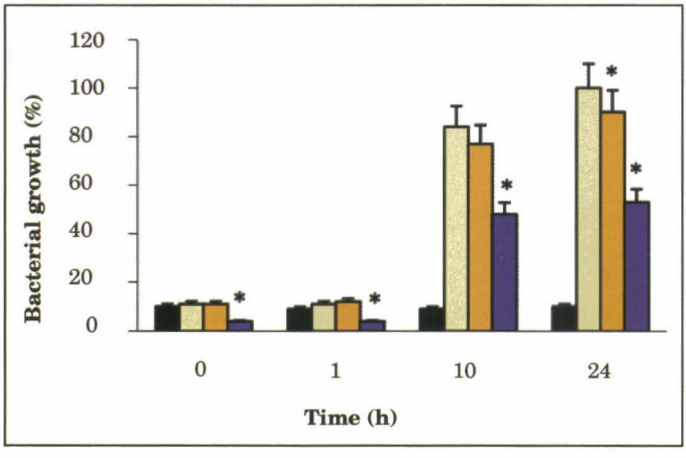
	% Bacterial Cell Death					
	Moon 10 h			Comb 10 h		
Bacteria (gram + or gram –)	**Starting Material**	**<500**	**>500**	**Starting Material**	**<500**	**>500**
Eschericia coli (-)	0	25.77	3.32	23.8	24.55	3.83
Serratia marcescens (-)	0	31.58	4.33	67.08	60.19	8.17
Chromobacterium violaceum (-)	0	0	0	0	0	16.24
Enterobactor aerogenes (-)	7.45	33.64	2.73	26.14	23.2	10.94
Proteus mirabilis (-)	0	0.81	0	62.3	34.46	17.06
Proteus vulgaris (-)	0	0	0	29.41	8.46	11.2
Pseudomonas aeruginosa (-)	0	34.74	0	0	6.25	0
Klebsiella pneumoniae (-)	0	23.1	0	0	0	2.51
Alcaligenes faecalis (-)	0	0	0	59.62	35.9	0
Bacillus subtilis (+)	0	0	0	11.24	7.87	0
Enterococcus faecalis (+)	0	0	0	6	4	0
Staphylococcus epidermis (+)	0	15.5	0	10.04	16.06	0
Staphylococcus aureus (+)	0	28.3	3.56	24.73	36.44	14.48
Micrococcus luteus (+)	0	22.77	13.4	4.11	9.68	0


Bacterial growth (%)
120
100
80
60
40
20
0
0
1
10
24
Time (h)

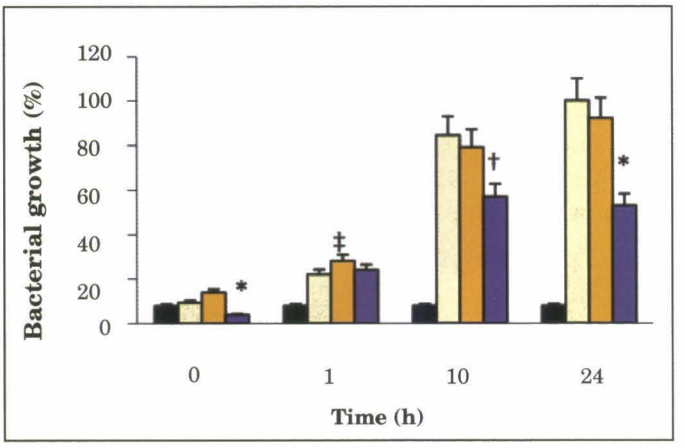
(A)


Bacterial growth (%)
120
100
80
60
40
20
0
0
1
10
20
Time (h)

(B)


Bacterial growth (%)
120
100
80
60
40
20
0
0
1
10
24
Time (h)

(C)



(D)

Fig 11.3. Bacterial Lysis Assay of the Fractionated Jellyfish Fluids. These figures show a more detailed analysis of time points as presented in **Table 1** for the Moon jellyfish assayed against *P. aeruginosa* (**A**) and *S. aureus* (**B**) and the Comb jellyfish assayed against *P. aeruginosa* (**C**) and *S. aureus* (**D**) . Similar graphs were constructed for all strains tested (data not shown). (Bars indicate % bacterial growth ± SD). Negative control (nutrient broth) – (■); Positive control (bacteria alone) – (□); Jellyfish starting material – (■); Jellyfish 500 dalton fraction – (■). *P<0.001 as compared to bacteria alone; †P<0.01 as compared to bacteria alone; ‡P<0.05 as compared to bacteria alone

Activity Detected in 40% Solid Phase Fractions

Both Moon and Comb jellyfish were subjected to solid phase extraction using a Sep-Pak C18 column. Each fraction was tested for lysozyme activity. Most lytic activity was observed in fractions that were eluted in 40% acetonitrile (Fig 11.4). In this experiment *M. luteus* was used to detect the presence of lysozyme in both Comb and Moon jellyfish. In experiments conducted using Comb jellyfish, the greatest activity was observed between fractions 11 and 20 (Fig 11.4 top) whilst activity was seen between fractions 13 and 23 (Fig. 11.4 bottom) in the Moon jellyfish.

DISCUSSION

Our sterile streak studies were conducted on Comb jellyfish as those were the only jellyfish that were available at the time of the study. Each sterile streak was cultured on an agar plate (Fuhrman, 1999) and the resulting colonies that were formed were counted and scored. Our results show that there were virtually no bacteria present in or on the jellyfish. The results obtained were not very surprising as it was our expectation that jellyfish would be very good at protecting themselves from microbes. The ocean is populated with copious amounts of microbes/L sea water ($\approx 10^9$ bacteria and

≈10^{10} viruses, respectively) (Fuhrman, 1999; Suttle, 2005). It is therefore necessary for jellyfish to have some mechanism by which they protect and defend themselves. From previous work by Ovchinnikova and colleagues it was found that Moon jellyfish have an antimicrobial peptide with defensin like properties which block toxins (Ovchinnikova *et al.*, 2006). This AMP, aurelin, was found to be ≈4,000 Da and exhibited antimicrobial activity against both gram positive as well as gram negative bacteria.

Antimicrobial peptides have been found in a wide range of invertebrates both marine and terrestrial. In 2004 Ovchinnikova and colleagues (Ovchinnikova *et al.*, 2004) also found other AMPs in the polycheate, *Arenicola marina*. Both arenicin-1 and arenicin-2 were found to be 21 residues long and have exhibited activity against gram positive and negative bacteria as well as fungi. They found AMP activity was equal in all strains they tested, *i.e.* no microbe was more or less susceptible to treatment with the AMPs. In other works AMPs have been found in the Rainbow trout where oncorhyncin III was found to be 6.7 kDa (Fernandes *et al.*, 2003). This AMP was isolated from the skin in 20% acetonitrile via RP-HPLC. Oncorhyncin III was also found to be active against both gram positive and negative bacteria in submicromolar concentrations. The AMP of the bumblebee has been shown to be quite potent which makes it difficult to use therapeutically. Not only does melittin destroy microbes but it is also toxic to human cells (DeGrado *et al.*, 1982). Tunicates have also been found to possess AMP activity. In 2003 Tincu and colleagues (Tincu *et al.*, 2003) found an AMP (plicatamide) in *Styela plicata*. This particular AMP is an octapeptide which is found in the hemocytes of the tunicate. Plicatamide was shown to be a very potent, rapidly acting broad spectrum AMP. It was also found that this AMP worked best at neutral pH and worked predominantly by breaking down the bacterial cell wall by changing the concentrations of the influx and efflux of membrane ions (Tincu *et al.*, 2003). In *Drosophila* it has been shown that restoring the activity of a single AMP can result in resistance of the fruit fly to bacteria (Tzou *et al.*, 2002). In this experiment fly lines were generated that expressed a single AMP gene. The flies that lacked the immune defense (*imd*) gene were highly susceptible to gram negative infection however when this gene was reintroduced into the flies they were completely resistant to infection by gram negative bacteria. Many AMPs have been found in a host of other organisms, (*i.e.* mussels, crabs, shrimp, frogs and moths) all of which have been associated with wound healing, chemotaxis and mediators of inflammation (Bals, 2000a; Bals, 2000b; Bang *et al.*, 1997; Benkendorff *et al.*, 2001; Boman, 2003; Dimarcq *et al.*, 1998).

After purification of both the Comb and Moon jellyfish samples, SDS PAGE analysis was conducted. Both jellyfish samples showed similar bands from the 500 dalton fractions which had a higher molecular weight band of

≈40 kDa. It is may be likely that this high molecular weight protein is lysozyme as further tests from bacterial lysis assays using *M. luteus* has shown lytic activity for both species of jellyfish. In addition, large lysozymes have been found in other marine invertebrates such as oysters and clams (Xue *et al.*, 2004; Zhao *et al.*, 2007b). The SDS gels were subjected to both silver stains as well as Coomassie blue staining but the silver stains were used because the bands were more visible via this method of staining. Silver staining allows a very small quantity (nanograms) of protein to be visible on the gel when this method is used whereas Coomassie blue is not as sensitive for showing the presence of proteins.

The samples that were collected from the various stages of purification were used to conduct bacterial lysis assays using several strains of gram positive and gram negative bacteria. In general, the Comb jellyfish samples seem to be more efficient at eliminating pathogens (especially gram negative microbes). Data collected from assays conducted on the Moon jellyfish also revealed that there was more killing activity on gram negative microbes than on gram positive bacteria. These results were a little surprising as gram negative bacteria are usually much harder to eliminate because they have LPS in addition to their peptidoglycan wall (Toke, 2005; Tomasz, 2006; Zasloff, 2002b). The presence of LPS provides that extra protection and makes the bacteria more resistant to potentially harmful toxins (Toke, 2005; Tomasz, 2006; Zasloff, 2002b). While this result was startling it is very promising as it provides the opportunity for the development of antibacterial agents which could prove to be quite potent against gram negative bacteria. Other data suggest that the AMP aurelin has been found in the Moon jellyfish and is active against the gram negative bacterium *E. coli* as well as the gram positive bacterium *L. monocytogenes* (Ovchinnikova *et al.*, 2006). The graphs in Fig 11.3 are a representation of antibacterial assays conducted on all the strains of bacteria. In all instances the most activity was seen in the 500 dalton jellyfish fractions even up to 24 h after bacteria were exposed to the samples. In most cases bacterial growth was inhibited by 60% when compared to the positive control.

Since a part of the antibacterial activity detected was suspected to be due to the presence of lysozyme, assays were conducted using *M. luteus* to measure the amount of killing. In order to obtain purer samples to work with, solid phase extraction was conducted on jellyfish samples with varying concentrations of acetonitrile. Previous methods suggest that lysozyme will elute in acetonitrile (Hetru & Bulet, 1997). The samples that were obtained from the stepwise separation using 10, 40 and 80% acetonitrile were tested and as shown in Fig 11.4 activity was detected in the 40% fractions. This result was consistent for both the Comb and Moon jellyfish. Further analysis is currently being done to characterize the molecule.

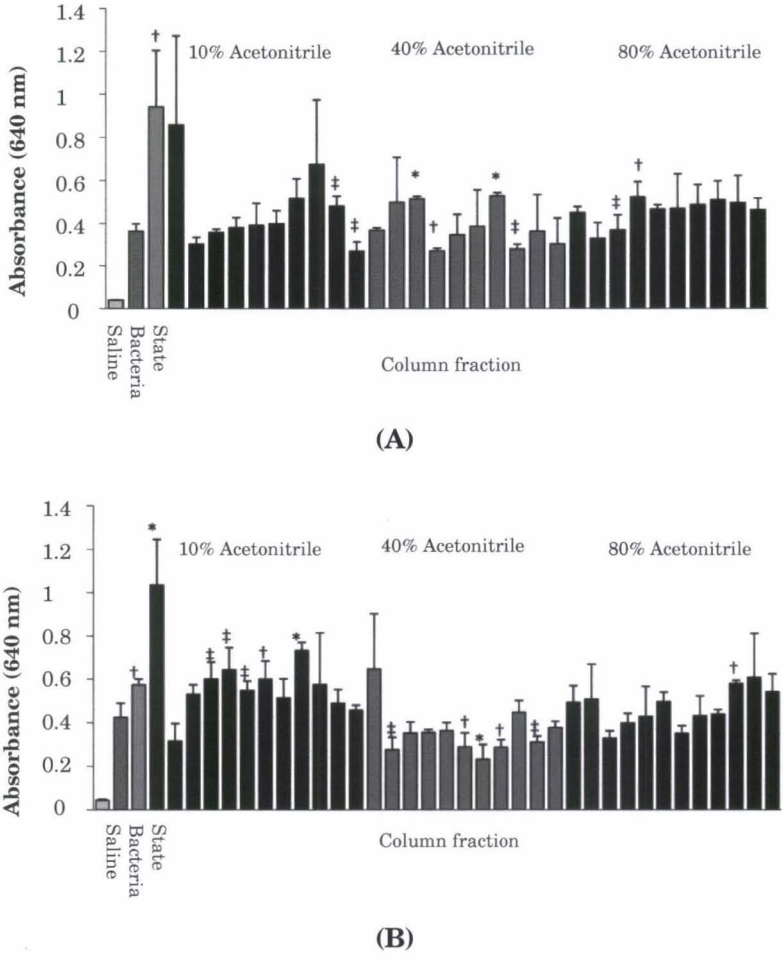


Fig 11.4. Detection of Lysozyme Activity. Assays conducted on fractions collected from solid phase extraction (Sep-Pak C18 column) of Comb (**top**) and Moon (**bottom**) jellyfish. Activity was detected in fractions eluted in 40% acetonitrile. Saline – (▩); Bacteria alone – (■); Jellyfish starting material – (□); 10 and 80% Acetonitrile – (■); 40% Acetonitrile – (■) $^{*}P<0.001$ as compared to bacteria alone; $^{\dagger}P<0.01$ as compared to bacteria alone; $^{\ddagger}P<0.05$ as compared to bacteria alone

Muramidase is an enzyme that is widely distributed throughout the animal kingdom. Its biological function has been found to be self defense from bacterial infection by lysing the peptidoglycan wall. Numerous studies have been conducted on organisms to detect and characterize lysozyme and its functions. In work done on bivalves by Ito and colleagues it was found that this bivalve lysozyme was four times as potent as chicken lysozyme when assayed against *M. luteus* (Ito *et al.*, 1999). Not only was it found that

lysozyme is involved with self defense but studies by Jolles *et al.*, (1975) showed that it may also act as a digestive enzyme (Bachali *et al.*, 2002). In other works done by Xue and colleagues and Hikima and colleagues on the eastern oyster and kuruma shrimp respectively, it was found that lysozyme was active against both gram positive and negative bacteria. Interestingly, lysozyme displayed activity against *Vibrio*, a gram negative bacterium (Hikima *et al.*, 2003; Xue *et al.*, 2007). Vibrios are one of the major pathogens that plague shellfish and other organisms within the seafood industry. The presence of lysozyme in these organisms acts as one of the major modes of defense by limiting microbial invasion, clearing the microbes from the organisms. Muramidase activity has also been detected in scallops, Japanese flounder, starfish and butterflies (Bachali *et al.*, 2004; Hikima *et al.*, 2001; Xue *et al.*, 2007; Zhao *et al.*, 2007a; Zhao *et al.*, 2007b). The lysozymes from these organisms also display activity against gram positive and negative bacteria.

Overall, this work has shown that both the Comb and Moon jellyfish exhibit natural antibacterial properties against both gram negative and gram positive bacteria (with the more potent activity being against gram negative bacteria). These results are promising as this may provide a new avenue by which therapeutics can be developed not only for human medicine but also for agriculture and aquaculture. Future directions of these studies are to characterize lysozyme as well as the other potential AMPs by means of molecular and biochemical methods.

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12

Plants of the Genus: *Commiphora*-their Chemistry

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ABSTRACT

Commiphora is a genus consisting of about 165 to 185 plant species coming under the family Burseraceae. This review includes discussion about the phytoconstituents present in a collection of twenty four species belonging to this genus Commiphora along with their extraction, isolation and characterization methods in short. Structures of all the two hundred thirty compounds reported are appended.

Key words : Burseraceae, *Commiphora*, diterpene, guggulsterone, pinene, sitosterol, terpenes

INTRODUCTION

Commiphora is the name of a genus consisting of a number of interesting plant species under the family Burseraceae. Out of nearly 200 species, here, in all 24 important species belonging to this genus have been selected and described keeping in view the chemical aspects only which includes works related to extraction, isolation and characterization of chemical constituents present. To highlight certain important and relevant aspects about the Burseraceae family to which the genus belongs, a few paragraphs are included.

Burseraceae is a family having nearly 20 genera and more than 500 species of flowering plants which include both trees and shrubs. As per literature, the family originated in North America during the Paleocene (~ 65 Mya), when the earliest fossils of the Sapindales, the order in which

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the family belongs at present, are found (Weeks *et al.,* 2005). During the Early to Middle Eocene (~ 53 Mya) family members dispersed to eastern Laurasia (*i.e.* Europe and Asia) and the continents in the Southern Hemisphere and finally it has become native to the tropical regions of Africa, Asia and the Americas.

Most Burseraceae members have fleshy, edible fruits and the mechanism of seed dispersal is mainly via animal vectors such as birds like hornbills, oilbirds, fruit pigeons etc. (endozoochoric dispersal) and that is how, most Burseraceae members were able to expand their range so efficiently across the globe (Weeks *et al.,* 2005). Controversy does exist in the statements regarding the exact numbers of genera and species the Burseraceae family imbibes. The actual numbers differ according to the time period in which a given source is written describing this family.

However, the Burseraceae trees and shrubs are characterized by non-allergenic fragrant resins that are present within the plant tissue inside the vertical resin canals and ducts in the bark and in the leaf veins. These resins have been used since long to make candles, incense and even torches in the past. Thus, Burseraceae is also known as the torchwood family or the incense tree family. Such non-allergenic resins have medicinal value also and thus have been used in Chinese herbal and Indian Ayurvedic medicines to treat several ailments.

The following review regarding *Commiphora* aims at assembling the research works of different authors related to phytoconstituents isolated and characterized from various plants belonging to this genus. For easy availability, the structures of all the reported compounds have been number wise clustered and presented just after the narrative portion of the article.

Commiphora abyssinica (Berg) Engl

The latex obtained from *Commiphora abyssinica* [Syn: *Commiphora madagascarensis* Jacq] was reported to contain 6.5% of steroids. Further fractionation by column chromatography followed by mass spectrum analysis revealed the presence of cholest-5-en-3β-ol **1**, Δ5-campestan-3β-ol **2** and β-sitosterol **3** (Cagnoli *et al.,* 1968).

The essential oil of *Commiphora abyssinica* (Berg) Engl was extracted in hexane and more than twenty different constituents have been isolated from it using column and preparative chromatographic techniques. Such compounds include α-pinene **4**, limonene **5**, cuminaldehyde **6**, cinnamaldehyde **7**, eugenol **8**, m-cresol **9**, formic acid **10**, acetic acid **11** and palmitic acid **12**; nine sesquiterpene hydrocarbons (δ-elemene **13**, β-elemene **14**, α-copaene **15**, β-bourbonene **16**, germacrene D **17**, caryophyllene **18**, humulene **19**, γ-cadinene **20** and δ-cadinene **21**); the sesquiterpene alcohol

(elemol **22**) and furanosesquiterpenoids such as furanodiene **23**, furanodienone **24**, isofuranogermacrene **25**, curzerenone **26** and lindestrene **27** (Brieskorn & Noble, 1982).

Commiphora africana (A.Rich) Engl

Volatile oil obtained by the steam distillation of aromatic resin collected from *Commiphora africana* was examined by capillary GC and GC/MS. Constituents reported were α-pinene **4** (high concentration), α-thujene **28**, sabinene **29**, myrcene **30**, car-3-ene **31** and p-cymene **32**. Some of these are important markers (Provan *et al.*, 1987).

The leaves of *Commiphora africana* yielded essential oils which were analyzed by GC, GC/MS and C-NMR. The two major compounds identified in the oil are α-oxobisabolene **33** (61.6%) and γ-bisabolene **34** (10.0%) (Ayedoun *et al.*, 1997).

Methanolic extract of the bark of the plant *Commiphora africana* gave a homogenous product through chromatographic separation which after crystallization from methanol furnished needles of dimethylterephthalene (benzene-1-dicarboxylic acid dimethylester) **35** (Choudhury *et al.*, 2000).

Bioassay-guided fractionation of a crude extract from *Commiphora africana* led to the isolation of the dihydroflavonol glucoside, phellamurin **36** (Ma *et al.*, 2005).

Commiphora confusa

The resin of *Commiphora confusa* afforded two new dammarane triterpenes, (3R,20S)-3,20-dihydroxydammar-24-ene **37** and (3R,20S)-3-acetoxy-20-hydroxydammar-24-ene **38** along with two known triterpenes, cabraleadiol 3-acetate **39** and α-amyrin **40** (Dekebo *et al.*, 2002a).

The steam distilled resin residue of *Commiphora confusa* yielded (20S)-3β-acetoxy-12β,16β-trihydroxydammar-24-ene **41**, (20S)12β,16β-trihydroxy dammar-24-ene-3β-O-β-glucopyranoside **42**, (20S)-3β-acetoxy-12β,16β,25-tetrahydroxydammar-23-ene **43** and (20S)-3β,12β,16β,25-pentahydroxy-dammar-23-ene **44**. The known compounds β-amyrin **45**, 3-β-amyrinacetate **46**, 2-methoxyfuranodienone **47**, 2-acetoxyfuranodienone **48**, (20R)-3β-acetoxy-16β-dihydroxydammar-24-ene **49**, 3-β-hydroxydammar-24-ene **50**, 3-β acetoxydammar-24-ene **51** and β-sitosterol **3** were also obtained in a similar way from the same extract after acetone extraction following column chromatography on silica gel using a gradient of ethyl acetate in n-hexane (Manguro *et al.*, 2003a).

Commiphora cyclophylla Chiov

The liquid resin obtained spontaneously by cutting the woody parts of *Commiphora cyclophylla* Chiov. occurring in Southern Ethiopia when

examined was found to contain primarily monoterpene hydrocarbons and no oxygenated derivatives, with limonene **5** as the major component (Abegaz *et al.*, 1989).

Commiphora dalzielli

Seven dammarane triterpenes namely cabraleadiol-3-acetate **39**, β-amyrin **45**, lupeol **52,** epilupeol **53,** cabraleone **54**, cabraleadiol **55** and isofouquierone **56** were isolated from the petroleum ether extract of stem bark of *Commiphora dalzielli* by column chromatography over silica gel using petroleum ether containing increasing amounts of ethyl acetate. Subsequently purification was done by circular preparative TLC (Waterman *et al.*, 1985).

Commiphora erlangeriana

The resin of *Commiphora erlangeriana* [Syn: Dhunkal in Ethiopia and Somalia] was known to be poisonous to humans and animals and had traditionally been used as an arrow poison. Phytochemical studies on this plant material identified four major lignans (erlangerins A **57,** erlangerins B **58,** erlangerins C **59** and erlangerins D **60**) which closely resemble the structure of podophyllotoxin. It was hypothesized that the well known poisoning effect of the resin could in part be due to its direct toxicity of constituents on mammalian cells (Habtemariam, 2003).

Commiphora erythraea (Ehrenb) Engl

The downward chromatostrip procedure followed by gas chromatographic analysis could determine the monoterpene hydrocarbon composition of essential oils obtained from *Commiphora erythraea* [Syn: Opopanax or Bisabol in Mediteranean region and Ethiopia, Arabia and Somalia] based on comparison of retention times with known compounds. The major component, ocimene **61** was identified with trace amounts of α-pinene **4**, sabinene **29**, car-3-ene **31,** myrcene **30**, limonene **5**. Column chromatography followed by gas chromatography of essential oil of *Commiphora erythrea,* facilitated identification of three components namely, α-oxobisabolene **33**, β-bisabolene **62** and γ-bisabolene **34** (Ikeda *et al.,* 1962).

Furanodienone **24** was obtained from the hexane extract of *Commiphora erythrea* gums by column chromatography on silica gel with petroleum ether (Maradufu, 1982).

Commiphora kua var. kua Vollesen

Commiphora kua var. kua Vollesen [Syn: *Commiphora flaviflora*], a tree growing wild in Kenya, Ethiopia and Somalia, is a plant in which a number of compounds were found during various investigations.

Three labile C_{22} octanordammarane triterpenes, namely 16-hydroperoxymansumbin-13(17)-en-3-one **63**, 16-hydroperoxymansumbin-13(17)-en-3β-ol **64** and 16-hydroperoxy-3,4-seco-mansumbin-3(28),13(17)-dien-3-oic acid **65** were obtained from the petrol extract of the stem bark of *Commiphora kua*. These three compounds rapidly degraded to give three break down products such as mansumbin-13(17)-en-3,16-dione **66**, 3β hydroxymansumbin-13(17)-en-16-one **67** and 16-oxo-mansumbin-3(28),13(17)-dien-3-oic acid **68** (Provan *et al.,* 1992).

Commiphora kua var. kua volatile oil obtained from the same species gave compounds such as α-pinene **4**, p-cymene **32**, α-thujene **28**, β-pinene **69**, limonene **5**, sabinene **29**, terpinene-4-ol **70**, car-3-ene **31** and myrcene **30**. In the residue after steam distillation and after ethyl acetate extraction followed by column chromatography, two known furanosesquiterpenoids were identified; 2-O-acetyl-8,12-epoxygermacra-1(10),4,7,11-tetraene **71** and 2-O-methyl- 8,12-epoxygermacra-1(10),4,7,11-tetraene **72** besides, a known compound xanthorrhizol **73**, and a new one *i.e.* 2-methyl-5-(5'-hydroxy-1',5'-dimethyl-3'-hexenyl)phenol **74** (Manguro *et al.,* 1996).

Three active compounds, namely mansumbinone **75**, mansumbinoic acid **76** and picro-polygamain **77** have been purified from an extract of *Commiphora kua* (Battu *et al.,* 1999).

The petrol extract of the resin reportedly yielded four known compounds which were identified as mansumbinone **75**, mansumbinol **78**, (16S, 20R)-dihydroxydammar-24-en-3-one **79**, T-cadinol **80** respectively. Besides, two new octanordammarane triterpenes, namely 15α-hydroxymansumbinone **81** and 28-acetoxy-15α-hydroxymansumbinone **82** were isolated on silicated column chromatography with n-hexane with increasing amount of ethyl acetate (Dekebo *et al.*, 2002a).

A novel bisabolene, 6-hydroxy-2-methyl-5-(5'-hydroxy-1'(R),5'-dimethylhex-3'-enyl)-phenol **83**, together with two new dammarane triterpenes, 3β,16β,20(S),25-tetrahydroxydammar-23-ene **84** and 3β-acetoxy-16β,20(S),25-trihydroxydammar-23-ene **85** were reported from ethyl acetate extract of *Commiphora flaviflora* by column chromatography over silica gel. In addition, three known compounds such as 2-methyl-5-(4'(S)-hydroxy 1'(R),5'-dimethylhex-5'-enyl)-phenol **86**, 3β,16β,20(R)-trihydroxydammar-24-ene **87** and its acetate derivative, 3β-acetoxy-16β,20(R)-dihydroxydammar-24-ene **88** and β-amyrin **45** were also identified (Manguro *et al.,* 2003b).

Commiphora glandulosa

Commiphora glandulosa Schinz, [Syn: *Commiphora pyracanthoides* Engl] a tree growing in the arid parts of Southern Africa, was reported as a rich source of triterpene acids, both free and as glycosides. Five free acids (commic acid A **89**, commic acid B **90**, commic acid C **91**, commic acid D **92** and

commic acid E **93**) were reported to have been isolated from the ethereal fraction of the resin (Thomas *et al.*, 1960).

The structures of commic acid C and commic acid D were elucidated as (2β,3β-dihydroxyolean-12-ene-23-oic acid) **91** and (2β,3β-dihydroxyurs-12-ene-23-oic acid) **92** respectively from *Commiphora pyracanthoides* Engl (Thomas, 1961).

Commiphora guidottii Chiov

The GC/MS analysis of the essential oil of *Commiphora guidotti*, [Syn: Sweet Myrrh, Bissabol in Hindi, Habag-hady, Hebbakhade in Somali] isolated by steam distillation of its gum resin, upon characterization revealed the presence of several sesquiterpene hydrocarbons namely car-3-ene **31**, α-santalene **94**, β-santalene **95**, epi-β-santalene **96**, β-bergamotene **97**, β-farnesene **98**, α-bisabolene **99**, β-bisabolene **62** and furanodiene **23** (Craveiro *et al.*, 1983).

The ethyl acetate extract of the resin of *Commiphora guidottii* Chiov. gave after purification on silica gel column a sesquiterpene (+)-T-cadinol **80** (Claeson *et al.*, 1991).

Two new sesquiterpenes, namely, cadinanetriol (4β,5α,10β-trihydroxycadinane) **100** and guaiane (6β,10β-dihydroxy-4(15)-guaiene) **101** and two were first time reported *i.e.* 3α-hydroxy-T-cadinol **102** and 3-oxo-T-cadinol **103** were also isolated along with two already known compounds such as (-)-oplopanone **104** and eudesme **105** (Andersson *et al.*, 1997).

Commiphora guillaumini H. Perrier

1,2-Dioleoylglycerol **106**, an ant attractant was identified by supercritical chromatography in the arils of *Commiphora guillaumini* Perr using CO_2 as carrier fluid and benzoylamidopropyl nucleosil as stationary phase (Schmeer *et al.*, 1996).

Commiphora holtziana Engl

Three known sesquiterpenes, namely, (1E)-3-methoxy-8,12-epoxygermacra-1,7,10,11-tetraen-6-one **107**, *rel*-2Rmethyl-5S-acetoxy-4R-furanogermacr-1(10)Z-en-6-one **108,** (1(10)E,2R*, 4R*)-2-methoxy-8,12-epoxygermacra-1(10),7,11-trien-6-one **109** and one novel furanogermacrene, 1,2-epoxyfurano-10(15)-germacren-6-one **110** have been identified from the ethanol extract of a resinous exudates of *Commiphora holtziana* from Kenya (Cavanagh *et al.*, 1993).

Commiphora incisa Chiov

A diethylether extract of *Commiphora incisa* Chiov when eluted through a column packed with silica, resulted in isolation of two aryltetralin lignans *i.e.* polygamain **111** and isomeric picropolygamain **100** (Provan *et al.*, 1985).

The resin of *Commiphora incisa* Chiov [Syn: *Commiphora candidula* Sprague] collected in Kenya and extracted with diethyl ether gave after purification on silica gel column two known lignans (polygamain **111** and picropolygamain **100**) and four triterpene derivatives *i.e.*, mansumbinone **101**, 3,4-*seco*-mansumbinoic acid **112**, mansumbinol **103** and 16(S),20(R)-dihydroxydammar-24-en-3-one **104** respectively (Provan *et al.*, 1986).

The diethyl ether extract of *Commiphora incisa* gave two new compounds when eluted over a column of silica gel using 5% ethylacetate in petrol, gave lα-acetoxy-9,19- cyclolanost-24-en-3ß-ol **113** and with 20% ethanol gave 29-nor-lanost-8,24-dien-lα. 2α,3β-triol **114** on precipitation with methanol (Provan *et al.*, 1988).

Commiphora merkeri Engl

A new pentacyclic triterpene, 2α,3β,23-trihydroxyolean-12-ene **115**, was isolated from the roots of *Commiphora merkeri* (Fourie *et al.*, 1989).

Commiphora molmol

The hexane extract of the essential oil of myrrh, *i.e. Commiphora molmol* Engl, on fractionation gave three new furanogermacrenes namely, 2-methoxy-4,5-dihydrofuranodiene-6-one **116**, 5-acetoxy-2-methoxy-4,5-dihydrofuranodiene-6-one **117** and 3-methoxy-10-methylenefuranoger-macra-1-ene-6-one **118** (Brieskorn *et al.*, 1980).

The non-polar fraction of hexane extract of myrrh, the resin of *Commiphora molmol* Engl, gave, the new furanoeudesmane **119** and furanoeudesma-1,3-diene **120** by column chromatography. Other compounds isolated included α-copaene **15**, bourbonene **16**, furanodiene **23**, β-elemene **14** and lindestrene **27**. From the polar fraction of the essential oil, another new furano-sesquiterpene of the eudesmane type was isolated by liquid chromatography, identified as furanoeudesma-1,4-diene-6-one **121** along with two more compounds, namely curzerenone **26** and furanodiene-6-one **122** respectively (Brieskorn *et al.*, 1983).

Structural investigations after hydrolysis and degradations of the resin revealed the presence of galactose **123**, arabinose **124**, 4-O-methyl-glucuronic acid **125**, and arabino-3,6-galactanprotein fractions and protein. In the crude gum, the two aldobiuronic acids, namely 6-O-(4-O-methyl-D-glucuronosyl)-D-galactose **126** and 4-O-(4-O-methyl-D-glucuronosyl)-D-galactose **127** were reported (Wiendl *et al.*, 1995).

Hexane extract of *Commiphora molmol* on fractionation through combination of silica gel column chromatography and semi-preparative HPLC, yielded three known sesquiterpenes namely furano-eudesma-1,3-diene **120** (>90%), isofuranogermacrene **25** and furanodiene **23** (Dolara *et al.*, 1996).

The non-polar fraction of myrrh resin from *Commiphora molmol* was extracted with hexane and was then separated by column chromatography. With a combination of mass spectrometry and H-NMR different compounds characterized included sesquiterpenes; furanodiene **23**, furaneudesma-1,3-diene, **120** methoxyfuranodiene **128**, acetoxyfuranodiene **129**, curzerenone **26**, furanodiene-6-one **122** & methoxyfuranoguaia-9-ene-8-one **130**, furanogermacrene-3 **131**, furanogermacrene-1 **132** and furanogermacrene-2 **133** (Dolara *et al.*, 2000).

Commiphora mukul (Hook ex Stocks) Engl

Commiphora mukul, also known as the Indian myrrh tree, is distributed through out the arid areas of northwest India namely Rajasthan, Gujarat, Karnataka and also found in Pakistan (Baluchistan) and Bangladesh. Guggulu (Sanskrit) is the gum resin exudates from this tree *Commiphora mukul* is an article of commerce in India. Considerable amount of work has been done on this plant.

Composition of the gum resin from *Commiphora mukul* (syn. *Balsamodendron mukul* Hook ex Stocks), commonly known as "guggul", was studied by Bose and Gupta. Complete hydrolysis of the gum resin revealed the presence of, D-galactose **123**, L-arabinose **124**, L-fucose **134**, 4-O-methyl-D-glucuronic acid **125** and aldobiouronic acid **135**. Paper chromatographic separation of the neutral fraction furnished three sugars, namely 2,4,6-tri-O-methyl-D-galactose **136**, 2,3,4-tri-O-methyl-D-galactose **137**, 2,4-di-O-methyl-D-galactose **138** and an acidic sugar *i.e.*, 2,3,4-tri-O-methyl-D-glucuronic acid **139** (in the ratio 1:6:2:3). Furthermore, hydrolysis of methylated *Commiphora mukul* gum furnished 2,3,4,6-tetra-O-methyl-D-galactose **140**, 2,3-di-O-methyl-L-arabinose **141**, 2,3,4-tri-O-methyl-D-galactose **137**, 2,4-di-O-methyl-D-galactose **138** and 2,3,4-tri-O-methyl-D-glucuronic acid **139** (in the ratio 1:1:1:2:1). It was established that the degraded gum is a branched polysaccharide (Bose *et al.*, 1964).

In one study, *Commiphora mukul* was extracted with alcohol and the extract, after removal of the solvent, was partitioned between water and ether. Two crystalline compounds were isolated from the unsaponifiable portion of the ether-soluble residue and identified as myricyl alcohol **142** (m.p. 83–4°C) and β-sitosterol **3** (m.p. 137–8°C) (Amjad *et al.*, 1967).

The monocyclic diterpenoids α-camphorene **143** and cembrene **144** were isolated from gum resin of *Commiphora mukul* Engl (Indian gum gugul) along with a diterpene alcohol, allylcembrol **145** from the overground parts (Rucker, 1972).

Chromatography of petroleum ether soluble fraction of gum resin exudates gave a diterpene hydrocarbon ($C_{20}H_{32}$) (cembrene) **144**, a diterpene alcohol ($C_{20}H_{34}O$) (allylcebrol) **145**, (+)-sesamin **146**, cholesterol **147** and two new isomeric $C_{21}H_{28}O_2$ steroids, which were identified as 4,17(20)-(trans)-pregnadiene-3,16-dione **148** (guggulsterone, Z-isomer) and 4,17(20)-(cis)-pregnadiene-3,16-dione **149** (guggulsterone, E-isomer). These two steroids were assigned trivial names, Z- and E-guggulsterone respectively. Ethyl acetate fraction gave additional three new sterols and a long-chain aliphatic triol. The three new sterols have been designated as guggulsterol-I **150**, guggulsterol-II **151** and guggulsterol-III **152** (Patil *et al.*, 1972).

From more polar ethyl acetate fraction of the extract of the same gum-resin exudates octadecan-1,2,3,4-tetrol **153**, nonadecan-1,2,3,4-tetrol **154** and eicosan-1,2,3,4-tetrol **155** were identified (Patil *et al.*, 1973).

Guggulsterol IV **156** and guggulsterol V **157** were isolated from the neutral fraction after saponification of the chloroform extract of guggul gum (Purushothaman *et al.*, 1976).

The major flavonoid components of the flowers of *Commiphora mukul* were identified as quercetin **158**, quercetin-3-O-α-L-arabinoside **159**, quercetin-3-O-β-D-galactoside **160**, quercetin-3-O-α-L-rhamnoside **161**, quercetin- 3-O-β-D-glucuronide **162**, ellagic acid **163** and pelargonidin-3,5-di-O-glucoside **164** respectively (Kakrani, 1981).

In another study, four compounds namely 20α-hydroxy-4-pregnen-3-one **165**, 20β-hydroxy-4-pregnen-3-one **166**, 16β-hydroxy-4,17(20)Z-pregnadien-3-one **167**, 16α-hydroxy-4-pregnen-3-one **168** were obtained after chromatographic fractionation and analysis (Bajaj *et al.*, 1982).

The seed oil from *Commiphora mukul* contained linoleic **169**, oleic **170**, stearic **171** and palmitic acid **12**. The unsaponifiable matter was found to contain sitosterol **3**, stigmasterol **172**, cholesterol **147**, campesterol **173**, and α-spinasterol **174** (Kakrani, 1982).

The presence of guggultetrol-20 **176** in the components of saponified resin was established by Kumar and Dev by direct comparison with synthetic compounds D-xylo (2S, 3S, 4R-configuration), *e.g.* D-xylo-octadecane-1,2,3,4-tetrol (D-xyloguggultetrol-18) **175** (Kumar *et al.*, 1987).

From the benzene fraction of guggul resin, a waxy solid was obtained which was identified as a mixture of esters of homologous long chain tetrols **177** and ferrulic acid **178** (Satyavati, 1991). Myrrhanol A **187** and Myrrhanone A **188** were isolated from aqueous methanolic extract of *Commiphora mukul* gum resin (Kimura *et al.*, 2001).

E-guggulsterone **149** and Z-guggulsterone **148**, the two stereoisomers of guggulsterone, were identified by densitometric analysis at 250 nm in a HPTLC method using alumnium plates precoated with silica gel using toluene–acetone (9:1) as a solvent system (Agarwal *et al.*, 2004).

Commiphora myrrha (Nees) Engl Var

The hexane fraction of the crude gum of *Commiphora myrrha* [Syn: *Commiphora molmol* Engl. ex Tschirch] yielded two new furanosesquiterpenoids, namely 2-O-acetyl-8,12-epoxygermacra-1(10),4,7,11-tetraene **71** and 2-O-methyl-8,12-epoxygermacra-1(10)-4,7,11-tetra-ene **189** when eluted with solvents *i.e.* petroleum ether and 3% CH_2Cl_2 in hexane, respectively (Maradafu, 1982).

The pure components of an oil mixture from the plant were separated by analytical HPLC in a 5 μm Hypersil column using isooctane or hexane as eluents. Four compounds namely furanodiene **23**, isofuranogermacrene **25**, lindestrene **27**, furanoeudesma-1,3-diene **120** were identified (Maradufu *et al.*, 1988).

The ethyl acetate extract of *Commiphora myrrha* was subjected to column chromatography over silica gel when eluted with chloroform-methanol mixture with increasing methanol content, yielded furanogermacra-1*E*,10(15)-dien-6-one **190**, 2-methoxyfuranogermacra-1(10),4-diene **191**, T-cadinol **80**, 3α-eudesm-4(15)-ene-1β,6a-diol **192** and some new compounds (Zhu *et al.*, 2003).

Commiphora opobalsamum (L.) Engl

A new triterpenoid, cycloartane-24-en-1a,2a,3b-triol **193**, an aliphatic alcohol glycoside, octadecane-1,2S,3S,4R-tetrol-1-O-a-L-rhamnopyranoside **194**, and two new sesquiterpenoids, eudesmane-1b,5a,11-triol **195** and guaia-6a,7a-epoxy-4a,10a-diol **196**, along with six known compounds, namely guaianediol **197**, myrrhone **198**, dihydropyrocurzerenone **199**, 2-methoxy-5-acetoxyfuranogermacr-1(10)-en-6-one **200**, 1(10)E,2R,4R)-2-methoxy-8,12-epoxygermacra-1(10),7,11-trien-6-one **109** and curzerenone **26** were obtained from petroleum ether extract of *Commiphora opobalsamum* on column chromatography over silica gel and sephadex LH-20 columns (Shen *et al.*, 2007).

Commiphora pubescens

From hexane extracts of *Balsamodendron pubescens* roots, β-sitosterol **3** and cedrelone **201**, (m.p. 203–4°C) and 4,7-dimethoxy-5-methylcoumarin (siderin) **202** were isolated (Balawant *et al.*, 1979).

Commiphora rostrata

In *Commiphora rostrata*, 22 oxygenated alkane compounds had been identified by GC and mass spectrometry. Some identified members were:

2-octanone **203**, 2-nonanone **204**, 2-decanone **205**, 2-undecanone **206**, 2-decanol **207**, 2-dodecanone **208**, 2-undecanol **209**, 2-tridecanone **210**, tridecanal **211**, 2-dodecanol **212**, 2-tetradecanone **213**, tetradecanal **214**, 2,2-dimethylnonanol **215**, 2-pentadecanone **216**, 2,2-dimethyldecanol **217**, 2,2-dimethylundecanol **218**, 2,2-dimethyldodecanol **219** and two unknown compounds (McDowell, 1988).

Commiphora sphaerocarpa

The pulverized petrol extracted resin was subjected to column chromatography over silica gel with increasing polarities using n-hexane and ethyl acetate. It yielded two compounds which were purified by preparative TLC and recrystallized from n-hexane - $CHCl_3$ (95:5). They were finally analyzed by X-ray crystallography and identified as 2-methoxy-8,12-epoxygermacra-1(10),7,11-trien-6-one **220** and furanodienone **24** (Dekebo *et al.,* 2000).

Column chromatography of an extract of the plant on chromatographic separation yielded a novel terpene, which showed the presence of a trisubstituted furan ring in ^{1}H-NMR and was characterized as (1E)-8,12-epoxygermacra-1,7,10,11-tetraen-6-one **221** (Dekebo *et al.,* 2002b).

Commiphora tenuis

After steam distillation, the oil obtained from the exudates of *Commiphora tenuis* was analyzed by GC/GC-MS and the main triterpenes identified were 3β-O-acetoxyolean-12-en-28-oic acid **222** and (1(10)E,4E)-8,12-epoxygermacra-1(10),4,7,11-tetraen-6-one **223** (Asres *et al.,* 1998).

Commiphora terebinthina Vollesen

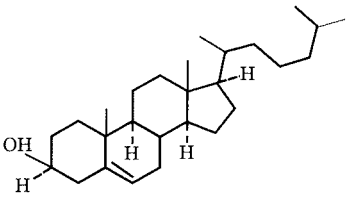
The resin from this plant consisted of monoterpene hydrocarbons with limonene **5** as the major component (Abegaz *et al.,* 1989).

Commiphora wightii (Arnott.) Bhanol

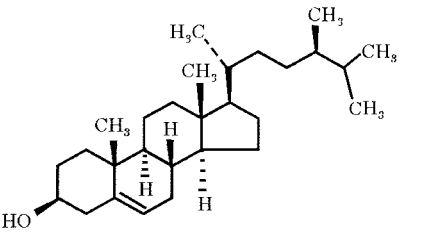
An ethyl acetate extract of *Commiphora wightii* was subjected to column chromatography with a solvent mixture of chloroform-methanol with increasing methanol content yielded eight fractions and with acetone-hexane mixture yielded nine fractions. The obtained ferulates on hydrolysis gave alcohols which were identified to be a mixture of (Z)-5-tricosene-1,2,3,4-tetraol **224** and (Z)-5-tetracosene- 1,2,3,4- tetraol **225** (Zhu *et al.,* 2001).

In another study, guggulsterone M **227**, dehydroguggulsterone M **228** and guggulsterol Y **229** were isolated from the methanolic extract of the plant by column chromatography (Meselhy, 2003).

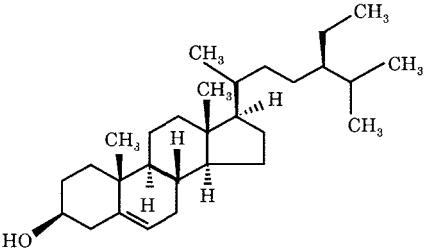
The ethanolic extracts of the plant when chromatographed on silica gel using hexane-ethyl acetate mixtures of increasing polarity, gave 3-O-(1",8",14"- trimethylhexadecanyl) naringenin **226**. Ethanolic extract upon separation with silica get column gave a new flavone named muscanone **230** along with naringenin **226** (Fatope *et al.*, 2003).



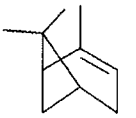
Cholest-5-en-3β-ol **(1)**



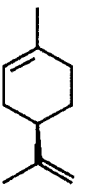
Δ5-campestan-3β-ol **(2)**



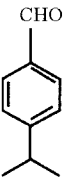
β-sitosterol **(3)**



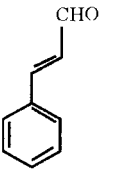
α-pinene **(4)**



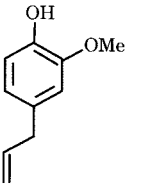
Limonene **(5)**



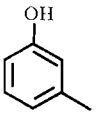
Cuminaldehyde **(6)**



Cinnamaldehyde **(7)**



Eugenol **(8)**



m-Cresol **(9)**

HCOOH

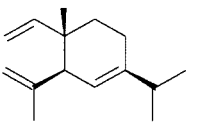
Formic acid **(10)**

CH_3COOH

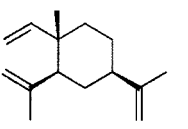
Acetic acid **(11)**

$CH_3(CH_2)_{14}COOH$

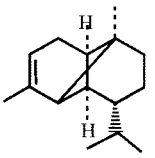
Palmitic acid **(12)**



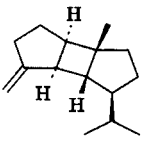
δ-elemene **(13)**



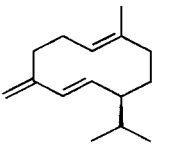
β-elemene **(14)**



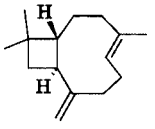
α-Copaene **(15)**



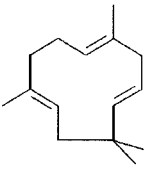
β-bourbonene **(16)**



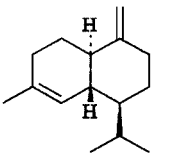
Germacrene D **(17)**



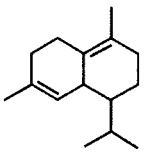
Caryophyllene **(18)**



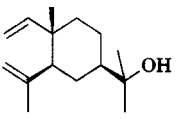
Humulene **(19)**



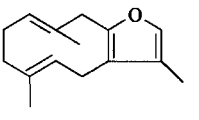
γ-Cadinene **(20)**



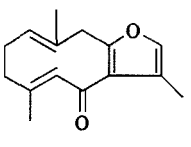
δ-Cadinene **(21)**



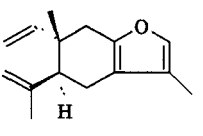
Elemol **(22)**



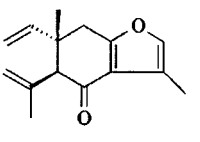
Furanodiene **(23)**



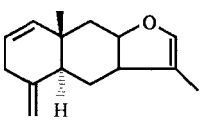
Furanodienone **(24)**



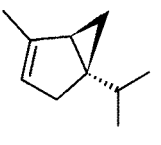
Isofuranogermacrene **(25)**



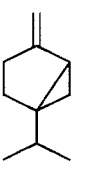
Curzernone **(26)**



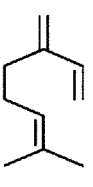
Lindestrene **(27)**



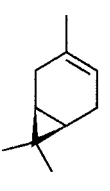
α-Thujene **(28)**



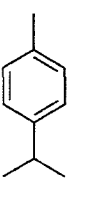
Sabinene **(29)**



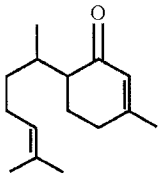
Myrcene **(30)**



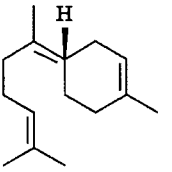
Car-3-ene **(31)**



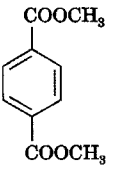
p-Cymene **(32)**



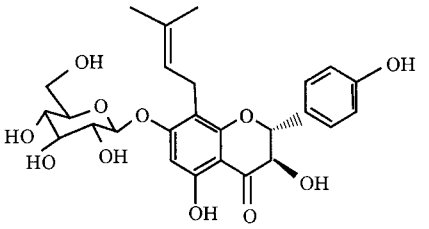
α-Oxobisabolene **(33)**



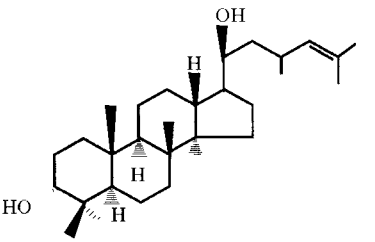
γ-Bisabolene **(34)**



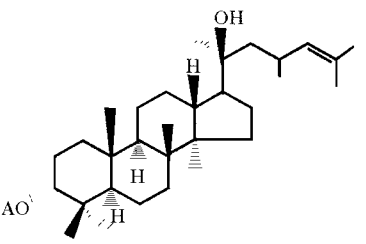
Dimethylterphthlene **(35)**



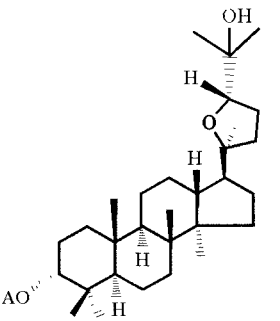
Phellamurin **(36)**



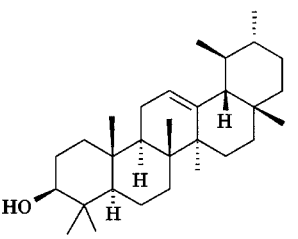
(3R, 20S)-3,20-dihydroxydammar-24-ene **(37)**



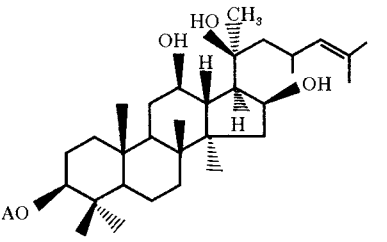
(3R,20S)-3-acetoxy-20-hydroxydammar-24-ene **(38)**



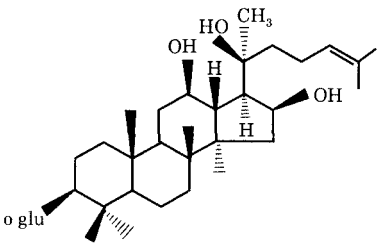
Carbraleadiol-3-acetate **(39)**



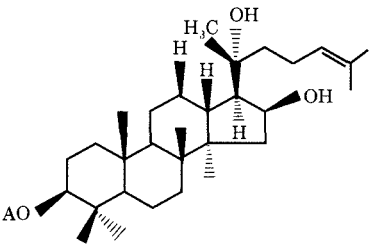
α-Amyrin **(40)**



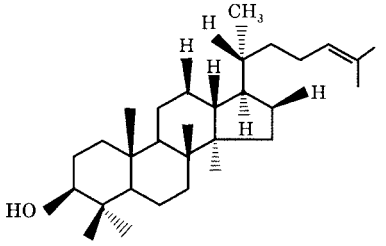
(20S)-3β-acetoxy-12β,16β-trihydroxydammar-24-ene **(41)**



(20S) 12β, 16β-trihydroxydammar-24-ene-3β-O-β-glucopyranoside **(42)**

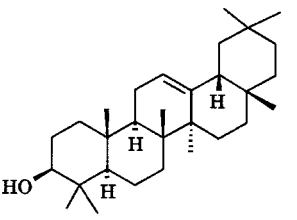


(20S)-3β,12β,16β,25-tetrahydroxydammar-23-ene **(43)**

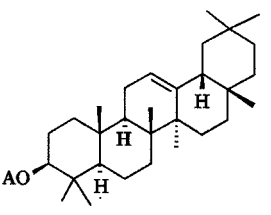


(20S)-3β,12β,16β,25-pentahydroxydammar-23-ene

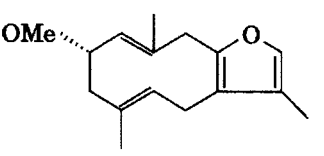
(44)



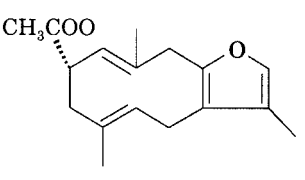
β-amyrin **(45)**



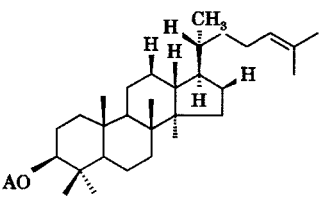
3-β-amyrinacetate **(46)**



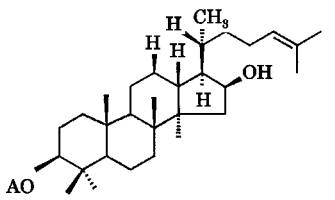
2-methoxyfuranodienone **(47)**



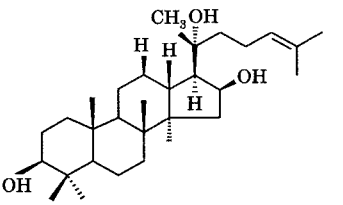
2-acetoxyfuranodienone **(48)**



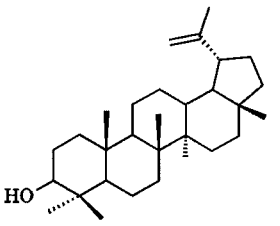
(20R)-3β-acetoxy-16β-dihydroxydammar-24-ene **(49)**



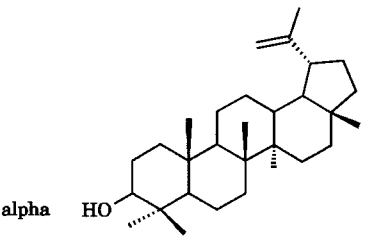
3β-hydroxydammar-24-ene (**50**)



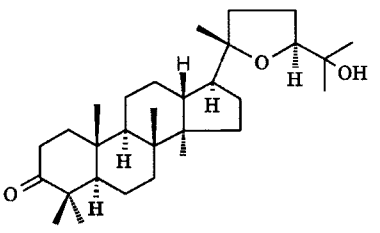
3β-acetoxydammar-24-ene (**51**)



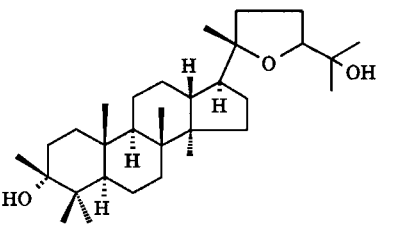
Lupeol (**52**)



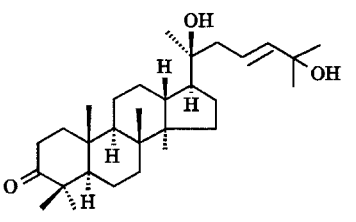
Epilupeol (**53**)



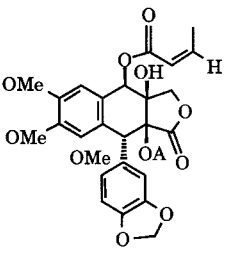
Carbraleone (**54**)



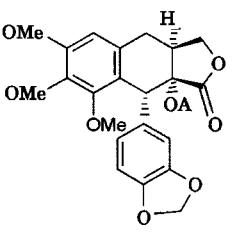
Carbraleadiol (**55**)



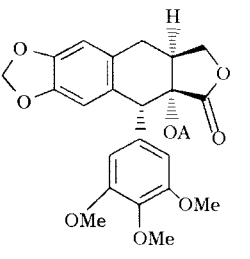
Isofouquierone (**56**)



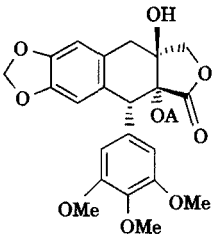
Erlangerins A (**57**)



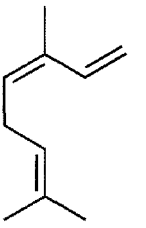
Erlangerins B (**58**)



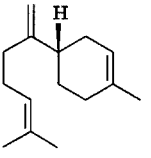
Erlangerins C (**59**)



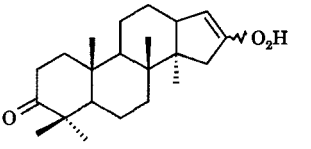
Erlangerins D **(60)**



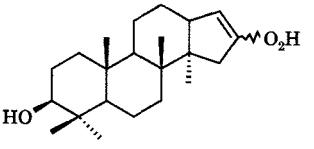
Ocimine **(61)**



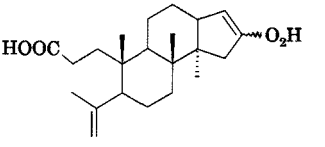
β-bisabolene **(62)**



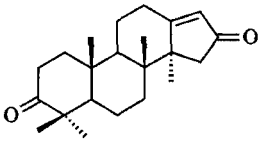
16-hydroperoxy-mansumbin-13(17)-en-3-one **(63)**



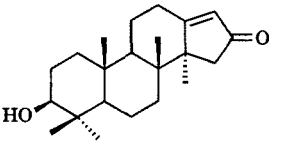
16-hydroperoxymansumbin-13(17)-en-3β-ol **(64)**



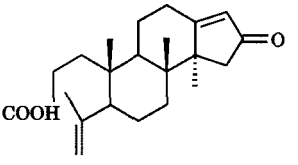
16-hydroperoxy-3,4-seco-mansumbin-3(28),13(17)-dien-3-oic acid **(65)**



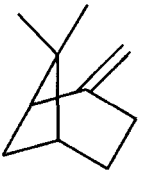
Mansumbin-13(17)-en-3,16-dione **(66)**



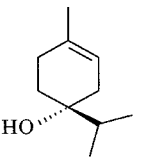
3β-hydroxy-mansumbin-13(17)-en-16-one **(67)**



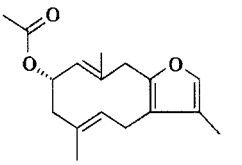
16-oxo-mansumbin-3(28),13(17)-dien-3-oic acid **(68)**



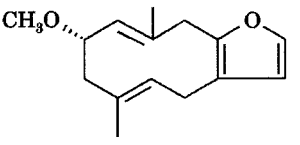
β-piene **(69)**



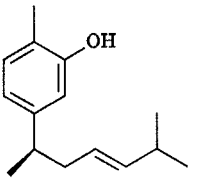
Terpinene-4-ol (**70**)



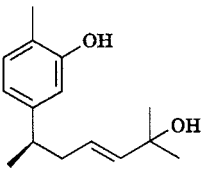
2-O-acetyl-8,12-epoxygermacra-1(10),4,7,11-tetraene (**71**)



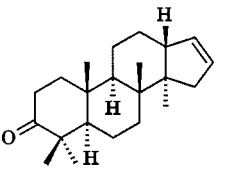
2-O-methyl-8,12-epoxygermacra-1(10), 4,7,11- tetraene (**72**)



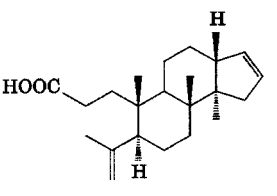
Xanthorrhizol (**73**)



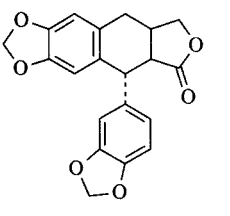
2-methyl-5-(5'-hydroxy-1', 5'-dimethyl-3'-hyxenyl) phenol (**74**)



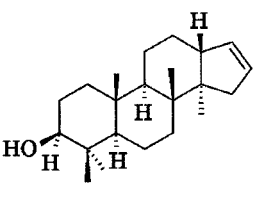
Mansumbinone (**75**)



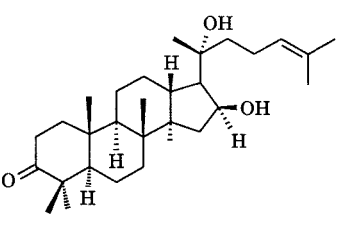
Mansumbinoic acid (**76**)



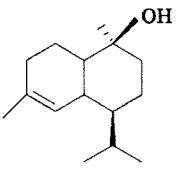
Picropolygamain (**77**)



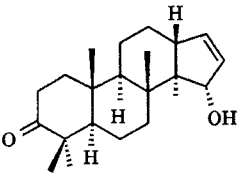
Mansumbinol (**78**)



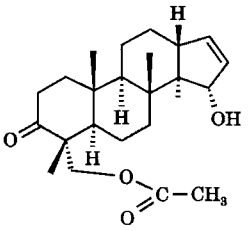
(16S,20R)-dihydroxydammar-24-en-3-one (**79**)



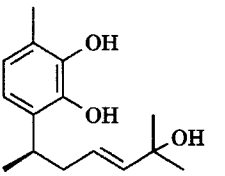
T-cadinol (**80**)



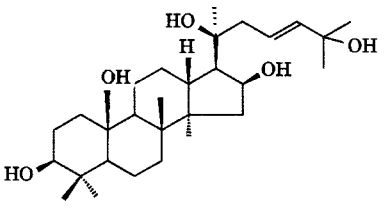
15α-hydroxymansumbinone (**81**)



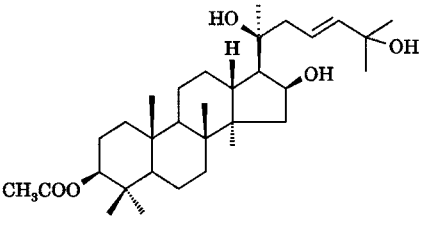
28-acetoxy-15α-hydroxymansumbinone (**82**)



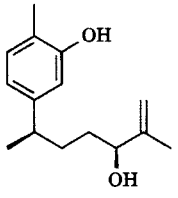
6-hydroxy-2-methyl-5-(5'-hydroxy-1'(R),5'-dimethylhex-3'enyl)-phenol (**83**)



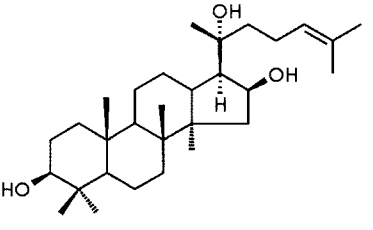
3β-16β,20(S),25-tetrahydroxydammar-23-ene (**84**)



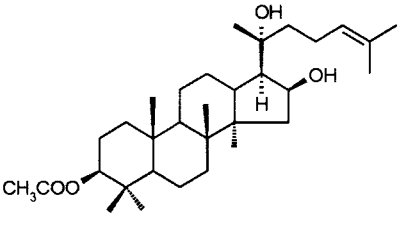
3β-acetoxy-16β,20(S),25-tetrahydroxydammar-23-ene (**85**)



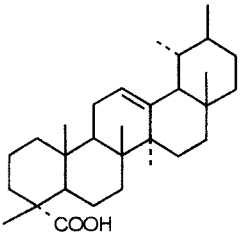
2-methyl-5-(4'(S)-hydroxy 1'(R),5'-dimethylhex-5'enyl)-phenol (**86**)



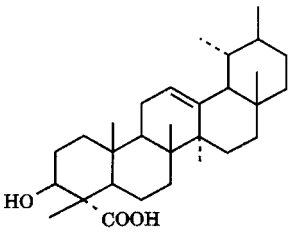
3β-16β,20(R)-trihydroxydammar-24-ene (**87**)



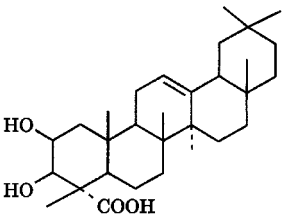
3β-acetoxy-16β,20(R)-dihydroxydammar-24-ene (**88**)



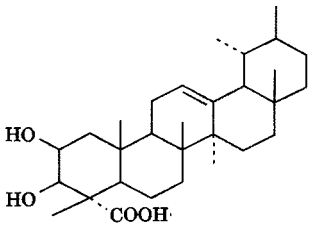
Comic acid A (**89**)



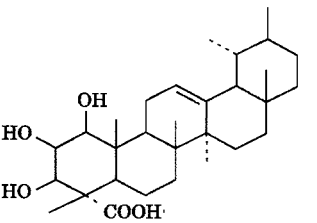
Comic acid B (**90**)



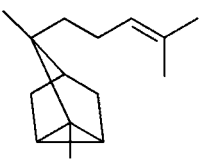
Comic acid C (**91**)



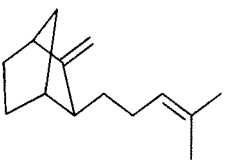
Comic acid D (**92**)



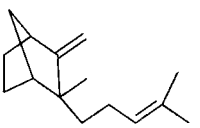
Comic acid E (**93**)



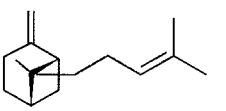
α-Santalene (**94**)



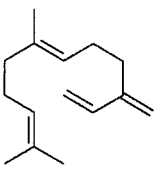
β-Santalene (**95**)



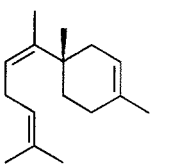
Epi-β-Santalene (**96**)



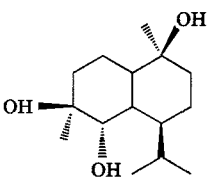
β-bergamotene (**97**)



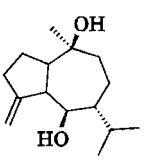
β-farnesene (**98**)



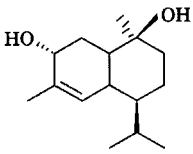
α-bisabolene (**99**)



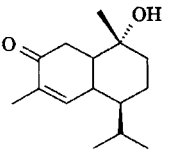
Cadinanetrio (4β,5α,10β-trihydroxycadinane) (**100**)



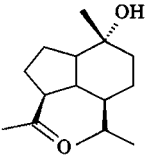
Guaiane (**101**)



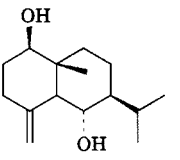
3α-Hydroxy-T-cadinol **(102)**



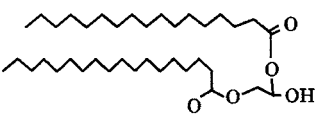
3-Oxo-T-cadinol **(103)**



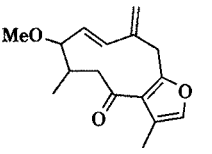
(–)-Oplopanone **(104)**



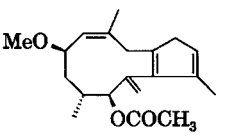
Eudesme **(105)**



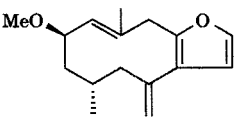
1,2-dioleoylglycerol **(106)**



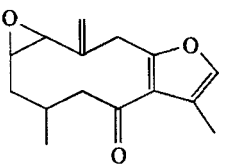
(1E)-3-methoxy-8,12-epoxygermacra-1,7,10,11-tetraen-6-one **(107)**



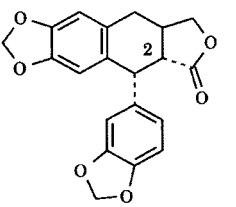
rel-2Rmethyl-5S-acetoxy-4R-furanogermacr-1(10)Z-en-6-one **(108)**



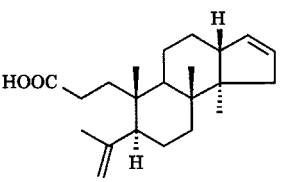
(1(10)E,2R*,4R*)-2-methoxy-8,12-epoxygermacra-1(10),7,11-trien-6-one **(109)**



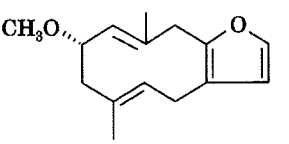
1,2-epoxyfurano-10(15)-germacren-6-one **(110)**



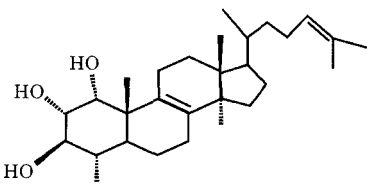
Polygamain **(111)**



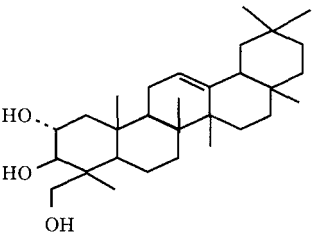
3,4-*seco*-Manumbinoic acid **(112)**



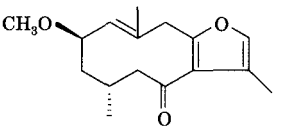
1α-acetoxy-9,19-cycolanost-24-en-3β-ol **(113)**



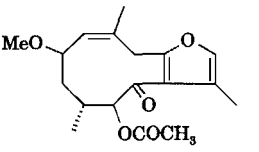
29-nor-lanost-8,24-dien 1α,2α,3β-triol **(114)**



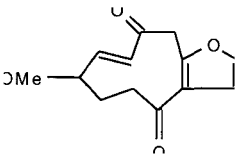
2α,3β,23-trihydroxyolean-12-ene **(115)**



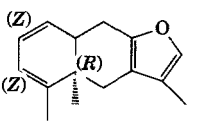
2-methoxy-4-,5-dihydrofurnodiene-6-one **(116)**



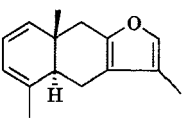
5-acetoxy-2-methoxy-4-5, dihydrofuranodiene-6-one **(117)**



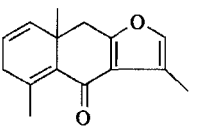
3-Methoxy-10-methylenefuran-ogermacra-1-ene-6-one **(118)**



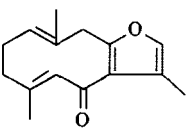
Furanoeudesmane **(119)**



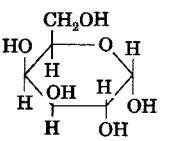
Furanoeudesma-1,3-diene **(120)**



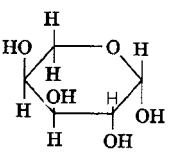
Furanoeudesma-1,4-diene-6-one **(121)**



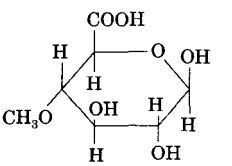
Furanodiene-6-one **(122)**



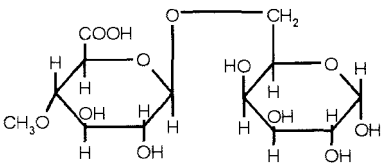
D-Galactose **(123)**



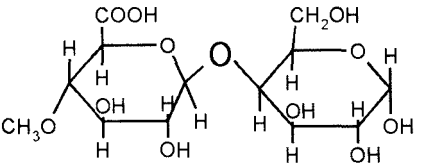
L-arabinose **(124)**



4-O-methyl-glucuronic acid **(125)**

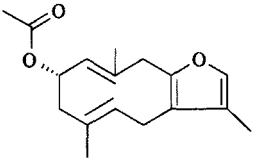


6-O-(4-O-methyl-D-glucuronosyl)-D-galactose **(126)**

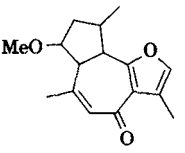


4-O-(4-O-methyl-D-glucuronosyl)-D galactose **(127)**

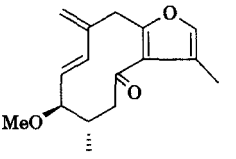
Methoxyfuranodiene **(128)**



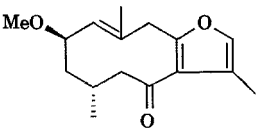
Acetoxyfuranodiene **(129)**



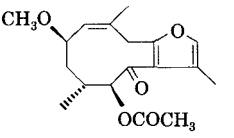
Methoxyfuranoguaia-9-ene-8-one **(130)**



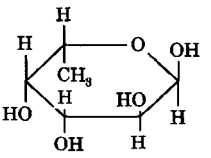
Furanogermacrene-3 **(131)**



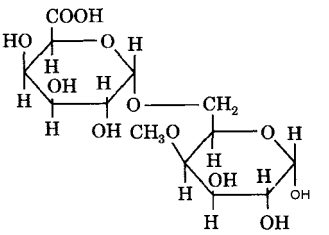
Furanogermacrene-1 **(132)**



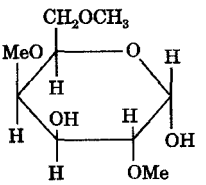
Furanogermacrene-2 **(133)**



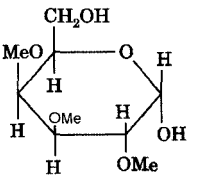
L-fucose **(134)**



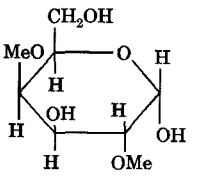
Aldobiouronic acid **(135)**



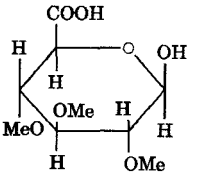
2,4,6-tri-O-methyl-D-galactose **(136)**



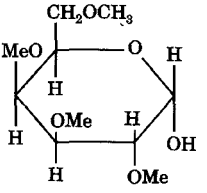
2,3,4-tri-O-methyl-D-galactose **(137)**



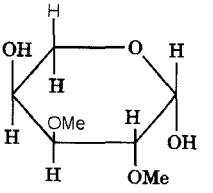
2,4-di-O-methyl-D-galactose (**138**)



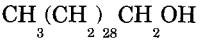
2,3,4-tri-O-methyl-D-glucuronic acid (**139**)



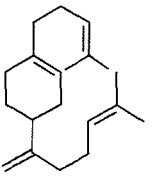
2,3,4,6-tetra-O-methyl-D-galactose (**140**)



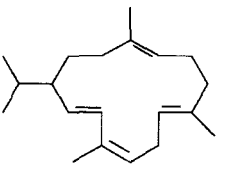
2,3-di-O-methyl-L-arabinose (**141**)



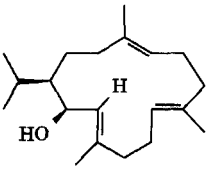
Myricyl alcohol (**142**)



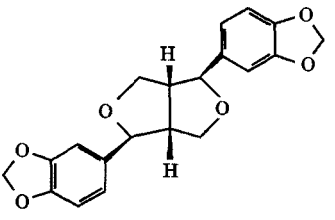
α-camphorene (**143**)



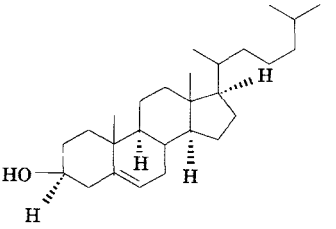
Cembrene (**144**)



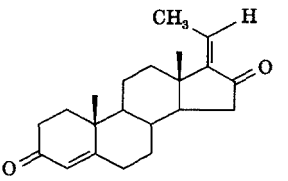
Allylcembrol (**145**)



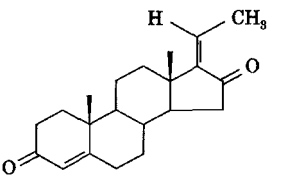
(+)-sesamin (**146**)



Cholesterol (**147**)

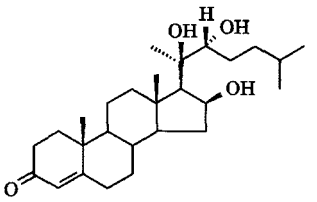


4,17(20)-(*trans*)-pregnadiene-3, 16-dione (Z-Guggulsterone) **(148)**

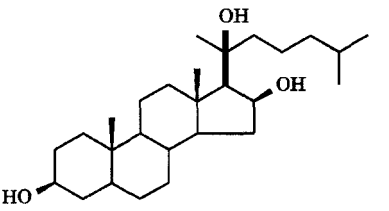


4,17-(20)-(*cis*)-pregnadiene-3,16-dione (E-Guggulsterone)

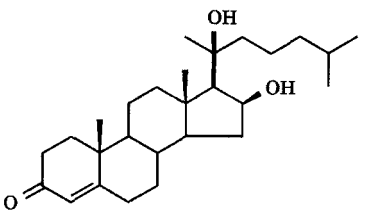
(149)



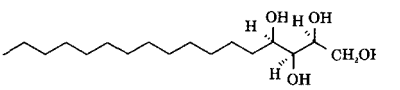
Guggulsterol-1 **(150)**



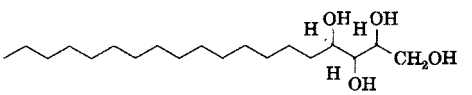
Guggulsterol-II **(151)**



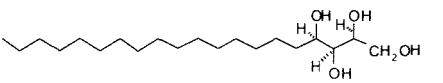
Guggulsterol-III **(152)**



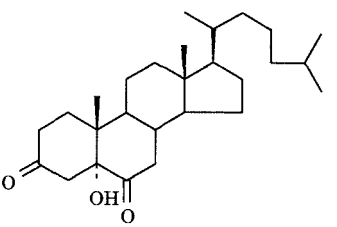
Octadecan-1,2,3,4-tetrol **(153)**



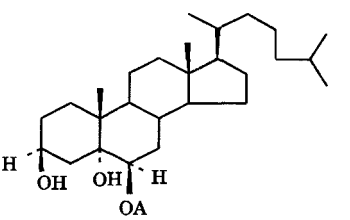
Nonadecan-1,2,3,4-tetrol **(154)**



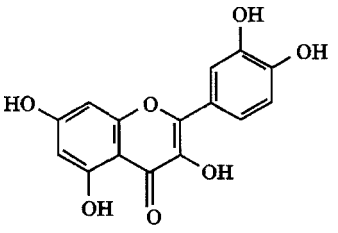
Eicosan-1,2,3,4-tetrol **(155)**



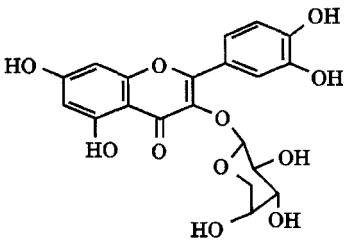
Guggulsterol-IV **(156)**



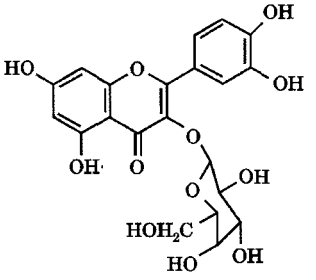
Guggulsterol-V **(157)**



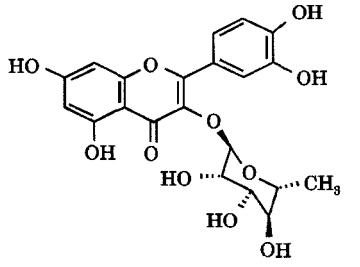
Quercetin (**158**)



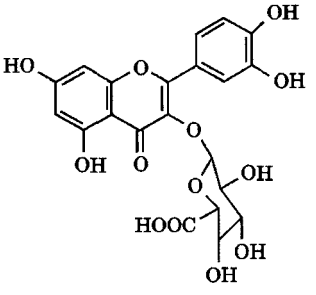
Quercetin-3-O-α-L-arabinoside (**159**)



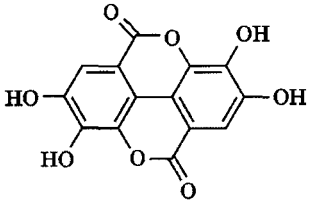
Quercetin-3-O-β-D-galactoside (**160**)



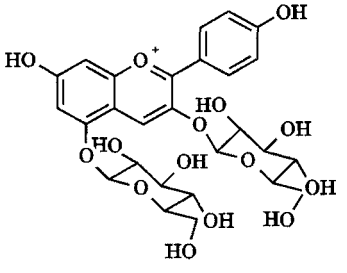
Quercetin-3-O-α-Lrhamnoside (**161**)



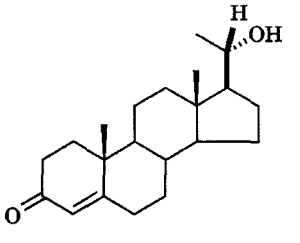
Quercetin-3-O-β-D-glucuronide (**162**)



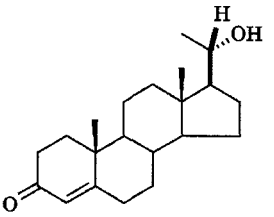
Ellagic acid (**163**)



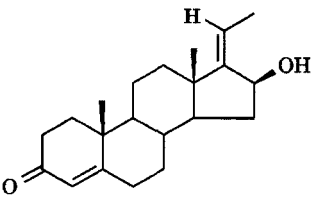
Pelargonidin-3,5-di-O-glucoside (**164**)



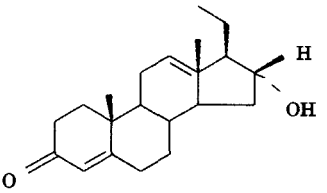
20α-hydroxy-4-pregnen-3-one(**165**)



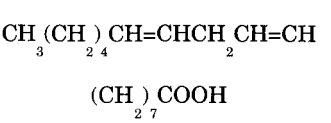
20β-hydroxy-4-pregnen-3-one **(166)**



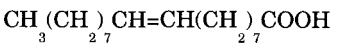
16β-hydroxy-4,17(20)Z-pregnadien-3-one **(167)**



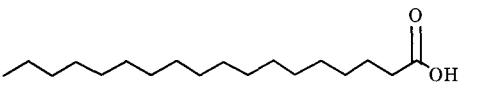
16α-hydroxy-4-pregnen-3-one **(168)**



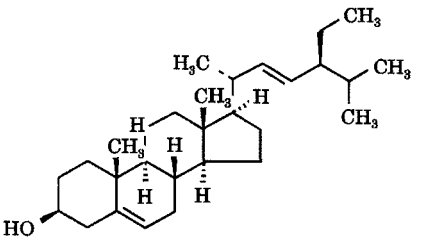
Linoleic acid **(169)**



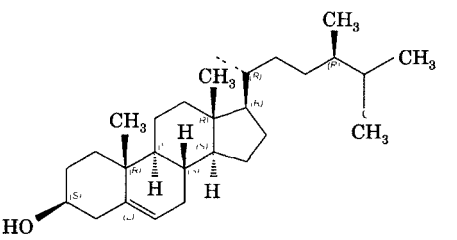
Oleic acid **(170)**



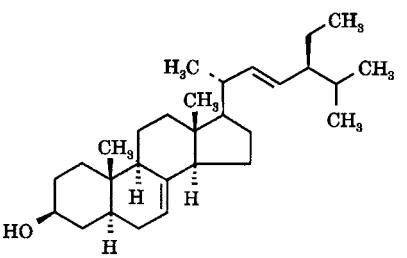
Stearic acid **(171)**



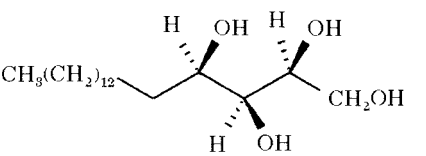
Stigmasterol **(172)**



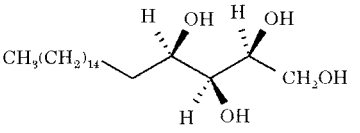
Campesterol **(173)**



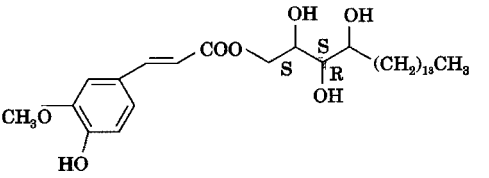
α-Spinasterol **(174)**



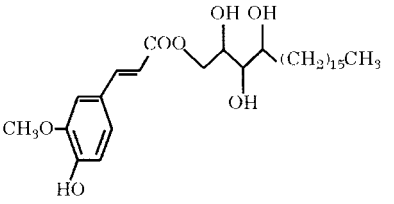
D-*xyol*-octadecane-1,2,3,4-tetrol **(175)**



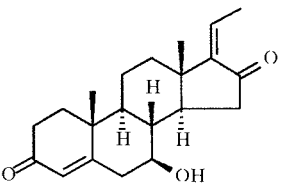
Guggulterol-20 **(176)**



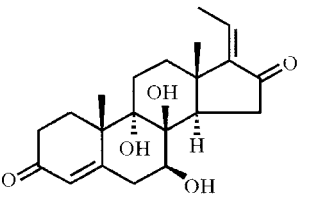
Long chain tetrols **(177)**



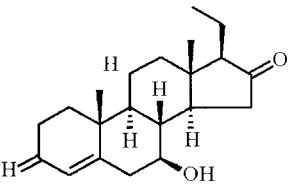
Ferrulic acid **(178)**



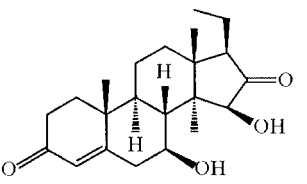
7β-hydroxypregna-4,17(20)-trans-diene-3,16-dione **(179)**



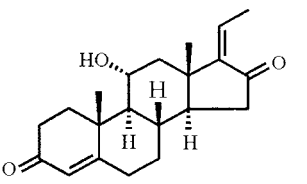
7β-hydroxypregna-4,17(20)-cis-diene-16-dione **(180)**



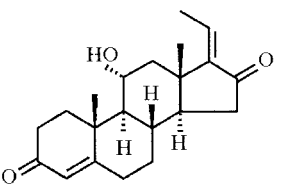
7β-hydroxypregn-4-ene-3,16-dione **(181)**



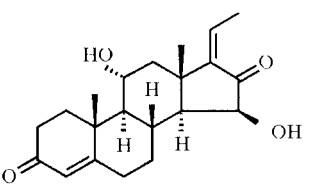
7β-15β-dihydroxypregn-4-ene-3,16-dione **(182)**



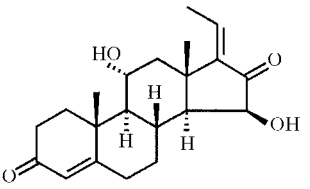
11α-hydroxypregna-4,17(20)-*trans*-diene-3,16-dione **(183)**



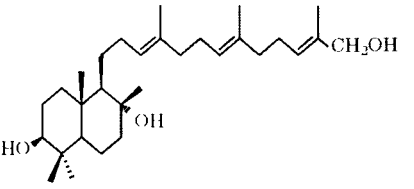
11α-hydroxypregna-4-17(20)-*cis*-diene-3,16-dione **(184)**



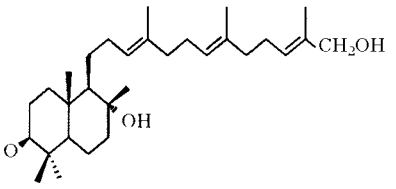
11α,15β-dihydroxpregna-4,17(20)-*trans*-diene-3-16-dione **(185)**



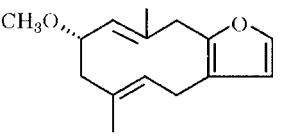
11α,15β-dihydroxypregna-4,17(20)-*cis*-diene-3,16-dione **(186)**



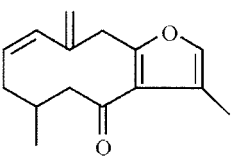
Myrrhanol A **(187)**



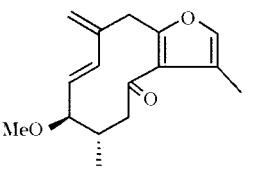
Myrrhanone A **(188)**



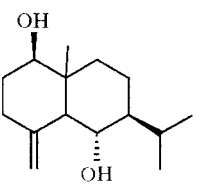
2-O-methyl-8,12-epoxygermacra-1(10),4,7,11-tetraene **(189)**



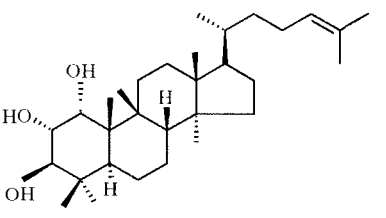
Furanogermacra-1*E*,10(15)-dien-6-one **(190)**



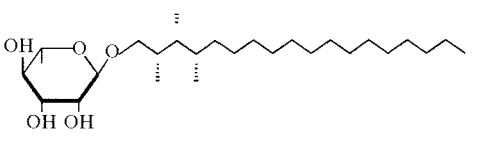
2-methoxyfuranogermacra-1(10),4-diene **(191)**



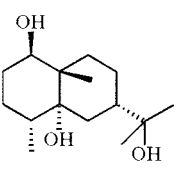
3α-eudesm-4(15)-ene-1β,6a diol **(192)**



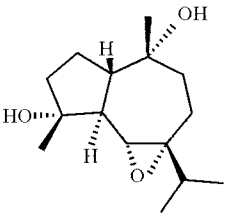
Cycloartane-24-en-1a,2a,3b-triol **(193)**



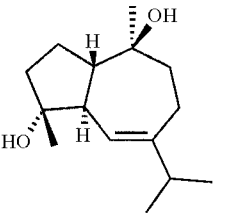
Octadecane-1,2S,3S,4R-tetrol 1-O-a-L-rhamnopyranoside **(194)**



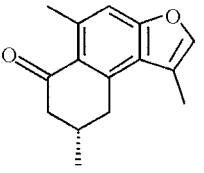
Eudesmane-1b-5a,11-triol **(195)**



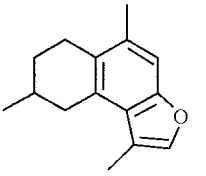
Guaia-6a,7a-epoxy-4a,10a-diol (**196**)



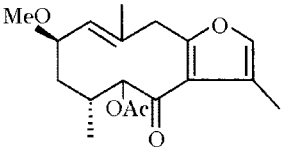
Guaianediol (**197**)



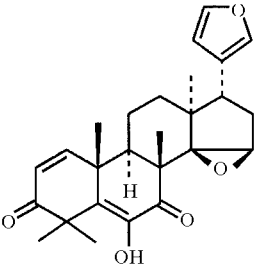
Myrrhone (**198**)



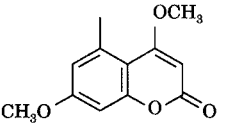
Dihydropyroxurzerenone (**199**)



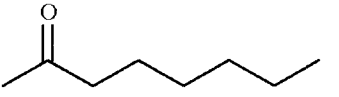
2-methoxy-5-acetoxyfuranogermacr-1(10)-en-6-one (**200**)



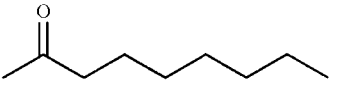
Cedrelone (**201**)



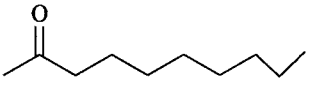
Siderin (**202**)



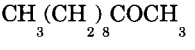
2-octanone (**203**)



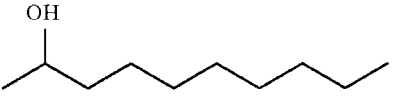
2-nonanone (**204**)



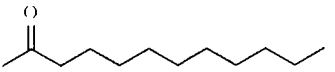
2-decanone (**205**)



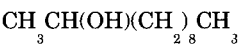
2-undecanone (**206**)



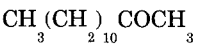
2-decanol (**207**)



2-dodecanone **(208)**

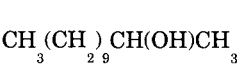


2-undecanol **(209)**

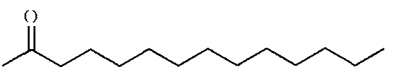


2-tridecanone **(210)**

Tridecanal **(211)**

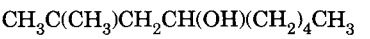


2-dodecanol **(212)**

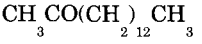


2-Tetradecanone **(213)**

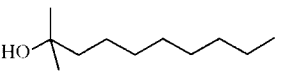
Tetradecanal **(214)**



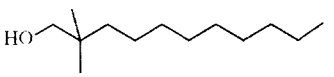
2,2-Dimethylnonanol **(215)**



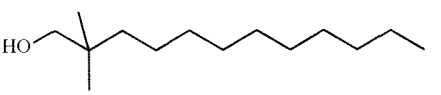
2-Pentadecanone **(216)**



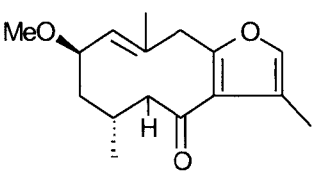
2,2-Dimethyldecanol **(217)**



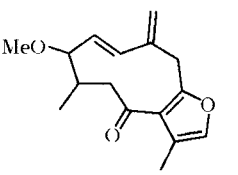
2,2-dimethylundecand **(218)**



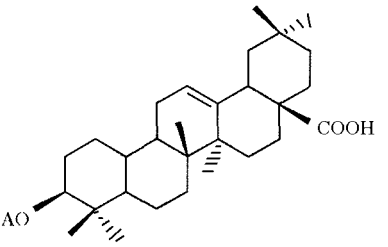
2,2-Dimethyldodecanol **(219)**



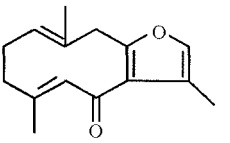
2-methyoxy-8,12,epoxygermacra-1(10),7,11-trien-6-one **(220)**



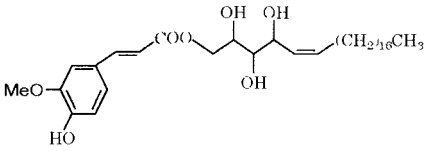
(1E)-3-methoxy-8,12-epoxygermacra-1,7,10,11-tetraen-6-one **(221)**



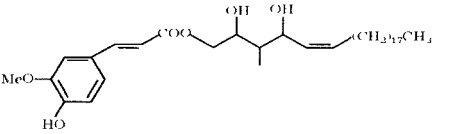
3β-O-acetoxyolean-12-en-28-oic acid **(222)**



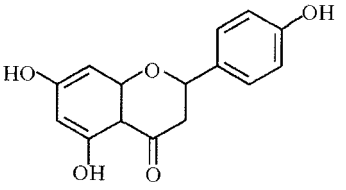
(1(10)E,4E)-8-12-epoxygermacra-1(10),4,7,11-tetraen-6-one **(223)**



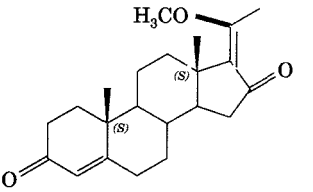
Z-5-tricosene-1,2,3,4-tetraol **(224)**



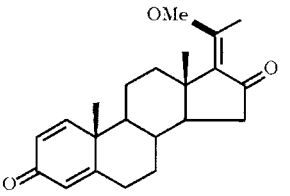
Z-5-tetracosene-1,2,3,4-tetraol **(225)**



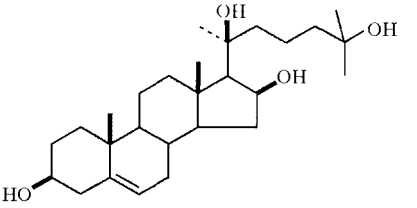
3-O-(1",8",14"-Trimethylhaxadecanyl) Naringenin **(226)**



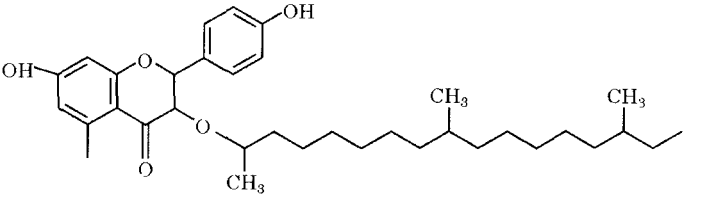
Guggulsterone-M **(227)**



Didehydroguggulsterone-M **(228)**



Guggulsterol Y **(229)**



Muscanone **(230)**

CONCLUSIONS

In this review article, research works involving the crude extracts of 24 different plants of Commiphora genus and the phytoconstituents isolated from them have been reported. A large variety of reported secondary metabolites of such plants, their isolation procedures in short, characterization techniques etc. have been included in this article. Furthermore, names and structures of such varied plant products which include aliphatic alcohols, free acids, monoterpene hydrocarbons, diterpene hydrocarbons, diterpene alcohols, monocyclic diterpenoids, various sesquiterpenes like sesquiterpene hydrocarbons, sesquiterpene alcohols, furanosesquiterpenoids, various triterpenes like pentacyclic triterpenes, dammarane triterpenes, other compounds like lignans, steroids, flavones, flavonoids, carbohydrates etc. have been appended at the end. Although, many of such isolated derivatives are biologically active molecules, their pharmacological and other biological activity aspects have not been considered here to keep the extent of the article with in limit.

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Abegaz, B., Dagne, E., Bates, C. and Waterman, P.G. (1989). Chemistry of the Burseraceae. Part 12. Monoterpene-rich resins from two Ethiopian species of *Commiphora*. *Flavour and Fragrance Journal,* **4**: 99-101.

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13

Secondary Metabolites and Biological Activities of some Gentianaceae species from Serbia and Montenegro

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ABSTRACT

Some plant species belonging to the genera Gentiana, Gentianella, Centaurium and Swertia have been studied for xanthone, flavonoid and secoiridoid compounds. Different analytical techniques such as TLC, HPLC, UV-VIS, MS and NMR have been used. Two new xanthone compounds have been isolated from the roots of Swertia punctata while one new xanthone was isolated from rhizomes and roots of Gentiana dinarica. Xanthone-C-glucoside lancerin isolated from the aerial parts of Gentiana utriculosa was detected in Gentiana species for the first time. Biological activity has been investigated for some plant extracts as well as for isolated compounds. Extract of Gentiana lutea flowers and isolated xanthone isogentisin showed antimicrobial activity against Myccobacterium bovis while extracts of Gentianella austriaca and isolated polyphenolic compounds possessed radioprotective and antiradical activity. Gentiakochianin and gentiacaulein isolated from the aerial parts of Gentiana kochiana exhibited in vitro antiglioma activity in both C6 rat glioma and U251 human glioma cell lines. The extract of the aerial parts of Gentiana kochiana and gentiacaulein strongly inhibited rat microsomal MAO A. Behavioural examinations on mice showed that administration of the extract significantly decreased immobility score

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in a forced swimming test and strongly inhibited ambulation and stereotypy in an open-field test. The fact that G. lutea is endangered species prompted us to investigated possibility for its cultivation in mountains regions of Serbia. Chemical evaluation of two, three and four years old plants were performed. Xanthone compounds and secoiridoids were also detected in in vitro cultured plants and hairy roots.

Key words : *Gentiana, Gentianella, Swertia, Centaurium*, xanthones, biological activities, *in vitro* propagation, chemotaxonomy

INTRODUCTION

Plants that belong to the family Gentianaceae are cosmopolitan in distribution and comprises about 1600 species classified into 87 genera (Albert & Struwe, 2002). The largest genera are *Gentiana* (360 species), *Gentianella* (250 species) and Swertia (135 species), while genus *Centaurium* contain 50 species.

In Serbia and Montenegro, 11 species of the genus *Gentiana* (*G. lutea, G. punctata, G. cruciata, G. asclepiadea, G. pneumonanthe, G. kochiana, G. dinarica, G. verna, G. tergestina, G. utriculosa, G. nivalis*), 6 *Gentianella* species (*G. austiraca, G. bulgarica, G. crispata, G. praecox, G. axillaris, G. ciliata*) and 4 species of the genus *Centaurium* (*C. erythraea, C. uliginosum, C. pulchellum, C. tenuiflorum*) were found (Jovanovic-Dunjic, 1977). *Swertia punctata* is the only species of the genus *Swertia* occurring in Serbia, while *S. perennis* was found in Montenegro.

Gentian root has a long history of use as a herbal bitter in the treatment of digestive disorders. The essential active principles are the bitter tasting secoiridoid glucosides contained in the herb. These bring about a reflex stimulation of the taste receptors, leading to increased secretion of saliva and the digestive juices. Gentian root is therefore considered to be not simply a pure bitter, but also as a restorative and tonic (LaGow, 2005). Approved indications by Commission E are dyspeptic complains, loss of appetite and flatulence (Blumenthal, 1998). Traditionally, it is taken internally in the treatment of liver complaints, indigestion, gastric infections and anorexia (Tasic *et al.*, 2004). Some studies showed that the root possesses cholagogue, anthelmintic, anti-inflammatory and antimicrobial activity (Öztürk *et al.*, 1998; Pontus *et al.*, 2006). Also, the roots are used in the industry of beverages, for liqueurs and as a base for various bitters. In the most of European Pharmacopoeias, beside the *Gentiana lutea,* the roots of *Gentiana pannonica*, *Gentiana punctata* and *Gentiana purpurea* are enclosed and therefore considered as officinal plants too.

Gentianella species have been used in a traditional medicine of South America as substitutes of *Gentiana* preparations for the treatment of digestive and liver problems (Lacaille-Dubois, 1996; Nadinic *et al.*, 1999).

Similar to *Gentiana* species, Centaury increases gastric secretion and salivation because of the typical bitter reaction. Antiphlogistic and antipyretic effects have been studied in various animal experiments. In folk medicine it is used for fever, diabetes, worm infestation, and as a hypotensive. It is also used externally in the treatment of wounds (LaGow, 2005; Tasic *et al.,* 2004).

About 20 species of the genus *Swertia* have been used in Chinese traditional medicine for the treatment of hepatic, choleretic and inflammatory diseases. In India, *Swertia chirata* Buch-Ham, commonly known as chirayata is *oficinal* in Indian pharmacopoeia. The extract is used as a bitter stomachic, febrifuge, anthelmintic, antimalarial and antidiarrhoeal. *S. purpurascens* is used in Pakistan as a substitute of *S. chirata* while *S. japonica* Makino is an important bitter stomachic in Japan (Pant *et al.,* 2000). *Swertia* species are not used in traditional medicine of Serbia.

It is well known that *Gentiana*, *Gentianella*, *Swertia* and *Centaurium* species synthesize secoiridoids, xanthones and flavone-*C*-glucosides as the main secondary metabolites (Hostettmann-Kaldas *et al.,* 1981). Secoiridoid compounds swertiamarin, gentiopicrin and sweroside, together with their derivatives, are the most characteristic iridoids from Gentianaceae.

Xanthones are also present in many species but their distribution is not universal in the family which make them as useful systematic markers (Jensen & Schripsema, 2002). The grade of substitution of xanthones is characteristic for a genus, and the oxidation pattern is uniform within particular section (Meszaros, 1994). Along with their taxonomic importance, xanthones display various pharmacological effects which might explain the growing interest in this class of compounds, demonstrated by the large number of the newly isolated and synthesized derivatives during the last decade (Pinto *et al.,* 2005; Vieira & Kijjoa, 2005).

Continuing our chemosystematic study of the Gentianaceae family from Serbia and Montenegro (Šavikin-Fodulovic *et al.*, 2002), the present review covers our recent results regarding chemical composition, biological activities, chemotaxonomy and cultivation of some Gentianaceae species growing in Serbia and Montenegro.

MATERIALS AND METHODS

Plant Material

Plant material was collected on the mountain regions throughout Serbia and Montenegro during the time of flowering: *Gentiana lutea* at mountain Suvobor, Serbia (at ca. 800 m), *G. utriculosa* and *G. dinarica* at mountain Tara, Serbia (at ca. 1200 m), *G. kochiana* at mountain Komovi, Montenegro (at ca. 2000 m), *Gentianella austriaca* at mountin Kopaonik, Serbia (at ca. 1750 m), *G. albanica* and *G. crispata* at mountain Hajla, Montenegro (at ca. 1900 m), *G. bulgarica* at the slopes of the same mountain (at ca. 800 m) and *Swertia punctata* at mountain Stara Planina, Serbia (at ca. 1850 m). Voucher

specimens have been deposited in the herbarium at the Faculty of Biology, Botanical Garden "Jevremovac", University of Belgrade.

General Procedure

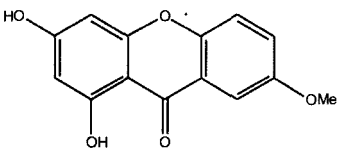
Methods of extraction and isolation were described in previously published papers (Menkovic *et al.,* 2000a; Menkovic *et al.,* 2002; Krstic *et al.,* 2004; Jankovic *et al.,* 2005). Silica gel and polyamide SC_6 (50–160 μm) were used for column chromatography. The UV spectra were measured on a Cintra 40 spectrometer. The NMR spectra were recorded using a Varian Gemini 2000 (^1H 200 MHz, ^{13}C 50 MHz) instrument. The mass spectra were obtained on a Finnigan MAT 8230 (EI, 70 eV and CI, 150 eV, isobutane) instrument. HPLC analyses were carried out on Agilent series 1100 with DAD detector, on reverse phase Zorbax SB-C18 analytical column 250 x 4.6 mm i.d., particle size 5 μm (Agilent). Mobile phase were MeCN (A) - H_2O containing 1% 0.1N H_3PO_4 (B), elution combination of gradient and isocratic modes: 98–90% B, 0–5 min, 90% B, 5–18 min, 90–85% B, 18–20 min, 85% B, 20–25 min, 85–70% B, 25–30 min, 70–30% B, 30–40 min, 30-0% B, 40-50 min. Flow rate 1 ml/min, injection volume 5 μl, detection at 260 and 320 nm.

RESULTS AND DISCUSSION

Chemical Constituents

The Genus Gentiana

Recent investigation (Menkovic *et al.,* 2000a) of chemical composition of the aerial parts pointed out the presence of 1,3,7-oxygenated xanthones isogentisin (**1**) and isogentisin-3-*O*-primeveroside (**2**), *C*-glucosides mangiferin (**3**), isovitexin (**4**) and isoorientin (**5**), and secoiridoids swertiamarin (**6**) and gentiopicrin (**7**).



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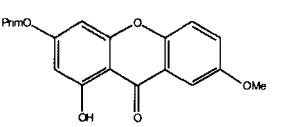

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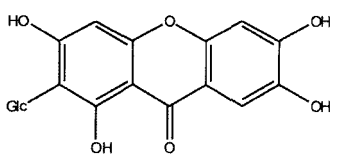

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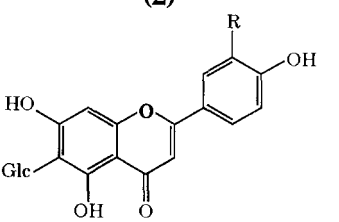

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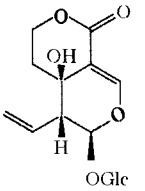

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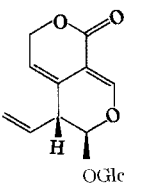
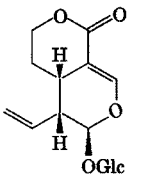


(7)

Gentiana dinarica (Fig 1) is endemic plant species growing on carbonate soils in subalpine and alpine regions (Tutin, 1972; Jovanovic-Dunjic, 1977). The LC-DAD analysis of the methanolic extract of the aerial parts indicated the presence of secoiridoids and flavonoids (Krstic *et al.*, 2004). Swertiamarin (**6**), gentiopicrin (**7**) and sweroside (**8**) were afforded, along with flavone-*C*-glucosides isoorientin (**5**) and isoorientin-4'-*O*-glucoside (**9**).



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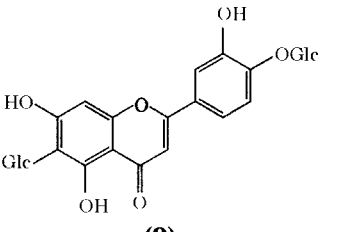
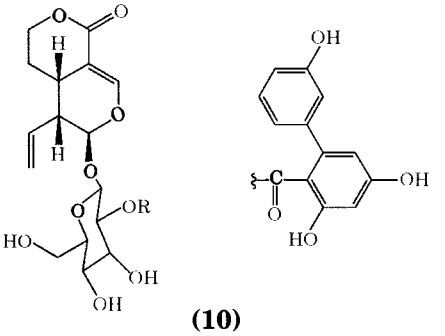
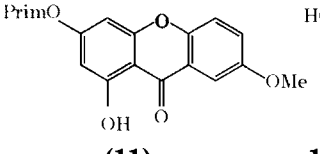


(9)

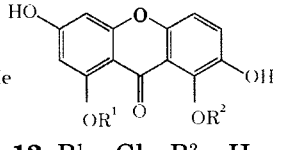
The roots contained the same secoiridoids and flavonoids as the aerial parts, in addition to amarogentin (**10**) and xanthone glycosides. Besides, xanthones gentianoside (**11**), norswertianin-1-*O*-glucoside (**12**), norswertianin-1-*O*-primeveroside (**13**) and norswertianin-8-*O*-primeveroside (**14**) were also isolated, the latter being identified as a new compound.



(10)

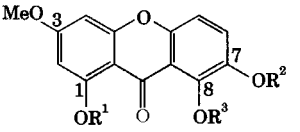


(11)



12: R^1 = Glc; R^2 = H
13: R^1 = Primv; R^2 = H
14: R^1 = H; R^2 = Primv

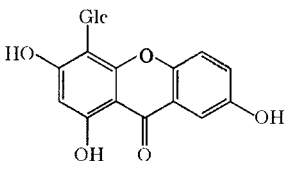
Gentiana utriculosa is an annual plant species found in Central Europe, mainly in the mountains of Italy and Balkan peninsula (Tutin, 1972). HPLC screening of the methanolic extract of the aerial parts revealed the presence of secoiridoids, flavonoids and xanthones (Jankovic *et al.,* 2008a). Among xanthone aglycones, gentiakochianin (**15**), gentiacaulein (**16**) and decussatin (**17**) were identified. Xanthone *O*-glycosides isogentiakochianoside (**18**), 1,8-dihydroxy-3-methoxy-7-*O*-primeveroside (**19**) and decussatin-1-*O*-primeveroside (**20**) were isolated. Lancerin, 1,3,7-trihydroxy-4-*C*-glucoside (**21**), was detected in the genus *Gentiana* for the first time, along with four known *C*-glucosides, mangiferin (**3**), isovitexin (**4**), isoorientin (**5**) and isoorientin-3'-*O*-glucoside (**22**).



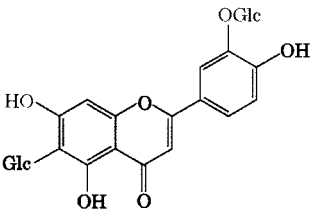
15: $R^1 = R^2 = R^3 = H$ **16**: $R^1 = R^2 = H$; $R^3 = Me$

17: $R^1 = H$; $R^2 = R^3 = Me$ **18**: $R^1 = Primv$; $R^2 = R^3 = H$

19: $R^1 = R^3 = H$; $R^2 = Primv$ **20**: $R^1 = Primv$; $R^2 = R^3 = Me$



(21)



(22)

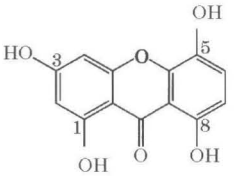
The Genus Gentianella

We have investigated chemical constituents of four species of *Gentianella* from Serbia and Montenegro, not studied previously: *G. albanica*, *G. austriaca*, *G. bulgarica* and *G. crispata* (Jankovic *et al.,* 2005) (Fig 13.1). According to the Flora Europaea (online database http://rbg-web2.rbge.org.uk), *G. austriaca*, *G. bulgarica* and *G. crispata* are endemic plant species, while little is known about *G. albanica* from the botanical point of view.

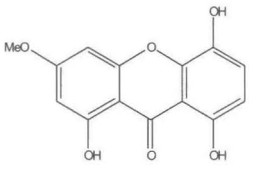
Fig 13.1. Some Gentianaceae species from Serbia and Montenegro

All species were characterized by the presence of xanthones, flavone-*C*-glucosides and secoiridoids typical for the genus. The predominant oxidation pattern of the xanthones found in genus *Gentianella* is 1,3,5,8-substitution. Demethylbellidifolin (**23**), bellidifolin (**24**) and corresponding 8-*O*-glucosides occur in all species studied so far. *Gentianella* species also contain xanthones with additional oxygenation at C-4, such as corymbiferin (**25**) and corymbiferin-1-*O*-glucoside, usually in admixture with the typical

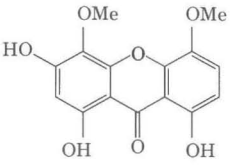
compounds. Along with these compounds, from the aerial parts of *G. austriaca* we isolated campestoside (**26**), a partially saturated analogue of the co-occurring demethylbellidifolin-8-*O*-glucoside. This compound was also found in *G. bulgarica*, but in *G. albanica* and *G. crispata* was not detected.



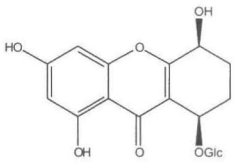
(23)



(24)

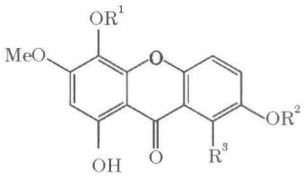


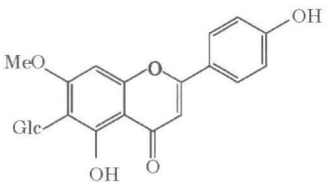
(25)



(26)

Moreover, our investigation of species from Serbia and Montenegro revealed the presence of 1,3,4,7- and 1,3,4,7,8-substituted glucosides (**27**, **28**), not found previously in European species. Co-occurrence of *C*-glucosides, such as mangiferin (**3**), isoorientin (**5**) and swertisin (**29**) is typical for species belonging to *Gentianella*.





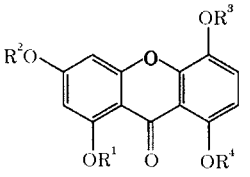
(29)

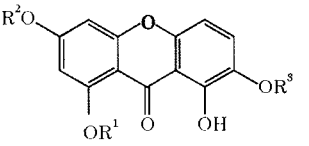
27: R^1 = Me; R^2 = Glc; R^3 = H
28: R^1 = Glc; R^2 = Me; R^3 = OH

The Genus Swertia

Among the European *Swertia* species, only *S. perennis* is officially accepted, whereas to *S. punctata* a provisional status has been assigned (Fig 1) (Tutin, 1972). On the other hand, Vladimirov and Tan (1998) claimed that *S. punctata* growing on the moisty terrains of Stara planina mountain is a well and precisely defined plant species differing from *S. perennis*. *S. punctata* is the only *Swertia* species found in Serbia (Jovanovic-Dunjic, 1977). It's population is scarce and endangered, and is registered in the Red Book of Serbian Flora (Jovanovic, 1999).

A detail characterisation of the chemical composition of the aerial parts and roots of *S. punctata* has been made (Pant *et al.,* 2000; Menkovic *et al.,* 2002). Aerial parts contain 1,3,5,8-oxygenated xanthones (**24**, **31**, **33**), along with *C*-glucosides mangiferin (**3**), isoorientin (**5**) and swertisin (**29**) (Šavikin *et al.*, 1996). Both 1,3,5,8- and 1,3,7,8-tetrasubstituted xanthones are present in *S. punctata* roots, with two newly isolated xanthone glycosides (**32**, **36**).





30: R^1 = H; R^2 = H; R^3 = Me; R^4 = H

31: R^1 = H; R^2 = Me; R^3 = Me; R^4 = H

32: R^1 = Primv; R^2 = H; R^3 = Me; R^4 = H

33: R^1 = H; R^2 = Me; R^3 = H; R^4 = Glc

34: R^1 = H; R^2 = H; R^3 = Me

35: R^1 = H; R^2 = Me; R^3 = Me

36: R^1 = Glc^6-Glc; R^2 = Me; R^3 = Me

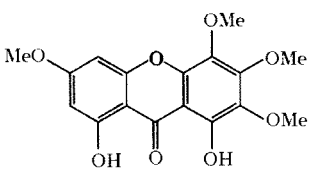
37: R^1 = Glc; R^2 = H; R^3 = H

Secoiridoids swertiamarin and gentiopicrin were also found in the whole herb.

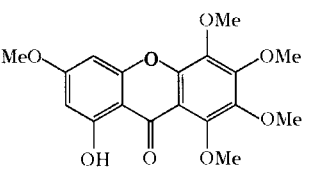
The Genus Centaurium

Secoiridoid glucosides of the sweroside-type have been reported as the main constituents in the aerial parts of *Centaurium* species. Swertiamarin is the main secoiridoid glucoside found in *C. erythraea*, while in aerial parts of *C. pulchellum,* sweroside is the most abundant compound (Van der Sluis, 1985).

Xanthones are the second major group of secondary metabolites in genus *Centaurium*. The characteristic oxygenation patterns found in these species are 1,3,5,8-, 1,3,7,8-, 1,3,5,6,8- and 1,3,5,6,7,8. Methylbellidifolin (**31**), decussatin (**17**), demethyleustomin (**38**) and eustomin (**39**) are present in large amounts in *C. erythraea* and *C. pulchellum* species (Jankovic *et al.,* 2000; Krstic *et al.,* 2003).



(38)



(39)

Biological Activities

Development of resistance by pathogens to many of the commonly used antibiotics provides an stimulus for further attempts to search for new antimicrobial agents to combat infections and overcome problems of resistance and side effects of the currently available antimicrobial agents. Therefore, in our search of antimicrobial activity from traditionally used medicinal plants, 96% ethanolic extracts of *Gentiana lutea* flowers, leaves and roots were screened against *Myccobacterium bovis* (Menkovic *et al.,* 1999). Flower extract showed weak bactericidal activity with minimal inhibitory concentration (MIC) value of 1 mg/mL, while leaves and roots extracts exhibited no activity at all. Xanthone isogentisin (**1**), component of flowers, possessed activity against *Myccobacterium bovis* (MIC 500 μg/mL). We also tested antimicrobial activity of *Gentiana lutea* extracts and isolated compounds against 16 microorganisms. Extracts of leaves and flowers as well as compounds mangiferin, isogentisin and gentiopicrin showed antimicrobial activity with MIC values ranging from 0.117-0310 mg/mL (Savikin *et al.,* 2007). Extract of *G. lutea* leaves showed better antimicrobial activity than the flower extract. Isogentisin and gentiopicrin showed similar activity while mangiferin was less active. The highest antimicrobial activity on *Escherichia coli* possessed gentiopicrin while the most active on *Candida albicans* was leaves extract. Other authors reported a fungistatic effect of the gentian extract as well as activity against *Streptococcus pyogenes* (Weckesser, 2007). Wound healing properties were also demonstrated (Öztürk *et al.,* 2006). An invention related to the use of a *Gentiana lutea* extract as a medicament in the treatment of bacterial infections was patented (Pontus *et al.*, 2006).

The inhibition of type A and type B monoamine oxidases by a number of xanthones has been obsereved (Suzuki *et al.,* 1980; Suzuki *et al.,* 1981), although without determining the exact IC_{50} value. Therefore, diethylether extract of aerial parts of *Gentiana kochiana*, as well as two dominant tetraoxygenated xanthones gentiacaulein (**16**) and gentiakochianin (**15**) were evaluated for CNS pharmacological activity in rodents (Tomic *et al.*, 2005). Extract and gentiacaulein strongly inhibited rat microsomal MAO A (IC_{50}= 0.22 μg/mL and 0.49 μm, respectively). Behavioural examinations on mice showed that 10 day s.c. administration of the extract (20 mg/kg) significantly decreased immobility score in a forced swimming test and strongly inhibited ambulation and stereotypy in an open-field test. The *ex vivo* MAO A activity in the crude brain mitochondrial fraction of mice treated with 20 mg/mL of the extract was significantly elevated, while that outside the brain nerve terminals it declined. Our study suggests some antidepressant therapeutic potential of *G. kochiana* that is presumably connected to the action of gentiacaulein.

Xanthones gentiakochianin and gentiacaulein were also identified as the active principles responsible for the *in vitro* antiglioma action of ether and methanolic extracts of *G. kochiana* (Isakovic *et al.,* 2008). Gentiakochianin and gentiacaulein induced cell cycle arrest in G_2/M and G_0/G_1 phases, respectively, in both C6 rat glioma and U251 human glioma cell lines, gentiakochianin being more efficient (IC_{50}<30 μm) than gentiacaulein (IC_{50}>50 μm). Since the interference with microtubule dynamics is one of the mechanisms for the blockade of cell division, we have measured the ability of these xanthones to affect microtubule polymerization. Gentiakochianin inhibited microtubule disassembly with the IC_{50} value of 18 μm, while gentiacaulein was inactive (IC_{50}>50 μm). Both xanthones reduced mitochondrial membrane potential and increased the production of reactive oxygen species in glioma cells, but only the effects of gentiakochianin were pronounced enough to cause caspase activation and subsequent apoptotic cell death.

Aerial parts of *Gentianella austriaca* were evaluated for antioxidative activity and protective properties on irradiated human peripheral blood lymphocytes *in vitro* (Leskovac *et al.*, 2007). Radioprotective effects of aqueous-ethanolic extract of *G. austriaca* against chromosomal damage induced by γ-rays were determined using micronucleus test. The results showed that *G. austriaca* displayed protective effects, decreasing the incidence of radiation-induced micronuclei by 35.56%, and significantly reduced lipid peroxidation for 30.88%. Further investigation have been done in order to identify active principles responsible for the reduction of the incidence of micronuclei. The radioprotective effects of water-soluble xanthones demethylbellidifolin (**23**), demethylbellidifolin-8-*O*-glucoside, bellidifolin-8-*O*-glucoside, and flavonoid swertisin (**29**) were measured (Jankovic *et al.,* 2008b). Among the examined compounds, the highest reduction by 27.92% in the incidence of micronuclei was observed in irradiated lymphocytes treated with swertisin. Bellidifolin-8-*O*-glucoside also showed radioprotective effects decreasing the incidence of micronuclei by 17.15%. Treatment of irradiated human lymphocytes with demethylbellidifolin-8-*O*-glucoside decreased the level of malondialdehyde (MDA) by 35.21%, while swertisin decreased the level of MDA by 25.65%. These results suggest that the antioxidative properties of the polyphenols tested may contribute to the radioprotective effects of *G. austriaca*

Chemotaxonomic Significance

As pointed out by Jensen and Schripsema (2002), the secoiridoid glucosides, with a predominance of swertiamarin and/or gentiopicrin appeared to be present in all species of *Gentianaceae* studied so far. Compounds sweroside,

swertiamarin and gentiopicrin together with their derivatives are the most characteristic iridoids in *Gentianaceae*.

Xanthones are not universally present in the family. Hitherto, they were detected in 121 species belonging to 21 genera (Jensen & Schripsema, 2002). The grade of substitution of xanthones is characteristic for a genus, and the oxidation pattern is uniform within particular section (Meszaros, 1994). They were found to be more useful chemotaxonomic markers in comparison to co-occurring secoiridoids and flavone-*C*-glucosides.

A detailed investigation of xanthone distribution has been made in the genus *Gentiana*, in particular in sections *Gentiana* L., *Calathianae* Froelich and *Megalanthe* Gaudin (Hostettmann & Wagner, 1977). The classification of species into these sections is in accordance with phylogenetic results based on molecular data (Gielly & Taberlet, 1996). In our study, xanthones isolated from *G. dinarica* (section *Megalanthe*) show the 1,3,7,8-oxygenation pattern characteristic for this section. It is noteworthy that only xanthone glycosides but not the aglycones were found in this species (Krstic *et al.*, 2004).

Xanthone glycosides with 1,3,7,8-oxygenation pattern are characteristic for the section *Calathianae*. Compounds **18** and **20** were reported as the constituents of three species of this section studied previously, such as *G. bavarica*, *G. nivalis* and *G. verna*. Along with these common glycosides, each species has its specific glycosides. For example, gentiabavarutinoside was found in high concentration in *G. bavarica*, while *G. verna* is characterised by swertianin-8-*O*-glucoside. On the other hand, compounds **19** and **21** have been found only in *G. utriculosa*. Xanthone-*C*-glucoside lancerin (**19**) is detected in the genus *Gentiana* for the first time, and further investigation of its distribution in other species of the genus could be interesting.

The predominant oxidation pattern of the xanthones found in genus *Gentianella* so far is 1,3,5,8-, and to a lesser extent, 1,3,7,8-substitution (Jensen & Schripsema, 2002). Five of the eight investigated *Gentianella* species contained xanthones with additional oxygenation at C-4, usually in admixture with the typical compounds. Xanthone compound lanceoside (**28**), detected in *G. albanica*, *G. bulgarica*, *G. austriaca* and *G. crispata* we analysed, is the first 1,3,4,7,8-oxygenated xanthone found in wild grown European *Gentianella* species (Jankovic *et al.*, 2005). According to Carbonnier *et al.* (1977), European species contain only 1,3,4,5,8-substituted xanthones, while the New Zealand representatives exhibit both 1,3,4,5,8- and 1,3,4,7,8-substitution pattern. At the same time, glucoside veratriloside (**27**) is the first 1,3,4,7-tetrasubstituted xanthone from the genus *Gentianella*. Tetrahydroxanthone glucosides (such campestroside) need special mention as such xanthones are rare and their occurrence is of a great biogenetic

significance. Campestroside (**26**), a partially saturated analogue of the co-occurring demethylbellidifolin-8-*O*-glucoside, was found in *G. austriaca* and *G. bulgarica* (Jankovic *et al.*, 2005). This compound was reported previously as the constituent of three *Gentianella* species, such as *G. campestris* (Kaldas *et al.,* 1978), *Gentianella germanica* and *Gentianella ramosa* (Hostettmann-Kaldas & Jacot-Guillarmod, 1978).

According to Neerja *et al.* (2000), 79 simple oxygenated xanthones have been isolated from the genus *Swertia* till the end of 1998. As far as the type of substitution is concerned, the xanthone complex of *S. punctata* is quite similar to that of the previously studied *S. perennis* (Menkovic *et al.,* 2002; Rivalle *et al.,* 1969; Hostettmann & Jacot-Guillarmod, 1976; Hostettman & Miura, 1977), but their distribution within plant is different. While in both aerial parts and roots of *S. punctata* xanthones with 1,3,5,8,-tetraoxygenation pattern were found, in *S. perennis* this type of xanthones were detected in the aerial parts only. Our results are in accordance with the previous suggestion for separation of *S. punctata* from *S. perennis*.

The co-occurrence of *C*-glucoflavones and *C*-glucoxanthone, mangiferin, is rather typical for some genera belonging to *Gentianaceae* (*Gentiana, Gentianella, Gentianopsis* and *Swerta*) (Jensen & Schripsema, 2002). The combination of the *C*-glucosides such as isovitexin and isoorientin is typical for genus *Gentiana*. Isovitexin and swertisin are the most common *C*-glucoflavones in genus *Swertia*, while in *Gentianella* species isoorientin and swertisin co-occurs.

In vitro Culture and Field Cultivation

The production of secondary metabolites was studied in shoots, roots and hairy roots of *Gentiana lutea* obtained *in vitro*. In shoots, both secoiridoid and γ-pyrone compounds were detected in amounts similar to those found in aerial parts of plants collected from nature. The most abundant secoiridoid was gentiopicrin (1.48% of dry weight) while mangiferin was the main compound among the γ-pyrones (2.32% of dry weight). The adventitious roots obtained *in vitro* showed a poor biosynthetic capacity in comparison with roots harvested in nature. Upon infection with *Agrobacterium rhizogenes*, nine hairy root clones were established which differed in the amount of secondary metabolites – some clones contained mostly xanthones and some are rich in secoiridoids (Menkovic *et al.,* 2000b).

Secoiridoid complex of *Gentiana punctata* cultured *in vitro* was also studied. The roots obtained *in vitro* were able to produce only small amounts of gentiopicrin (0.09%). On the other hand, the shoots of *G. punctata* produced both gentiopicrin and swertiamarin in significant amounts (2.42% and 0.47 %, respectively) (Menkovic *et al.,* 1998).

Shoot cultures of *G. punctata* were transformed with *Agrobacteroium rhizogenes* strain A4M70GUS and with *A. tumefaciens* clone C58C1 (pArA4b). The obtained hairy roots were cultured in liquid and solid medium. Similar amounts of gentiopicrin were found in hairy roots obtained by *A. rhizogenes* (0.62% in liquid and 0.61% in solid medium), while in hairy roots obtained by *A. tumefaciens* gentiopicrin was detected only in roots cultured in liquid medium (0.30%) (Menkovic *et al.,* 2000c). *In vitro* culture, regeneration and production of secoiridoid glucosides in *Gentiana* species was also reported by (Skrzypcyak *et al.,* 1993).

Shoot cultures of *Gentianella austriaca* were established from seedling epicotyls (Vinterhalter *et al.*, 2008). A characteristic feature of these cultures was precocious flowering, which appeared in all rapidly elongating shoots. Shoot cultures contained the same types of secondary metabolites as plants from nature. Xanthones were the major constituents, with demethylbellidifolin (**23**), demethylbellidifolin-8-*O*-glucoside and belidifolin-8-*O*-glucoside as dominant compounds. Secondary metabolite production was strongly affected by the presence of BA (benzyladenine) in the medium.

Centaurium erythraea and *C. pulchellum* were also introduced in *in vitro* culture. The quantitative distribution of xanthone compounds, demethyleustomin (**38**) and eustomin (**39**), as well as secoiridoids, swertiamarin and gentiopicrin, was studied in aerial parts and roots of *in vitro* obtained *C. erythraea* (Jankovic *et al.,* 1998; Jankovic *et al.,* 2000). The amount of secoiridoids was higher in the aerial parts than in the roots. The predominant secoiridoid in plants collected from natural habitats was swertiamarin (6.30% of dry weight), while in plants cultured *in vitro* gentiopicrin prevailed (4.92% of dry weight). The roots of all analyzed samples contained greater level of xanthones than the aerial parts. Roots of plants cultured *in vitro* contained greater amounts of **38** and **39** than plants grown in nature. It was also noticed that both BA and sucrose concentrations affected the production of secondary metabolites.

Secoiridoids gentiopicrin, swertiamarin and sweroside, and xanthones methylbellidifolin (**31**), demethyleustomin (**38**) and decussatin (**17**) were detected in shoots and roots of *C. pulchellum* cultured *in vitro* (Krstic *et al.,* 2003). Different sugars (glucose, fructose and sucrose) added in different concentrations in the medium influenced growth of the plants as well as the production of secondary compounds.

Inoculation of *C. erythraea* and *C. pulchelum* shoots proved to be suitable for initiation of hairy roots (Jankovic *et al.*, 2002). Five clones of hairy roots of *C. erythraea* and three of *C. pulchellum* were selected according to morphological differences. Hairy root clones of both species retained the capacity for the production of xanthones while secoiridoids were detected only in one clone of *C. pulchellum*. It is noteworthy that all clones of hairy

roots produced greater amounts of xanthone compounds than the roots of plants grown in nature. Spontaneous bud initiation was observed in hairy roots, and obtained transgenic shoots produced both secoiridoids and xanthones.

Due to excessive exploitation from the nature, the survival of *Gentiana lutea* in the nature is endangered. Orientation to large-scale production of this plant species could provide both protection of natural resources and market demands. During the last two decade a lot of efforts to improve Yelow Gentian growing technology have been made (Bezzi *et al.*, 1986; Bezzi *et al.*, 1996; Galambosi, 1996). We have cultivated *G. lutea* on Tara mountain in four year period; throughout measurements of the main morphological parameters as well as chemical analysis of the aerial and underground parts have been done (Radanovic *et al.*, 2007). Fresh and air-dried roots yields achieved in the fourth productive year were comparable to the best ones reported in literature - more then 15 t/ha of fresh and 4.4 t/ha of dried roots. The amounts of secondary metabolites (secoriridoid gentiopicrin and xanthone isogentisin) in roots varied during the age of plantation as well as during the vegetation period. The production of gentiopicrin is higher in younger plants (two-years-old) than in four-years-old plants (113.65 and 44.13 mg/g of dry weight, respectively). Moreover, the seasonal variation in the content of this metabolite is recorded, its amount decreased from June to October. Isogentisin showed different dynamic of accumulation. The amount of isogentisin increased with cultivation period, being 0.21 and 2.9 mg/g of dry weight in roots of two-years-old and four-years-old plants, respectively.

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14

Chitin and Chitosan : Extraction and Characterization

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ABSTRACT

Chitin and chitosan are naturally occurring polymers similar to cellulose in chemical structure but contain an amino group which can provide distinctive biological functions and offer great potential for many chemical modifications. Chitin could thus have even higher potential than cellulose in many fields. Due to this high potential of chitin this review is focused on the different sources of chitin and chitosan in nature, the methods of extraction from crustacean exoskeletons, squid pens, insects and fungi. Discussion of the factors affecting the extraction process and the quality of the final products chitosan is given. Chitosan hybrid materials have been also discussed. Through the sol/gel technique new chitosan/silica materials have been developed to improve the quality of the chitosan blend keeping its biocompatible and biodegradable nature.

Key words : Chitin, chitosan, extraction from crustacean, insect, fungi, enzymatic extraction, microwave assisted extraction, chitosan/silica hybrid materials

INTRODUCTION

Chitin is a polysaccharide consisting of β-(1,4)-linked-2-acetamido-2-deoxy-D-glucose, present in large quantities in the shells of crabs, shrimps, and other crustaceans. It is also present in the exoskeletons of insects and in the cell walls of bacteria. It is a white powder very similar to cellulose (Scheme 1). Chitin is second only to cellulose in abundance. About 100 billion tons of chitin are produced by nature annually, however most of this quantity is said not to be used (Rege *et al.*, 1999).

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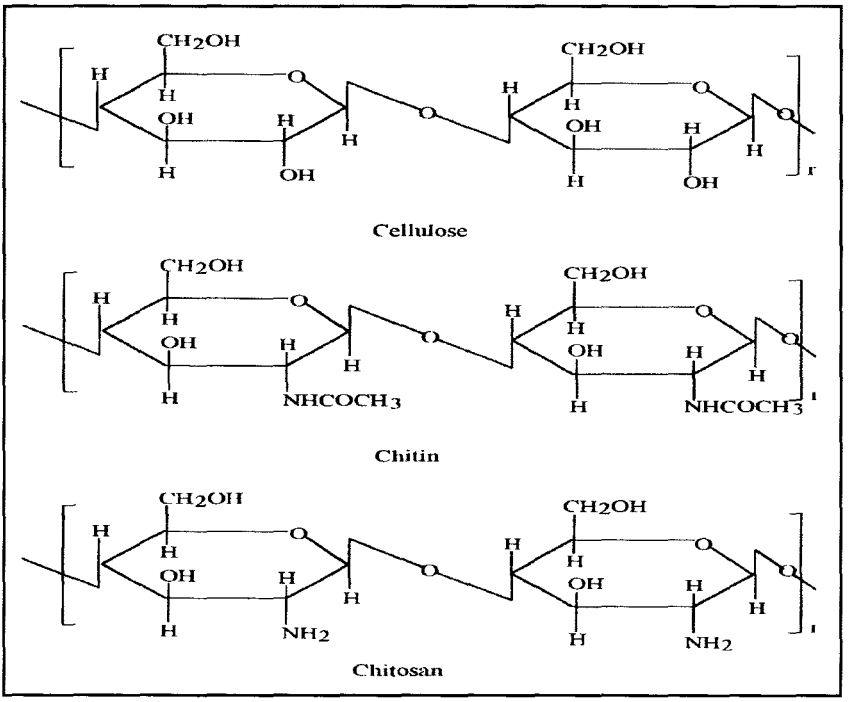
Because chitin has superior biocompatibility, it is used in an array of applications in biomedical field. Efforts are now being directed to investigate the substance's potential for use as anti-cancer agents, serum cholesterol lowering agents, and nonpolluting agricultural chemicals.

Chitosan is a deacetylated chitin found widely throughout the natural world. It has been the focus of wide-ranging research. The substance is drawing attention for its unique characteristics and is being employed in a diverse array of fields.



Scheme 1. Chitin, Chitosan and Cellulose structures

Chitosan has distinctive characteristics that include blocking the body absorption of fat, a property that is promoting the use of the substance for health and medical treatments of various kinds. It has been also reported that chitosan reduces high blood pressure, improves various internal disorders, reduces uric acid levels, and reduces blood sugar level.

Chitosan is soluble in water at pH lower than 6 and being polycationic, non-toxic and biodegradable finds numerous applications especially in the agriculture, food and pharmaceutical industries, such as food preservation (Chatterjee *et al.,* 2005; Tasi *et al.,* 1999) fruit juice clarification (Rolle *et al.,* 1999; Begin *et al.,* 1999; Tsai *et al.,* 2000; Jeon *et al.,* 2000; Ouattara *et al.,* 2000; Imeri *et al.*, 1988) water treatment especially for removal of heavy metals ions (Mitani *et al.,* 1992; Covas *et al.,* 1992; Chatterjee *et al.,* 2002);

sorption of dyes and flocculating agent. Chitosan and its derivatives can also be used as biological adhesive for its hydrogel-forming ability (Ono *et al.,* 2000) wound healing accelerator (Pruden *et al.,* 1970; Azad *et al.,* 2004) and also in cosmetic industries. Hirano and Nagao have suggested that Low molecular weight chitosan in an agar system inhibited a range of phytopathogenic fungi (Hirano *et al.,* 1989; Sekiguchi *et al.,* 1994) more effectively than high molecular weight chitosan inhibited the organisms. The depolymerized products of chitosan were found to be potent inhibitors of tumor-induced angiogenesis (Prashanth *et al.,* 2005). Chitosan is known to prevent the development of phage infections in cultures of various microorganisms (Kochkina *et al.,* 1995) it also exhibits antimicrobial activity against some strains of filamentous fungi (Sudrashan *et al.,* 1992), and bacteria.

Colloidal and Water Soluble Chitin

Colloidal chitin can be prepared by dissolving chitin into hot concentrated hydrochloric acid followed by pouring the solution into water (Kurita *et al.,* 2001). Chitin precipitates as a powdery material and can be then dispersed in water. Colloidal chitin can be used as a starting material for chemical reactions, as a substrate to assay chitin-degrading enzymes, and as a carbon and nitrogen source for chitin-digesting microorganisms. Water soluble chitin can be prepared by steeping the chitin in concentrated sodium hydroxide (40% w/w) and treating the slurry with crushed ice, an alkali chitin is formed. Under theses conditions, N-deacetylation proceeds rapidly in solution. An alkali chitin solution in 10% NaOH left at room temperature for 70 h gives a product with about 50% DAA that is soluble even in neutral water (Sannan *et al.,* 1976; Sannan *et al.,* 1975). Random acetylation of chitosan to a degree of acetylation of about 50% gives rise to a water-soluble product (Kurita *et al.,* 1989; Kurita *et al.,* 1991).

Crystalline Structure of Chitin

The crystalline structure of chitin was found to play an important role in determining its chemical reactivity and therefore it is of value to identify the type of crystalline structure of the polymer. Three crystalline forms are known for chitin: α-, β-, and γ-chitins. The most abundant form is the α chitin, where the molecules are aligned in an anti-parallel fashion. This molecular arrangement leads to the formation of strong intermolecular hydrogen bonding making α-chitin insoluble in most known solvents, less susceptible for the deacetylation reaction and swelling. β-Chitin is composed of parallel chains and thus has less intermolecular forces and consequently it deacetylates much easier using less drastic conditions. β-Chitin is less stable than the α form (Kurita *et al.,* 2001).

On dissolution or extensive swelling, β-chitin converts to α-chitin. This is not a reversible process and hence suggests that β-chitin is a metastable entity biosynthesized by specific mechanism different from that leading to the α-form. γ-Chitin is considered to be a mixture of or an intermediate form of the α- and the β-forms and has both parallel and anti-parallel arrangements.

EXTRACTION OF CHITIN FROM CRUSTACEANS

Chitin is found in the exoskeleton of crustaceans in combination with minerals such as $CaCO_3$ and proteins. The most common method of isolating chitin consists usually of demineralization followed by deproteination. Separation of the contaminated protein improves the quality of the final product.

Chemical Methods

Several authors have isolated chitin from different locations: Moroccan seaside (Rhazi *et al.,* 2000), Egyptian seaside (Abdou *et al.,* 2008), the Arabian Gulf near Kuwait (Alsagheer *et al.*, 2009) and others (Grigoryeva *et al.,* 2008).

Demineralization Step

The raw materials (shrimp shells, waste crab crust) are washed with water, dried and ground. Demineralization is generally performed with acid treatment including HCl, HNO_3, H_2SO_4, CH_3COOH, and HCOOH. HCl, seems to be the preferred reagent

Demineralization was found to be a critical step. A minimum of 0.70 M HCl was found to be optimal whereas excess of HCl leads to reduction of the molecular weight (Stevens *et al.,* 2002). Acidic treatment was also found to depend on the source and on the mineral content (counterions of carbonate or phosphate ions) of the different species and penetration of the shell by hydrochloric acid. Tolaimate *et al.* (1999) have investigated in detail the demineralization process for 12 crustacean species using a 0.55 M HCl bath. The process was repeated 2 to 5 times and the mineral content was determined by elemental analysis after the successive acidic baths. The mineral content varied according to the source from 1.7% for squid to 33.99 for lobster, shrimp 21.5% and crabs have 25.96 to 31.13%. Several procedures are present in the literature that deal with the concentration of the hydrochloric acid, the treatment temperature and the duration of the demineralization process (Tolaimate *et al.,* 2003; Mima *et al.,* 1982; Shimahara *et al.,* 1992; Shahidi *et al.,* 1991; Whistler *et al.,* 1962).

Deproteination

Deproteination is carried out using sodium hydroxide solution (2–5%) at 80–85°C. This treatment can be repeated several times. The absence of protein is indicated by the absence of color of the medium at the last treatment. The medium is left to settle, filtered, washed several times with water to neutrality, and then dried. Mild oxidizing agents may be used to remove traces of coloring pigments or protein, for example: $KMnO_4$/oxalic acid mixture or H_2O_2/HCl in a ratio of 9:1 (Hakman *et al.,* 1974).

Deacetylation of Chitin

Numerous sea sources of chitin have been investigated and the chitin content and crystallographic type of the extracted chitin compared. In this work the author's main target was to compare the different sea sources of chitin and show how the different process parameters play a role in the characteristics of the chitosan obtained. In this work two procedures were studied. The first was the standard Broussignac process (Broussignac *et al.,* 1968) which uses a mixture of solid potassium hydroxide as a deacetylation reagent (50% w/w), 96% ethanol (25% w/w) and monoethyleneglycol (25% w/w) which are essentially anhydrous conditions. This mixture was prepared by mixing the two solvents, and then solid KOH was added in small portions with stirring. The dissolution is exothermic which raises the temperature up to 90°C. Chitin was then added and the temperature was increased to the desired value. After the required period of heating, the reaction was cooled and the chitosan filtered and washed with water until neutrality. It was then dried at room temperature in an air stream. The reaction parameters of this process were varied using 500 mg of chitin suspended in 30 mL of mixed solvents.

The second procedure was proposed by (Kurita *et al.,* 1993) in which a suspension of chitin (0.5 g) was heated in 30 mL NaOH (50% by weight) under a nitrogen atmosphere with stirring. After the required time the solid was filtered off, washed with water to neutral pH, then with methanol, and finally with acetone, and dried. The chitin in this work was obtained from 12 different sea sources. The chitin content ranged from 7 to 40% depending on the source.

Chitin has been extracted from six different sources in Egypt. A comparison of percent of protein, chitin and minerals in the different sources obtained from the Moroccan and Egyptian sea sides (Rhazi *et al.,* 2000; Abdou *et al.,* 2008) are shown in Table 14.1.

Table 14.1. Chitins from Egypt and Morocco

Source	Egyptian sea side			Moroccan sea side		
	$CaCO_3$	Protein	Chitin	Mineral content	Protein	Chitin
Brown shrimp (*Penaeus azetecus*)	48.97	29.50	21.53			22
Pink shrimp (*Penaeus durarum*)	42.26	34.02	23.72	21.5		
Cuttlefish pens	88.48	6.12	5.40			20
Squid pens	4.74	46.23	49.00	1.70		40
Crab shells	66.58	16.68	16.73	26-31		10-16
Crayfish shells	63.94	15.46	20.60			36

It is perhaps interesting to note the variation of the individual content of the different sources of chitin in spite of the proximity of the two sources. Species in Egypt could have been harvested from the Red Sea as well as from the Mediterranean Sea, which could explain the changes in compositions.

The chitin obtained from these sources was converted into the more useful chitosan by steeping it in solutions of NaOH of various concentrations and for extended periods of time. Alkaline chitin was then heated in an autoclave which dramatically reduced the time of deacetylation. Chitin from squid pen did not require steeping in sodium hydroxide solution, and showed much higher reactivity toward deacetylation in the autoclave. After 15 min of heating a degree of deacetylation of 90% was achieved. It has been found that the degree of deacetylation (DDA) increases with increasing the steeping time from 1 to 4 days followed by heating for 1.0 h in the autoclave. The increased DDA values were as follows: from 88 to 92% for the brown shrimp and from 91 to 97.5% for the pink shrimp. Increasing the heating time in the auto clave from 05 to 3.0 h after steeping in 40% NaOH for 1 day led to an average increase in the DDA from 80 to 92. The sodium hydroxide concentration was found to be crucial for the deacetylation process and should be higher than 30% by wt. The viscosity of the obtained chitosan was found to decrease with the steeping and reaction time. In other words the viscosity decreases with increasing the DDA. X-ray diffraction (XRD) analysis showed that all the extracted chitin has relatively high crystallinity which deceases after deacetylation into chitosan.

A study was conducted to determine the baseline composition of commercial shrimp shell waste, its conversion to chitin by chemical treatment, and a correlation between chitin quality from different sites and from the most abundant species from India (Pradhan *et al.*, 2002). Commercial

shrimp shell waste with heads, collected from 4 sites (Thane, Ratnagiri, Cochin, Tutticorin), were analyzed for chitin, proteins, minerals, and other parameters. The most abundant shrimp species (*Penaeus indicus* and *Penaeus monodon*) were also analyzed for the same parameters. The basic composition from all 4 sites was compared, depending on the most abundant species at each source. Results reported that the chitin composition from *P. monodon* shells and the sites where it was abundant, *i.e.* Cochin and Tutticorin were greater than the other sites *i.e.* Thane and Ratnagiri. Chitin extracted from *P. indicus* shells was whiter vs. *P. monodon* shell chitin and hardly required any bleaching treatment to improve its color.

P. indicus shells contained more minerals than *P. monodon* shells. Proteins were more abundant in *P. monodon* vs. *P. indicus* shells.

A seasonal study of the chemical composition and chitin quality of shrimp shells obtained from northern deep water shrimp (*Pandalus borealis*) was conducted in northern Norway. The shells harvested from January to December in the Barents Sea were investigated (Rodde *et al.,* 2008). The average dry matter content of the samples of shrimp shells was 22 ± 2%, with no significant seasonal variation. The protein content was found to vary between 33% and 40% of the dry weight, the chitin content varied between 17% and 20%. The ash content was found to be relatively constant with an average value of 34 ± 2% of the dry weight and consisted mainly of calcium carbonate. One can observe that the mineral content in the northern deep water shrimp is lower than that in shrimp harvested from the Mediterranean Sea while the protein content is higher and the chitin content fairly similar.

A review dealing with the improvement of the extraction of chitin and chitosan from various sources, quality assessment and applications of these marine biopolymers was given as a tribute to the late Professor Hirano in acknowledgment of his contribution in the field of chitin and chitosan development (Stevens *et al.,* 2000).

The structure of chitosan prepared under different deacetylating conditions has been investigated (Harish Prashanth *et al.,* 2000). In that work, N-deacetylation of chitin was performed by alkali treatment under a nitrogen atmosphere and in the presence of thiophenol as an oxygen scavenger. Deacetylation under a nitrogen resulted in producing an anhydrous crystal lattice. Use of thiophenol resulted in a chitosan with higher degree of polymerization, higher viscosity, higher average molecular weight and higher order of structure. It also showed higher thermal stability due to reduced oxidative bond cleavage.

The utilization of crawfish processing wastes for carotenoids, chitin, and chitosan has been reviewed (No *et al.,* 1992). The recovered carotenoid

astaxanth finds applications in aquaculture and poultry industry. Crawfish shell, after initial pigment extraction is an excellent source of chitin. In general the protein from shellfish may be recovered using a base extraction or enzyme hydrolysis process. The carotenoid pigments are extracted into oil at 60°C or by using an enzyme-assisted process (Shahidi *et al.*, 1998). Shrimp shell waste samples from different freezing plants were studied as a source of chitin. The protein and minerals were removed with alkali and acid treatment respectively. The best processing conditions were deproteination with 2% NaOH and demineralization with 5% HCl at 50°C. The best conditions for scaling the process up to pilot plant level were established. The product purity was determined for the high-quality chitin (0% protein, 0.01% ash, 99.99% chitin) and standard grade chitin (0% protein, 0.9% ash, 99.13% chitin) (Pinelli *et al.*, 1998).

Multistage alkali treatment of chitin from Persian Gulf shrimp shells has proven to be superior compared to one stage treatment. In the multistage treatment the DDA reached 91% with improved biomedical qualities of the resultant chitosan (Yaghobi *et al.*, 2004).

Chitin was extracted from red shrimp processing waste. The effect of reaction time and temperature during heterogeneous alkali reaction on DDA and molecular weight (MW) of the resulting chitosan was elucidated, and the reaction conditions to obtain the desired DDA and MW chitosan product were established (Tsaih *et al.*, 2003). The highest DDA of the resulting chitosan after alkali deacetylation at 99°C and 140°C were 92.2 and 95.1% respectively. The DDA contents increased fast at the beginning of the reaction process then slowed overtime. The reaction rate and the rate constant of the deacetylation reaction decreased with increasing the DDA of the reactant. The MW of chitosan decreased along with the deacetylation time. The rate of chitosan degradation was > 43.6% in the initial stage, then decreased to approximately 20%/h. The degradation rate constant increased substantially in the latter stages.

Optimization of chitin extraction from shrimp shells was also conducted by Percot *et al.* (2003). The demineralization process was followed by measurement of the variation in pH of the supernatant liquid, and the increase in pH was related to the calcium release. The kinetics of demineralization was then followed and it was concluded that the demineralization time should be minimized to 15 min at ambient temperature using 0.25 M HCl (with a solid-to-liquid ratio of 1:40 w/v). Under these mild conditions, the DA (degree of acetylation) of the chitin obtained remained stable with minimal hydrolysis of the glycosidic bonds. The deproteination is conveniently performed in 1 M NaOH within 24 h at a temperature close to 70°C with no effect on the molecular weight or the

DDA. Under these conditions, the residual ash is below 0.01%, and the DDA is almost 95% (Percot *et al.,* 2003).

The deacetylation of α-chitin differs from the deacetylation of the β-chitin, since the latter has different crystalline structure (parallel alignment of the chains with relatively weaker intermolecular forces) (Minke *et al.,* 1978). This renders it much more reactive toward chemical reactions. β-Chitin is more easily solubilized in known solvents such as dimethyl acetamide/LiCl, N-methyl-2-pyrrolidone/LiCl, hexafluoro-isopropanol (Terbojevich *et al.,* 1988; Kumar *et al.,* 2000). In addition, β-chitin is more reactive than α-chitin, an important property in regard to its chemical derivatization for different applications. The kinetics of deacetylation of squid pens has been investigated by different authors (Pawadee *et al.,* 2003; Rodrigo *et al.,* 2007). Chitin was extracted from squid pen and its heterogeneous deacetylation was performed using various conditions. The reaction followed the pseudo-first-order kinetics during the initial period. The DAA increased with increasing temperature, NaOH concentration and time. In the temperature range 40–100°C, the apparent rate constant and the activation energy of the reaction ranged from 1.0×10^{-3} to 2.4×10^{-2} min^{-1} and from 5.4 to 11.9 Kcal/mol, respectively. Various optimum conditions for obtaining 90% DDA chitosan were predicted from the regression analysis. Tolaimate *et al.* (2003) have also investigated the preparation of chitin from squids and used two deacetylation methods, (Kurita's, 1993; Broussignac, 1968), to produce chitosan with adequate physicochemical characteristics (molecular weight and DDA). Kurita's process was found to produce chitosan with high molecular weight and within a large range of deacetylation degrees. The second process may be carried out to obtain chitosan with low degrees of acetylation faster but with lower molecular weights. The authors also investigated the repeated alkaline steps and compared it to the continuous process.

The squid pens, a rich source of β-chitin containing low contents of inorganic compounds, are available in considerable amounts as a refuse from the fishery industries in Brazil. Thus, the aim of that work was to use squid pens from *Loligo sanpaulensis* and *Loligo plei*, species found in the Brazilian coast, as the raw material for the extraction of β-chitin (Rodrigo *et al.,* 2007). The squid pens were submitted to the usual sequence of treatments used for chitin extraction, demineralization and deproteination, but due to its low content of inorganic compounds a two-step alkaline treatment was enough to produce β-chitin with low contents of ash (≤0.7%). Indeed, the contents of ash and metals, such as Ca (610.4 ppm), Mg (62.5 ppm), Mn (63.1 ppm) and Fe (61.8 ppm), are lower than those reported in most of the papers found in the literature. Also, the β-chitin extracted by employing only the alkaline treatment was more acetylated than the other

samples prepared in this work. Regardless of the treatment employed for the extraction of the β-chitin from the squid pens, its infrared spectra and X-ray diffraction pattern presented only minor differences, however they were clearly distinguished from commercial α-chitin.

Similar extraction of β-chitin from *Illex argentinus* squid pens (near the Argentinean coasts) was carried out by using chemical methods and the pens composition determined (Cortizo *et al.,* 2008). The extract from the squid pens represented 0.2% w/w of the squid *Illex argentinus.* It was found that the squid pens contained 1.0 wt.% (base dry) of ash, 2.3 wt.% lipids and lipoproteins, 64 wt.% protein and 31 wt.% chitin.

The chitin isolated from *Illex argentinus* squid pens has been characterized with several techniques. The polysaccharide exhibited a β-chitin structure according to the IR and ^{13}CNMR spectra. A high degree of crystallinity (74.9%) and degree of acetylation (96%) further characterized the β-chitin. The values achieved were in good agreement with other β-chitins isolated from squid species. High average viscosity molecular weight (above 2×10^6 g.mol^{-1}) were estimated from extrapolation of the existing intrinsic viscosity-molecular weight relationships for β-chitin in DMAc/LiCl 5% {[η] (cm^3.g^{-1}) = 0.24 M$^{0.69}$}.

Factors Affecting Chitin Extraction

Chitosan was prepared by carrying out the deacetylation in the presence of nitrogen atmosphere, and by adding 1% thiophenol (v/v) before refluxing with sodium hydroxide. Thiophenol was found to prevent excessive degradation of the chitosan during deacetylation (Percot *et al.,* 2002).

Deproteination was also conducted using mixed organic acids in order to reduce the harsh action of HCl (Charoenvuttitham *et al.,* 2006). A marked decrease in viscosity during the deproteination process was also established and a high molecular weight chitin could be obtained in just 15 min when using large excess of 0.25M HCl. The deproteination process was followed by analyzing the amino acids of the protein remaining in the reaction mixture.

Effect of Irradiation

Chitin was extracted from prawn shells, deproteination were found to be, and demineralization processes were conducted under the influence of a 25 kGy irradiation dose. It was found that the irradiation reduces the time of deproteination reaction by a factor of three (Mahlous *et al.,* 2007).

The best conditions for chitin extraction for deproteination: irradiation at the dose of 25 kGy, 1N NaOH, reaction temperature 85°C, and reaction

time 1 h. For the demineralization: irradiation dose 25 kGy, 1N HCl, room temperature, reaction time 3 h. For deacetylation: 60% NaOH reaction temperature 100°C, reaction time 120 min. These conditions allowed reaching a DDA levels of almost 93%.

Effect of Sonication

The effect of sonication during chitin extraction from freshwater prawn (*Macrobrachium rosenbergii*) shells on yield, purity, and crystallinity of chitin was investigated (Kjartansson *et al.,* 2006). Dry prawn shells were suspended for 4 h in 0.25 M HCl at 40°C while they were sonicated for 0, 1, and 4 h. Demineralized shells were lyophilized, re-suspended in 0.25M NaOH, and sonicated again for 0, 1, and 4 h. The yield of chitin decreased from 8.28 to 5.02% for non-sonicated and sonicated samples, respectively, which was attributed to losses of depolymerized materials in the wash water. Although the application of ultrasound enhanced the removal of proteins, the glucosamine content and the crystallinity indices of chitins were decreased with the time of sonication. No added value is achieved by sonication.

CHITIN AND CHITOSAN FROM INSECTS

Extraction of chitin from crab and shrimp is usually associated with problems due to protein contamination and inconsistent level of deacetylation which leads to variation of the physico-chemical characteristics of the chitosan from these sources. Application of chemicals could lead to ecological and health problems due to residual toxicity and carcinogenicity. Therefore, more attention is now being paid to the use of natural active components from plants and animals in pharmacological and biochemical applications. Insects represent one of the most successful groups of evolution accounting for nearly one million species. Insects are a large, unexplored and unexploited source of potentially useful compounds for modern medicine.

Chitosan was isolated from the larvae of housefly, *Musca domestica* (Hui *et al.,* 2008; Gyliene *et al.,* 2002). The larvae of housefly were washed with 15% (w/v) aqueous sodium chloride solution, freeze-dried and grounded into crude powder. The protein was removed by heating in 1M NaOH for 6 h then decolorized with potassium permanganate and oxalic acid. Deacetylation was conducted as usual using 40% NaOH at 70°C for 8 h. The average molecular weight of chitosan from housefly larvae was determined to be 426 kDa. The DDA level was 90.3%. The chitosan obtained from the housefly larvae showed strong antioxidant potency, efficient reducing power, and considerable ferrous ions chelating ability. Moreover, it exhibited significant antitumor activity against HeLa and S-180 tumor cell lines *in vitro*, in a dose-dependent manner. Ferrous ions are considered to be the most effective pro-oxidant present in food system. The high chelating effect of chitosan would be beneficial if it is formulated into foods. These *in vitro* studies

suggested that the chitosan from the larvae of the housefly could be effectively used as a natural antioxidant to protect the human body from free radicals and retard the progress of many chronic diseases. Furthermore, the chitosan with antitumor activity from the larvae of the housefly might provide useful information for the development of antitumor drugs.

Chitin in the α-form was isolated from beetle larva cuticle and silkworm (*Bombyx mori*) pupa exuvia by treatment with 1N HCl and 1N NaOH (Zhang *et al.*, 2000). Chitosan was prepared by treating the obtained chitin with 40% NaOH containing $NaBH_4$. The chitin and chitosan were analyzed by XRD, ^{13}C-NMR, cross-polarization, magic angle spinning nuclear magnetic resonance (CP/MAS-NMR), and scanning electron microscopy (SEM). Insect chitin was found to degrade faster than shrimp chitin when treated with 6 M HCl and the enzyme-chitinase. About 94% DDA was achieved after one step treatment with 40% NaOH for 4 h at 110°C. The wide-angle XRD patterns for the powdered chitin samples prepared from beetles, silkworms, and shrimp after pretreatment with 2 N HCl at 100°C, showed a similar diffraction pattern, *i.e.* similar 2θ values assigned to the diffraction planes (020), (110), and (101) of the simple type of the α-form orthorhombic crystal structure (Zhang *et al.*, 2000). The chitin isolated from shrimp shells exhibited a higher crystallinity (54%) than chitin from silkworm pupa exuviae (47%) or beetle larvae cuticles (56%). After 2 N HCl treatments, at both ambient temperature and at 100°C there was an increase in the degree of crystallinity in all the three samples (Table 14.2). This experimental result was explained as a consequence of the rapid erosion in the amorphous or non-crystalline regions of the chitin samples investigated.

Table 14.2 Residual protein content (%) in prawn shell as a function of reaction time in 1 N NaOH solution

Chitin isolated from	Before treatment with 2 N HCl	After treatment with 2 N HCl	
		RT	100°C
Silkworm pupa exuviae	47	54	60
Beetle larva cuticles	56	58	66
Shrimp shells	54	56	58

A more significant change in degree of crystallinity is seen in Table 14.2 for the insect chitin samples. A reasonable explanation for this is that because catechol compounds were confirmed to be present in the insect cuticle, they were in the insect chitin samples as well. These low molecular weight compounds affect the crystal structure of the chitin and were easily removed after the HCl treatment resulting in a relative increase in the crystallinity.

Chitin and chitosan were also isolated from honey bees (Varlamov *et al.,* 2002). The method included the extraction with CO_2 and treatments by means of proteases and alkali. The optimal process conditions (time, temperature, and pH) for the deacetylation of chitin were investigated. Water soluble chitosan oligomers were prepared using the chitinolytic enzymes which reduces the chitosan molecular weight.

Chitin was also isolated from chrysalides of silkworm (*B. mori*) (Paulino *et al.,* 2006). These chrysalides are the adult form of the larvae responsible for the production of silk threads, and the chrysalides themselves constitute a by-product from the silk industry that is of low cost and readily available. China and Brazil are the principal exporting countries of cocoons and raw silk (Paulino *et al.,* 2006). The complete metamorphosis of butterflies, moths, and some other insects involves four stages: egg, larva (caterpillar), pupa (chrysalides or cocoon), and adult. Silk is a continuous protein filament around each cocoon, and in the silk industry, it is freed by softening the cocoon in water. (Paulino *et al.,* 2006) have isolated chitin from the chrysalides of silkworm (*Bombyx mori*) using a modified method employed for the extraction of chitin from crustaceans. Two procedures of chitin and chitosan extraction were investigated and compared; the first used a closed reactor while the second one used an open system with a stirrer. In both methods the dried chrysalides were treated with 1M HCl for 20 min at 100°C. The product was filtered and washed repeatedly with deionized water until neutral. Deproteination was carried out using 1M NaOH at 80°C for 24 h. The hot solution was filtered, washed with water then several times with dilute Na_2CO_3 solution and dried. Yields of chitin ranged between 2.6 to 4.2% based on the dry weight of the chrysalides. Deacetylation was made using NaOH solution (40 wt. %) with $NaBH_4$ (0.83 g L^{-1}) as the reducing agent using a closed reactor and oven for heating. The average DDA of the obtained chitosan ranged from 78 to 97% depending on the time of heating. The chitosan obtained, although in low yield, gave a final product of high purity and porosity.

Early works by Polish workers dealing with the production of chitosan from silkworm *Bombyx mori* are reported in Polish Journals (Weclawowicz *et al.,* 1983; Cieniewska *et al.,* 1983). Here the ground larvae, or the starved and dried silkworm pupae, were defatted by extraction with CCl_4 and the residues were separated from the protein and Ca salts by boiling in water at 1:15 ratio for 6 h, treating with boiling 3% NaOH at 1:150 ratio for 3 h, and treating with 2% HCl at 1:150 ratio for 2 h. The product was again treated with boiling 3% NaOH at 1:100 ratio for 3 h, bleached with $KMnO_4$ and oxalic acid mixture for 3 min,

filtrated, and treated with $NaHSO_3$ solution, filtrated, washed, and dried. The chitin obtained was converted into chitosan in approx 80% yield by boiling in 50% NaOH for 6 h.

Insect chitin possessing a shell-like structure was prepared from the bumblebee corpses by treatment with 1M HCl and 1M NaOH (Majtan *et al.*, 2007). The bumblebee chitin was compared with crustacean (shrimp) chitin using elemental analysis, Fourier-transform infrared (FT-IR) and solid-state ^{13}C-(CP/MAS)-NMR spectroscopy and confocal microscopy (Table 14.3 & 14.4) Both chitins (bumblebee and shrimp) exhibited identical spectra, while the bumblebee chitin had a 5% lower degree of acetylation and was characterized by a fine membrane texture as shown in Fig 14.1.

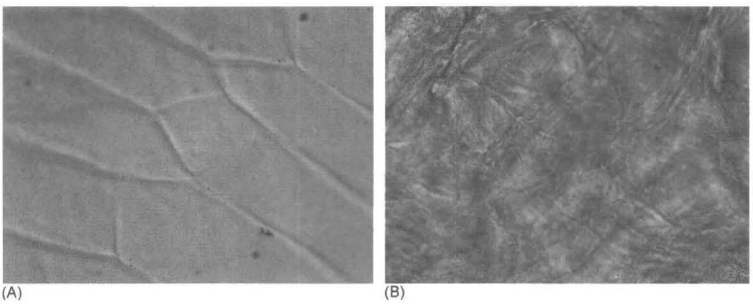


Fig 14.1. Confocal microscopy photographs of (*A*) bumblebee chitin and (*B*) shrimp chitin

The IR and ^{13}C NMR spectra of the bumble bee and crustacean are compared and are given in Table 14.3 and 14.4 respectively

Table 14.3. Assignments of the relevant bands of FT-IR spectra of bumblebee chitin and of shrimp α-chitin

Assignments	Wave number (cm^{-1}) Bumblebee chitin	Wave number (cm^{-1}) Shrimp α-chitin
ν (O–H	3444	3452
ν ($COCH_3$)	2933	2933
ν (C–H)	2891	2891
ν (C–O)	1659	1660
ν (C O of *N*-acetyl group)	1626	1626
δ (N–H of *N*-acetyl group)	1558	1558

ν: stretching; *δ*: bending

Table 14.4. ^{13}C CP/MAS-NMR spectral data of the bumblebee and crustacean chitin samples

^{13}C signal assignment	Bumblebee chitin	Shrimp α-chitin
C=O	173.34	172.76
C-1	103.91	103.91
C-4	82.75	82.78
C-5	75.49	75.49
C-3	73.08	73.09
C-6	60.59	60.60
C-2	54.87	54.78
CH_3	22.52	22.55

EXTRACTION OF CHITIN AND CHITOSAN FROM FUNGI

The traditional source of chitin is from shrimp and crab processing waste (Acosta *et al.,* 1993). However, the industrial isolation of the polymer is restricted due to the problems of seasonal and limited supply and environmental pollution while collecting large amounts of shell waste. Moreover, the conversion to chitosan at high temperature causes variability of product properties and chitosan quality, specially the molecular weight, and increases in the processing costs. At the same time, the waste liquid which contains base, proteins and protein degradation products from the industrial processing leads to the environmental pollution. Recently, some other sources, such as fungi, have begun to be employed to obtain chitosan. Production and purification of chitosan from the cell walls of fungi grown under controlled conditions offer greater potential for a more consistent product (Rane *et al.,* 1993; Suntornsuk *et al.,* 2002). The cell walls of Zygomycetes are characterized by the joint occurrence of chitosan and chitin, which have protective and supportive functions. Chitin, chitosan and β-glucan are the structural components, whereas mannoproteins, galactoproteins, xylomannoproteins and glucuronoproteins are the interstitial components of fungal cell walls. Proteinaceous and other cell components are removed along with interstitial components as a result of their solubility in alkaline medium. Chitosan is isolated from the remaining structural components (chitin and β-glucan) by acid extraction. The mycelia of various fungi including *Aspergillus niger* were used for the production of chitosan (Zhao *et al.,* 1999; Cai *et al.,* 2002).

In *A. niger*, the contents of chitosan reported range from 20 to 22% in dry mycelium (Zhao *et al.,* 1999). *A. niger* has often been used to produce citric acid. The final fermentation mash can contain 20 g/L mycelium. If the chitosan content of the mycelium was calculated according to 20%, the chitosan production yield would reach to 4.0 g/L. So, chitosan derived from

the waste mycelium of citric acid production plants could have wider availability. It is not only regarded as resource of chitosan, but also opens a new path for the integrated applications of the waste mycelium of citric acid production plant. The extractions of chitosan from the waste *A. niger* mycelium of citric acid production plant have mostly employed the traditional acid–alkali method. However, the chitosan obtained using this method was of poor quality and this technology produce environmental pollution. In this study (Zhao *et al.,* 1999), chitosan was prepared by treating the waste mycelium of citric acid with neutral proteases, such as lysozyme, snailase and the novel chitin deacetylase from *Scopulariopsis brevicaulis*, in order to utilize the waste resource, decrease the environmental pollution, and improve the quality of product. The optimum dosage of neutral protease and chitin deacetylase were 0.17 g (5100 units) per 100 g fresh mycelia and 1200 units per 100 g fresh mycelia respectively. The deproteination rate was 59.9%. The wt-average molecular weight (M_w), DDA and the content of glucosamine were 267.97 kDa, 73.6 and 84.4% respectively. Compared with chemical extraction methods, the M_w was three times higher while other parameters were very similar.

A new method has been developed for the extraction of chitosan from the zygomycetes cell wall (Zamani *et al.,* 2007). It is based on the temperature dependent solubility of chitosan in dilute sulfuric acid. Chitin is insoluble in cold or hot sulfuric acid; however the chitosan is soluble only in hot 1% sulfuric acid at 121°C within 20 min. The new method was developed to measure the chitosan content of the cell wall. The phosphate, protein, ash content, glucuronic acid, and degree of acetylation were then measured. The cell wall derivatives of the fungus *Rhizomucor pusillus* were examined by this new method. The results indicated that 8% of the biomass was chitosan. The phosphates constitute the major impurities in the biomass.

Microbiological processes were also used for chitin and chitosan production by *Cunninghamella elegans* (UCP 542) (*C. elegans*) grown in a new economic culture medium (Stamford *et al.,* 2007). The assay was carried out to evaluate the growth of the fungus using yam bean medium, in different times of growth (24, 48, 72, and 96 h), incubated at 28°C in an orbital shaker at 150 rpm. The polysaccharides were extracted by alkali-acid treatment, and characterized by IR spectroscopy, titration and viscosity. Yam bean (*Pachyrhizus erosus* L. Urban) is a leguminous plant native from the Amazon region and from Mexico semiarid region. The leguminous produces comestible tubercles and seeds with high level of protein and lipids. The tubercles were used as a good starch source for several industrial purposes (Stamford *et al.,* 2001). The main characteristic of yam bean is the simple manipulation and low nutrition requirements when compared with other similar cultures, and tuberous roots yields is up to 60 t/ha (Stamford *et al.,* 2007; Andrade *et al.,* 2000).

Cunninghamella elegans grown in the yam bean medium produced a high yield of biomass (24.3 g/mL) in 96 h. The use of biomass from fungi has demonstrated great advantages, such as: independence of seasonal factor, wide scale production, simultaneous extraction of chitin and chitosan, extraction process is simple and cheap resulting in reduction in time and cost required for production, and also absence of proteins contamination, mainly the proteins that could cause allergy reactions in individuals with shellfish allergies (Andrade *et al.,* 2000; Amorin *et al.,* 2001; Franco *et al.,* 2005; Andrade *et al.,* 2003).

The biomass contains chitosan (66 mg/g), and chitin (440 mg/g) after 72 h of growth. The DDA and the molecular weight were 6.2% and 3.25×10^4 g/mol for chitin and 85% and 2.72×10^4 g/mol for chitosan respectively. The results obtained suggest high biotechnological potential for yam bean as an economic source for the production of chitin and chitosan by *C. elegans*. Chitin and chitosan extracted from the mycelia biomass of *C. elegans* were used for the biosorption of heavy metals *e.g.* copper, lead and iron from aqueous solutions (Franco *et al.,* 2004). The growth curve of *C. elegans* was followed by the determination of the biomass, pH, glucose and nitrogen consumption. Chitin and chitosan were extracted by alkali-acid treatment and the yields were 23.8 and 7.8% respectively. The rate of metallic biosorption was dependent upon the concentration and pH metal solutions, and the best results were observed with pH 4.0. Chitosan showed the highest affinity for copper and chitin for iron adsorption. The results suggest that *C. elegans* (IFM 46109) is an attractive source of production of chitin and chitosan, with a great potential of heavy metals bioremediation in polluted environments.

A variety of culture and processing protocols using *Mucor rouxii* were studied for their effect on biomass yield and chitosan molecular weight (Arcidiancono *et al.,* 1992; Synowiecki *et al.,* 1997; Synowiecki *et al.,* 2003; White *et al.,* 1979; Wang *et al.,* 1999). The chitosan yield ranged 5–10% of total biomass dry weight and 30–40% of the cell wall. Of the culture parameters studied, length of incubation and medium composition affected the biomass production and molecular weight of the isolated chitosan. Modification of the processing protocol, including the type and strength of acids, and cell wall disruption in acid prior to refluxing were used to optimize the efficiency of chitosan extraction. The chitosan obtained directly from the fungal cell wall had a higher DDA than common chitosan from the chemical conversion process. A typical procedure for isolation of chitin and chitosan from the mycelia of *Mucor roxii* is as follows: deproteination of 3–10 g lyophilized mycelia with 2% (w/v) NaOH solution (30:1 v/w, 90°C, 2 h), separation of the alkali-insoluble fraction (AIF) by centrifugation (4000 g, 15 min), extraction of chitosan from AIF under reflux (10% v/v acetic acid, 40:1 v/w, 60°C, 6 h), separation of the crude chitin by centrifugation and precipitation of chitosan from the extract at pH 9.0,

adjusted with 4M NaOH solution. Crude chitin and chitosan were washed with water, ethanol and acetone and air dried at 20°C. During the cultivation period the biomass increased rapidly up to 48 h of growth and the final density reached during this time was 4 g dry mycelia per liter of the medium. The pH of medium drops from 4.5 to 4.1 after 96 h of the incubation, a value favorable to prevent undesirable microbial contamination of the medium. The main components of the mycelia can be seen in Table 14.5.

Table 14.5. Changes in the main components of the mycelia during growth of *M. rouxii*

	Component Growth time (h)			
	12	**24**	**48**	**96**
Water (%)	81.1 ± 2.2	82.9 ± 2.3	84.0 ± 0.9	81.0 ± 2.1
Proteins (% db) Deproteinized	63.7 ± 1.2	61.7 ± 0.5	60.1 ± 1.0	55.5 ± 0.0
Mycelia (% db) Residue insoluble in	11.8 ± 0.3	13.8 ± 0.46	16.45 ± 1.0	17.1 ± 1.2
CHCOOH (%db)	7.0 ± 0.9	7.7 ± 0.7	8.9 ± 0.3	9.6 ± 0.0
Chitosan (% db)	4.4I ± 0.5	6.1 ± 1.0	7.3 ± 0.10	7.0 ± 0.10

db, dry weight basis of the mycelia

Another work (Wang *et al.,* 1999) describes the extraction of chitosan from the Mycelia of *Mucor rouxii* as a source of chitin and chitosan which could be useful for medical and cosmetic purposes. The influence of growth time on the contents of chitosan and other main components of *Mucor rouxii* mycelia, as well as the yield of chitin and chitosan during the isolation process have been examined by the Chinese authors (Wang *et al.,* 1999). The mycelia yield increased rapidly up to 48 h of growth. The yield of the culture during this time was about 13 g of dry mycelia per L of medium. Chitin and chitosan isolation involved deproteination of the mycelia with 2% NaOH solution at 90°C for 2 h, extraction of chitosan with 10% acetic acid at 60°C for 6 h and subsequent precipitation with NaOH at pH 9.0. Therefore 2 - day old culture of the mycelia of *M. rouxii* may serve as a source of chitin and chitosan with yields of 8.8% and 7.4% on a dry weight basis of the mycelia respectively.

Work dealing with the isolation of chitin from *M. roxii* was published by Chatterjee *et al.* (2005). Three fermentation media were used to study the growth and production of chitosan from *M. rouxii*. The first medium is the molasses salt medium (MSM) which contains 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.001% $FeSO_4$, 0.001% $MgSO_4$, 0.2% yeast extract and molasses as carbon source. Molasses were added to the media to obtain sucrose concentrations

varying from 2 to 5%. The second medium is the yeast peptone glucose medium (YPG). YPG was made with yeast extract 0.3%, peptone 1% and glucose 2%. The third medium was the potato dextrose broth (PDB) which contains potato extract 20% and dextrose 2%. The pH of all media was adjusted to 5.0, and 50 ml of each medium was added to a 250 mL Erlenmeyer flask and sterilized by autoclaving at 121°C for 15 min. Inocula were prepared by growing the organism in potato dextrose agar (PDA) plates at 30°C for 3 days. Flasks containing the media were inoculated with one 5 mm diameter mycelium covered agar disk containing 6.4×10^6 spores/disk was used as inoculum and incubated at 30°C under submerged conditions (120 rpm) for different periods of time. At the end of the desired incubation period mycelia were harvested by filtration, dried by lyophilization and weighed. Mycelia and culture filtrates were stored at -20°C until use. Mycelia (biomass) were autoclaved at 121°C for 15 min after homogenizing in a blender with 1N NaOH (1:40, w/v). The alkali insoluble mass was washed thoroughly with water followed by ethanol and refluxed with 100 volumes of 2% acetic acid (v/v) for 24 h at 95°C. The slurry was centrifuged at 12,000 rpm for 45 min at 4°C. Chitosan was precipitated out from the supernatant by adjusting the pH to 8.5 with 1N NaOH; washed several times with chilled water and triturated with acetone.

The yield of chitosan isolated from *M. roxii* cultured in these three media under submerged conditions has been found to be almost the same, being 0.61 g/l for MSM, 0.51 g/l for PDB and 0.56 g/l for YPG respectively. Their physico-chemical properties such as ash, moisture, protein contents do not show much difference. However, their polydispersity and crystallinity were different. Chitosan from MSM was less polydispersed and more crystalline compared to the other two (Chatterjee *et al.*, 2005).

The DDA and the Cu^{2+} adsorption capacity of the fungal chitosan were measured and compared with those of authentic samples of chitosan prepared by common chemical methods from crustaceans. The Cu^{2+} adsorption capacity of the fungal chitosan was higher than that of chitin. Chitosan with the same DDA was independent of the molecular weight from the various sources (Chatterjee *et al.*, 2005). This provides an added advantage for this method beside the green chemistry involved.

Chitosan was also extracted from five different fungal cells with NaOH and acetic acid, with yields varying from 1.2 to 10.4% of the dry fungal cell weight (Miyoshi *et al.*, 1992). The DA (degree of acetylation) of the extracts measured by the colloidal titration method varied considerable depending on the individual species.

The cell wall of *Rhizopus oryzae* is composed of chitin and chitosan as was illustrated by IR spectrometry and thermogravimetry (Chen *et al.*, 1995).

The author claimed that the cell wall of *Rhizopus oryzae* is capable of replacing the shells of shrimp and crab as raw material for extracting chitosan.

Chitosan extraction of mycelia from *Absidia coerulea* ATCC 14076 was conducted under hot alkaline and acid treatments (Wu *et al.,* 2001). The alkaline treatment was carried out at 95°C and 121°C using 1N NaOH for various periods of time. Acid treatment was carried out using three acids HCl, formic and acetic acids for periods of 3, 6 and 12 h. All extractions were carried out using 15 mL of 2% acid solution at room temperature (23°C) and at 95°C. The highest yield of chitosan was obtained with alkaline extraction at 121°C for 30 min and HCl extraction at 95°C for 2 h. The chitosan yield ranged from 9.2 to 10.7% based on dry weight of the mycelia. The viscosity of the chitosan obtained decreased with increasing the alkaline heating time. The highest viscosity (2.69 cSt) was obtained with alkali treatment at 121°C for 30 min. (Kobayashi *et al.,* 1988) studied the effect of alkaline treatment on chitosan extractability from *Absidia butleri.*

Rhizopus oryzae (Paul *et al.,* 2005) is alternative source of chitin and chitosan. The yield, DDA, average molecular weight and color of chitosan extracted from three fungal isolates, *Absidia* sp. DR, *Absidia sp.2a1 and Rhizopus* sp. grown on three different growth media YPG (complex media), BG (semi-defined media), and TVB (defined medium) were compared. Results show that fungi grown on YPG gave rise to higher yield of chitosan per biomass (10% to 18% for *Absidia sp.* and 11% to 16% for *Rhizopus sp.*) and a higher molecular weight (2.30×10^5) as compared to BG (6.76×10^4) and TVB (7.37×10^4). The DDA for chitosan was found to be highest when extracted from fungi grown on TVB (84.5%) followed by BG (83.8%) and YPG (82.6%).

Chitinous material was extracted from mycelia of *Aspergillus niger* and *Mucor roxii* grown in yeast peptone dextrose broth for 15 and 21 days respectively (Wu *et al.,* 2005). The maximum glucosamine level determined in the mycelium of *A. niger* was 11.10% based on the dry weight and in the mycelium of *M. roxii* it was 20.13%. It has been found that *M. roxii* mycelia contained both chitin and chitosan, whereas *A. niger* contained only chitin. The yields of crude chitin from *A. niger* and *M. roxii* were 24.01 and 13.5%, respectively and the yield of chitosan from *M. roxii* was 12.49%. Significant amounts of glucan were associated with chitinous compounds from both species and could not be eliminated by the extraction method used. The degrees of acetylation were detected to be 76.53% and 50.07% for chitin from *A. niger* and *M. roxii* respectively, and 19.5% for *M. roxii* chitosan. The crystallinity of fungal chitin and chitosan was estimated and was found to be less than in corresponding materials from shrimp shells.

Chitosan was extracted from the mycelia of *Rhizopus oryzae* USDB 0602 at various phases of growth (Tan *et al.,* 1996). The growth phase which

produced the most extractable chitosan was determined to be the late exponential phase. In contrast to previous work on the screening of chitosan from fungal sources, mycelia of the fungi used in this study were harvested at their late exponential growth phase instead of at a fixed incubation time. The amount of extractable chitosan varied widely among the fungal strains. *Gongronella butleri* USDB 0201 was found to produce the highest amount of extractable chitosan per ml of substrate, followed by *Cunninghamella echinulata* and *Gongronella butleri* USDB 0428. However, in terms of yield of chitosan per unit mycelia mass, *C. echinulata* was the best strain among all fungi in this study. Therefore, besides *G. butleri* USDB 0201, *C. echinulata* can also be considered to be for use in the commercial production of chitosan.

Chitosan was extracted from *Absidia glauca* var. *paradoaxa* IFO 4007 in order to be used as a clarifying agent for apple juice (Rungsardthong *et al.*, 2006). The effectiveness of fungal chitosan was compared with that of commercial chitosan prepared from shrimp shells and it was found that fungal chitosan provides greater clarity for the apple juice. *Absidia glauca* var. *paradoaxa* IFO 4007was cultured in liquid medium at 24.8°C with an agitation speed 100 and 200 rpm. The harvested mycelia were treated with hot 2% sodium hydroxide to isolate the alkali-insoluble materials. The extraction of chitosan from the alkali-insoluble materials was carried out with 2% acetic acid at room temperature. The maximum chitosan extracted was 0.6 and 1.28 g/l at 100 and 200 rpm, respectively. The degree of deacetylation of the extracted chitosan was 86%. The viscosity of 0.1% chitosan in 0.5% acetic acid was 4.0 cP.

Free chitosan, 2 g/100 g mycelia from *Gongronella butleri* and 6.5 g/100 g mycelia from *Absidia coerulea* were isolated (New *et al.*, 2007) by 1M NaOH at 45°C for 13 h and 0.35 M acetic acid at 95°C for 5 h. Both myceliar matrices did not break down under these conditions. However, myceliar matrices could be decomposed by treatment with 11M NaOH and 0.35M acetic acid at 45°C for 13 h at 95°C for 5 h. Then the total chitosan extracted was 8–9 g/100 g mycelia from both fungi. According to these results, *G. butleri* has higher amount of complexed chitosan and *A. coerulea* has higher amount of free chitosan.

The choice of the proper chitosan extraction procedure is important for high yield production of fungal chitosan. It is essential to free the chitin/chitosan from its anchorage in the membrane and to the β-glucan. High concentration of NaOH is required in the first step of this solubilization, alpha-amylase enzyme has to be applied to separate the chitosan from the glucan fraction. Using these improved treatments a better quality of fungal chitosan can be produced. Fungal chitosan has a high degree of deacetylation, low viscosity, low molecular weight, high solubility and does not contain shrimp allergenic protein. With these properties, this fungal chitosan will find its way into the agricultural and especially the pharmaceutical industry.

An interesting study regarding the accumulation of chitinous material in *Agaricus bisporus* (Table mushroom) stalks was reported (Wu *et al.,* 2004). The chitinous material was extracted after alkali treatment and acid reflux of the alkali insoluble material and then analyzed for yield, purity, degree of acetylation DA, and crystallinity. The total glucosamine content in mushroom stalks increased from 7.14% dry weight (DW) at harvest day 0 to 1.0% DW and 19.02% DW after 15 days of storage at 4°C and 5 days of storage at 25°C, respectively. The yield of crude chitin isolated from stalks stored at 25°C for 5 days was 27.00% DW and consisted of 46.08% glucosamine and 20.94% neutral polysaccharides. The DA of fungal chitin varied from 75.8 to 87.6%, which is similar to commercially available crustacean chitin.

USE OF ENZYMES DURING THE EXTRACTION PROCESS

Proteolytic extract from *Carica papaya* was used to enhance the extraction of chitin from shrimp shell wastes, the quality of the chitin obtained was comparable to that obtained by conventional chemical method (Jasmine *et al.,* 2007).

In an attempt to use more environmentally friendly methods to extract chitin from prawn shrimp shell, lactic acid fermentation was used (Beaney *et al.,* 2005). The fermentation removed 690 g/kg and 770 g/kg of inorganic matter, 490 and 440 g/kg of protein and 540 and 770 g/kg of lipids from the shells at lab and pilot plant scales, respectively. Although the extraction of chitin was incomplete, the degree of acetylation (DA) of the chitin, the molecular weight and the DDA of the chitosan were similar to those obtained chemically.

Chitin can be deacetylated by the enzyme deacetylase. Five fungal strains have been compared for their ability to produce the extracellualr chitin deacetylase (Win *et al.,* 2000). Three strains, *Colletotrichum lindemuthianum*, *Asergillus alliaceus* and *Aspergillus nidulans* produce extracellualr deacetylase that can act on natural (10% deacetylated) chitin whereas the extracellualr enzyme from *Absidia coerulea*, *Mucor rouxii* and *Absidia glauca* cannot be used with natural chitin but can act only on partially deacetylated chitin (max. activity with around 60% DDA). The enzymes have further been characterized by their optimal temperature and pH for enzymatic deacetylation, their thermostability, and their sensitivity to the reaction product. The best enzyme producing strains are *Absidia coerulea* and *Colletotrichum lindemuthianum*. *Absidia* has the advantage that it can be produced easily (maximum activity was produced within 3 days) but cannot act on natural chitin. *Colletotrichum* has the advantage that it can act on natural chitin but is less attractive due to its plant pathogenicity and its slow growth (max activity was produced after 5 days). A synthetic medium containing standard amounts of inorganic salts, glucose and lactose appeared to be an efficient carbon source for the application of

Absidia. Growth was optimal at 30°C and a pH in the neutral range above 4.5. Enzyme levels were significantly enhanced if chitin was included in the medium. This inducer of enzyme activity is most effective at a degree of deacetylation of about 60%.

The catalytic properties of chitin deacetylase from *Mucor roxii* were studied with the aim of using the results to control the properties of chitosan prepared by enzymic deacetylation (Kolodziejska *et al.*, 1999; Malesa-Ciecwierz *et al.*, 1997). The enzymes present in *Mucor roxii* mycelium are able to hydrolyze not only colloidal chitin but also chitosan of low acetylation degree. The maximum chitinolytic and chitosanolytic activities were at pH 6.1. The chitosanolytic activity of the extract decreases significantly below pH 6.0 and above 7.7 (Kolodziejska *et al.*, 1996).

A crude deacetylase extracted from the mycelium of *Mucor roxii* exhibited maximum activity at pH 5.8 with both water soluble and acid soluble chitosan. The extracellualr enzyme of the culture medium exhibited maximal activity at pH 4.8. The deacetylase in the crude extract was stable over the pH range of 4.1–8.9 at 25°C and retained 85–100% of activity at 40°C. The extract was most active towards acid-soluble chitosan at 50°C and pH 5.8. Ca^{2+}, Mn^{2+}, and Zn^{2+} had no effect on activity, while EDTA, Fe^{2+}, and Fe^{3+} caused partial inhibition of the enzyme. *Mucor roxii* may be a convenient source of deacetylase, because the biomass, after the enzyme isolation, may be used for isolating chitosan.

Chitin deacetylase, (EC 3.5.1.4), the enzyme that catalyzes the hydrolysis of acetamido groups of N-acetyl-D-glucosamine of chitin, has been purified to homogeneity from the culture filtrate of the fungus *Colletotrichum lindemuthianum* and further characterized (Tsigos *et al.*, 1996). The enzyme as compared to all other corresponding enzymes, exhibits different properties, *e.g.*, increased thermostability, different pH optimum, and is not inhibited by acetate. Furthermore, initial results on the effectiveness of chitin deacetylases from *Mucor rouxii* and *Colletotrichum lindemuthianum* on chitin and chitosan substrates are also presented.

The kinetics of deacetylation of chitosan by chitin deacetylase, an enzyme isolated from *Absidia orchidia* was investigated (Jaworska *et al.*, 2003). The experiments were performed at pH 2.0 and a temperature of 50°C in citric buffer (0.2 M) for chitosan concentrations ranging from 0.1 to 4.0 g/L. The concentration of chitin deacetylase was increased from 3 to 10 μg/mL.

Chitin deacetylase is an enzyme presented mainly in the mycelium of fungi belonging to Zygomycetes and after separation, it can be used for enzymatic deacetylation of chitin or chitosan (Jaworska *et al.*, 2003). Enzymatic deacetylation of chitosan was investigated only on a small laboratory scale, although this process can play a more important role in the future. Loosening of chitin crystal structure due to chemical treatment

in the process of deacetylation makes the acetyl groups more accessible for the enzyme. The process for the production of chitosan with a very low degree of acetylation degree (close to 0%) and medium or high molecular weight can follow two consecutive steps: chemical and enzymatic deacetylation.

A one-step extraction of chitin from red crab shell waste, cofermentation with *Lactobacillus paracasei* subsp. *tolerans* KCTC-3074, a lactic-acid-producing bacterium, and *Serratia marcescens* FS-3, a protease-producing bacterium, has been reported (Jung *et al.,* 2006). Fermentation with single strain (L. 3074 or FS-3) was studied. At day 7, the pH in L. 3074, FS-3, and L. 3074+FS-3 (1:1) treatment was decreased from 6.90 to 3.30, 5.88, and 3.48, respectively. Ash content in the residue after fermentation treatment of crab shells in L. 3074 and L. 3074+FS-3 (1:1) treatment drastically decreased from 41.2% to 3.19 and 1.15%, respectively. In L. 3074+FS-3 (1:1) cofermentation, the level of demineralization was the highest value of 97.2%, but the level of deproteination in the cofermentation was 52.6% on day 7. Protein content in the treatment of FS-3 alone reduced from 22.4 to 3.62%. These results indicated that cofermentation of the shells using the two strains is efficient and applicable for the one-step extraction of crude chitin from red crab shell waste.

The same authors conducted a successive two-step fermentation process from red crab shell wastes for biological extraction of chitin. A combination of the 1st step with a lactic acid bacterium *Lactobacillus paracasei* subsp. *tolerans* KCTC-3074 and a 2nd step with a protease producing bacterium *Serratia marcescens* FS-3, and vice versa was reported (Jung *et al.,* 2007). In the 1st step fermentation with KCTC-3074, the pH decreased rapidly from pH 6.90 to 3.31 and TTA increased rapidly to 10.99 in 5 days. On day 7 in the 2nd step fermentation with FS-3, the pH dropped further to 2.82 and TTA also dropped to 1.71. In the 1st step fermentation using FS-3, the pH decreased slightly from pH 6.90 to 5.89, and TTA was low at 1.50 for 5 days. On day 7 in the 2nd step fermentation with KCTC-3074, the pH value was 3.62, and TTA increased to 8.95. The successive fermentation in the combination of FS-3 and KCTC-3074 gave the best results in co-removal of $CaCO_3$ and proteins from crab shells. In this combination, the rates of demineralization and deproteination were 94.3% and 68.9%, respectively, at the end of fermentation. To date, this is the 1st report on successive fermentation for the biological extraction of chitin from crustacean shells.

Recently, studies of biological process for chitin production have been reported using organic acid producing bacteria and enzymes for the demineralization and deproteination of crustacean shells. Deproteination processes have been reported for chitin production mainly from shrimp using Enzymatic processes (Synowiecki *et al.,* 2000) and microbes such as *Lactobacillus* (Rao *et al.,* 2002; Rao *et al.,* 2001; Rao *et al.,* 2000) *Pseudomonas*

aeruginosa K-187 (Oh *et al.,* 2000), and *Bacillus subtilis* (Yang *et al.,* 2000). Also, demineralization processes have been reported for crayfish using *Lactobacillus pentosus* 4023 (Bautista *et al.,* 2001). In these biological processes, demineralization and deproteination occur simultaneously but incompletely.

MICROWAVE ASSISTED DEACETYLATION OF CHITIN

Chemical N-deacetylation of chitin, during chitosan preparation from seafood waste usually requires very drastic processing conditions involving the use of concentrated alkali, high temperatures, and lengthy treatments. A novel method to prepare chitosan from squid pen (*Loligo* spp.) waste was investigated, aiming to decrease the long processing times typically required to achieve N-deacetylation (Goycoolea *et al.,* 1997). Finely ground dry squid pen meal was dispersed in a 30% solution of NaOH, subjected to microwave radiation (approximately 2.45 GHz) for 22 min, washed with cold water, and dried. The physicochemical characteristics of chitosan thus obtained, were compared to those of the materials obtained under conventional heterogeneous (NaOH 50%; 90°C: 6 h, bubbling N_2) and homogeneous (NaOH 11.1%; 0; 25; 77 h) conditions. The microwave-treated chitosan was soluble in dilute acetic acid. It had a DA of approximately 12.8%, as assessed by UV spectroscopy. This was lower than that of chitosan obtained under conventional heterogeneous and homogeneous conditions; the DDA level was 16.2 and 36.8%, respectively. The microwave-accelerated deacetylation reaction produced a slight decrease in intrinsic viscosity ($[\eta] \approx 695$ mL/g), with respect to the conventional products ($[\eta] \approx 821$-1102 mL/g), indicative of chain degradation. X-ray diffraction analysis revealed that the crystalline structure of the microwave-treated squid pen chitosan was similar to that of the conventionally produced material. Both differed from the typical chitosan L-2 polymorph diffraction pattern, with loss of 100 equatorial reflections at a lattice angle 2θ around 10.6° indicative of a more amorphous configuration. Solid-state CP-MAS 13C NMR spectra, confirmed that deacetylation in the microwave-treated sample proceeded to a greater extent than for a homogeneous conventionally treated sample. Also those similar polymorphic structures were obtained on both materials, since identical chemical shifts and peak features were observed for different C signals.

Preparation of chitosan by microwave heating (2450 MHz 700 W) was also studied by (Bofen *et al.,* 1997). Chitosan was produced from the reaction of chitin with 45% NaOH aqueous solution. The preparation time was considerably shortened, compared to ordinary method by water bath at 85°C. The DDA was > 80%, and viscosity (3.80-9.00 Pa.s) of chitosan was higher than that of chitosan prepared by the water bath method. Viscosity decreased

with increasing DDA. Various methods for determining the degree of deacetylation were compared.

Chitosan was prepared rapidly by using 34% (wt/wt) NaOH solution under microwave radiation (Ding *et al.,* 2003). The molecular weight and DDA were also measured. The quality of the chitosan can be easily controlled, and the reaction time is quite short using microwave irradiation.

CONCURRENT PRODUCTION OF CHITIN FROM SHRIMP SHELL AND FUNGI

The control of fungal fermentation processes to produce quality chitin makes fungal mycelia an attractive alternative source. However crustacean shells constitute the traditional and current commercial source of chitin. Therefore, the exploitation of both of these sources to produce chitin in a concurrent process could be advantageous and has been reported (Teng *et al.,* 2001). Three proteolytic *Aspergillus niger* strains (0576, 0307 and 0474) were selected for screening for protease activity from among 34 zygomycete and deuteromycete strains. When fungi and shrimp shell powder were combined in a single reactor, the release of protease by the fungi facilitated the deproteination of shrimp-shell powder and the release of hydrolyzed proteins. The hydrolyzed proteins in turn were utilized as a nitrogen source for fungal growth, leading to a lowering of the pH of the fermentation medium, thereby further enhancing the demineralization of the shrimp-shell powder. The shrimp-shell powders and fungal mycelia were separated after fermentation and extraction of chitin with 5% LiCl/DMAc solvent. Chitin isolates from the shells were found to have a protein content of less than 5%, while chitin isolates from the three fungal mycelia strains had protein content in the range of 10–15%. The relative molecular weights as established by GPC for all chitin samples were in the 105 Dalton range. All samples displayed characteristic profiles for chitin in their FTIR and solid-state NMR spectra. All chitin samples evaluated with MTT and Neutral Red assays with three commercial cell lines did not display cytotoxic effects.

CHITOSAN HYBRID MATERIALS

A large variety of composite materials based on organic and inorganic materials have been reported in the recent years. By mixing polymers and inorganic materials (ceramics or their precursors) at the molecular level, it is possible to create hybrid materials. These materials are known as creamers (Schmidt *et al.,* 1994). They can exhibit useful properties of both components: heat resistance, retention of mechanical properties at high temperature, low thermal expansion coefficient from ceramics, toughness, ductility, and ease of processing from the high polymers (Mark *et al.,* 1985;

Ahmad *et al.,* 2001; Ahmad *et al.,* 2007). The properties of the resulting hybrid can vary from soft and flexible to brittle and hard materials depending on the chemical structure and the proportions of organic and inorganic components. A successful approach for preparing such hybrid materials has been *in situ* polymerization of metal alkoxides in organic polymer matrices via the sol-gel process (Brinker *et al.,* 1990). The hydrolysis and polycondensation of a metal alkoxides $M(OR)_n$, where M can be Si, Ti, Zr, Ce etc, can produce rigid inorganic networks. The silica is considered to be the most favorable inorganic component because of low reactivity of its alkoxide (Myers *et al.,* 1995), though many other metal oxides such as titania and zirconia have been used to reinforce the organic polymers. The chemistry of the sol-gel process involves hydrolysis and condensation processes:

Hydrolysis: $M(OR)_n + nH_2O \rightarrow M(OH)_n + nROH$

Condensation: $M(OH)n \rightarrow MO_{n/2} + n/2H_2O$

The hydrolysis reaction through the addition of water replaces alkoxide group (-OR) with hydroxyl group (-OH). As the number of alkoxane bonds increases, the individual molecules are bridged which jointly aggregate in the form of a sol. When the sol particles aggregate or inter-knit into a network, a gel is formed. Upon drying, the trapped volatiles (alcohol and water) are driven off and the network shrinks further as condensation occurs. The microstructure of the inorganic network is dependent on the kinetics of the hydrolysis/condensation reactions and these are largely controlled by the type of solvent, pH of the solution, the nature of alkoxy group and the water to metal oxide ratio (Brinker *et al.,* 1990).

The sol-gel method allows *in situ* development of an inorganic ceramic network within a polymeric matrix producing hybrid materials at the molecular level and a large number of organic-inorganic hybrids have been prepared (Al-Sagheer *et al.,* 2008; Carlos *et al.,* 2003). In order to develop interaction between the disparate phases, different techniques have been used. The organic polymer is suitably modified to include the appropriate functional groups to link the chain with an inorganic network. Suitable organosilanes such as γ-glycidoxypropyltrimethoxysilane (GPTMS) (Liu *et al.,* 2004), 3-isocyanatopropyl-triethoxysilane (ICPTES) (Sliva *et al.,* 2005) and 3-aminopropyl triethoxysilane (APTEOS) (Chen *et al.,* 2007) been have used as compatibilizers along with tetra-ethoxysilane (TEOS).

Being a natural chiral compound, chitosan is a multifunctional polymer containing large numbers of amino groups together with hydroxyl groups capable of assembling with template molecules such as amino acids through hydrogen bonds (Aburto *et al.,* 2004; Xia *et al.,* 2005). The poor solubility of chitosan however limits its application and processing convenience.

Considerable efforts have been made to develop water soluble chitosan to improve these drawbacks. Versatile modifications can be performed on hydroxyl and amino groups on glucosamine units of chitosan to tailor its physico-chemical properties (Ding *et al.,* 2003; Sashiwa *et al.,* 2003). On the other hand poor mechanical strength might also be critically important in some applications (Francis *et al.,* 2000; Zeng *et al.,* 1998) Synthesis of chitosan-silica hybrids therefore may provide another approach to improve its physico-chemical properties (Mizushima *et al.,* 1992; Suzuki *et al.,* 1997).

In 1997, Retuert *et al.* (1997) reported the synthesis of partially deacetylated chitin/silica hybrid via a sol-gel process using TEOS as a precursor. The resulting polymer hybrids were compatible with each other over a wide range of composition. GPTMS with an epoxy group and three methoxysilane groups has also been used as cross-linking agent. Chitosan-silica hybrids were obtained by adding GPTMS in chitosan-acetic acid aqueous solution. Developing covalent bonds between the silica domain and chitosan chains increases mutual compatibility, and moderates the rate of the silanol condensation reaction to prevent phase separation during film formation (Liu *et al.,* 2004). The chitosan-silica hybrid materials obtained showed improved stability in water to elevate the potential of chitosan as a biomaterial and as separation material. An enantioselective chitosan/GPTMS hybrid membrane was prepared in an aqueous phase by the sol-gel method using chitosan as the bulk polymer (Jiang *et al.,* 2006). These hybrid membranes had the advantage of lowering the swelling degree as well as improving the separation properties. Chao *et al.* (2008) used GPTMS as the cross-linking agent and NaCl particles as the porogen, to prepare a chitosan-silica porous hybrid membrane. Five different compounds *i.e.* histidine, glutamic acid, tyrosine, L-DOPA, and p-aminobenzoic acid were individually grafted onto the chitosan/GPTMS membrane. The grafted chitosan/GPTMS membranes were linked to genipin for affinity adsorption of tyrosinase from a crude *Agaricus bioporrus* solution.

Recently, the synthesis of amine-functionalized cross-linked sol-gel derived hybrids in which the siliceous backbone is covalently bonded to poly(ether) chains by means of urea or urethane cross-links originating from ICPTES, named as di-ureasils and di-urethanesils, respectively, has been carried out (Bermudez *et al.,* 1999; Stathatos *et al.,* 2002; Bekiari *et al.,* 2000; Fu *et al.,* 2004; Goncalves *et al.,* 2004; Carlos *et al.,* 1999; Carlos *et al.,* 2001). Silva *et al.* introduced ICPTES to prepare chitosan-silica hybrids in which covalent bonded bridges, essentially comprising of urea, linked to chitosan and the poly (siloxane) network. Fig 14.2 shows the preparation of the chitosan-siloxane hybrids (Sliva *et al.,* 2005).

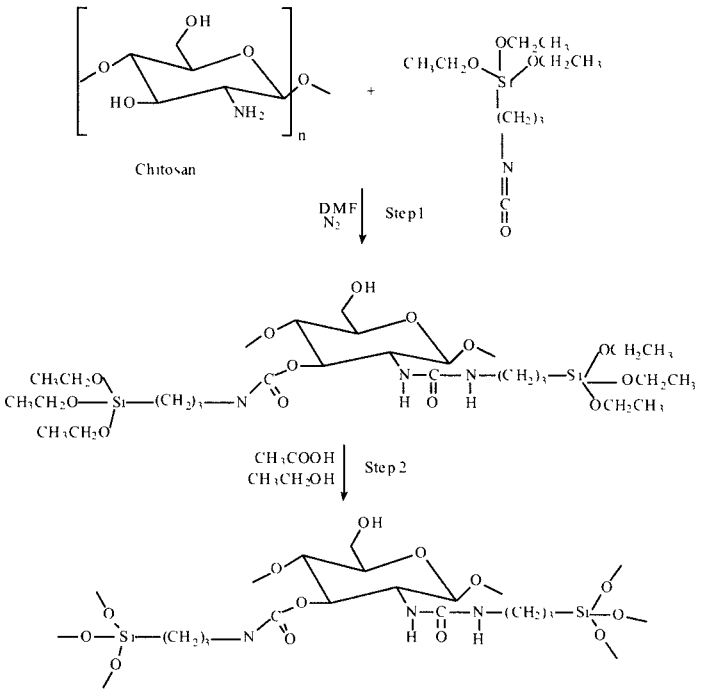


Fig 14.2. Scheme proposed for preparation of bonded chitosan-siloxane hybrids

The chitosan-siloxane hybrids also show interesting photoluminescent features. The photoluminescence spectra show the presence of a new band with higher energy and longer lifetime, compared to the characteristic emission of pure low molecular weight chitosan. This band associated with electron-hole recombination arising from silicon-related defects at the surface of the siliceous nano-domain. These hybrids are bioactive materials, the apatite formation was shown to depend on the amount and arrangement of silanol groups. Yeh *et al.* (2007) improved the hybrids of chitosan/silica by preparing hybrid materials using a mixture of tetraethoxysilane/ vinyltriethoxysilane (TEOS/VTES) and chitosan. They proved that hydrogen bonds emerge between chitosan and SiO_2 in hybrid material.

Chen *et al.* (2007a, b) used APTEOS to cross-link with chitosan to prepare the hybrid membrane. The amorphous region in the hybrids increased with increasing APTEOS content and reached a maximum when APTEOS content was 10%. These hybrids exhibited a low degree of swelling in ethanol/ water mixture while their solubility and diffusion selectivity increased with increasing ethanol content. Compared to pure chitosan, the hybrid membrane pervaporation properties, permeation flux and water permselectivity increased markedly with increasing APTEOS content. Li *et al.* (2008) employed the same hybrids of chitosan/silica using APTEOS as

coupling agent for protein reorganization. The model template protein, bovine serum albumin, was covalently immobilized on chitosan. APTEOS and TEOS were used to assemble and polymerize the polysaccharide-protein complex via sol-gel process in aqueous solution at room temperature. The protein-imprinted surface possessed high affinity toward template protein. The complementary nature of the hydrophilicity/hydrophobicity was a major factor affecting imprinting and template recognition. Easy preparation of the imprinted material, high affinity and good re-usability increases its application as a biosensor.

Toa *et al.* (2007) prepared chitosan/TiO_2 hybrid film by the sol-gel method using chitosan, acetic acid, tetrabutyl titanate and butyl alcohol mixture to get a better tensile strength through the response surface method. Chen *et al.* (2003) have reported glucose biosensors based on an organically modified sol-gel/chitosan composite in which ferrocene acted as mediator. They found that the biosensor was not good if ferrocene alcohol or acetone solution was dropped directly on the surface of the glass carbon electrode. This led to accumulation of the ferrocene at the margin of the electrode surface after solvent evaporation. This resulted in non-uniformity of the mediator at the electrode surface. Tan *et al.* (2005) produced an amperometric glucose biosensor based on glucose oxidase immobilized in sol-gel chitosan-silica hybrids. The composite film was prepared from chitosan and methyltrimethoxysilane (MTOS) on the surface of Prussian blue-modified glass carbon electrode. This biosensor had high sensitivity, good repeatability and reproducibility, rapid response, good selectivity, and long-term stability. It can be used successfully to determine the glucose concentration in real human blood samples. Development of an optical biosensor based on immobilization of 3-methyl-2-bezothiazolinone hydrazone in hybrid nafion/ sol-gel silicate film and tyrosinase in chitosan film for detection of phenolic compound has also been described (Abdullah *et al.,* 2006). These hybrids can provide a moderate hydrophobic environment for dye immobilization and permits permeation of the analyte/enzymatic product into material structure where recognition can occur.

Kang *et al.* (2008) developed another glucose biosensor based platinum nanoparticles-deposited carbon nanotubes in sol-gel chitosan-silica hybrid. This hybrid was produced by mixing MTOS with the carbon nano tube-Pt nanoparticles-chitosan solution. With the immobilization of glucose oxidase into the sol-gel, the glucose biosensor was fabricated. Tan *et al.* (2005) developed an amperometric cholesterol biosensor based on multi-walled carbon nanotubes and an organically modified sol-gel/chitosan hybrid composite film which was used to determine the free cholesterol concentration in human blood samples.

Recent research work (Yuan *et al.,* 2007; Libby *et al.,* 2003; Wu *et al.,* 2007; Li *et al.,* 2006) has demonstrated that polymer-zeolites hybride

membranes can be utilized for the preparation of a direct methanol fuel cell. Wang *et al.* (2008) selected chitosan as bulk polymer owing to its high proton conductivity, excellent alcohol barrier properties, facial chemical modification and low cost to prepare a series of chitosan/zeolite hybrid membranes for a direct methanol fuel cell. By adding plasticizer (sorbitol) in the membrane casting solution and/or elevating solvent evaporation temperatures during membrane fabrication, the glass transition (Tg) and crystallinity of the chitosan/zeolite hybrid membrane were markedly decreased. The delicate tailoring of interfacial morphologies in organic-inorganic hybrid membranes improved permeation property and consequently lead to an improved performance in suppressing methanol crossover. Wang *et al.* (2008) found that zeolite significantly influences the free volume characteristics of chitosan in hybrid membranes and their performance was highly dependent on the zeolite particles, pore size and hydrophilic/hydrophobic nature. Incorporation of hydrophobic zeolites increases the diffusion resistance of methanol, and consequently decreased the methanol permeability, whereas incorporation of hydrophilic zeolites decreases the diffusion resistance of methanol and decreases the methanol permeability. They found that the prepared membranes showed low methanol permeability and high proton conductivity for direct methanol fuel cell.

CONCLUSIONS AND SUMMARY

Chitin and Chitosan are natural polymers found in abundance in nature similar to cellulose in structure except for the presence of an acetamide or amino groups in chitin and chitosan respectively instead of a hydroxyl group in C2. Chitin can be extracted from many renewable sources in nature as the exoskeleton of crustaceans, insects and fungi. Chemical methods of extraction involve demineralization with dilute acids usually HCl followed by deproteination using dilute sodium hydroxide. Chitin exists in three crystalline structures; α, β and γ. In the α structure the chains are aligned in an anti-parallel way, this molecular arrangement leads to the formation of strong intermolecular hydrogen bonding making α-chitin insoluble in most known solvents, less susceptible for the deacetylation reaction and swelling. β-Chitin is composed of parallel chains and thus has less intermolecular forces and consequently it deacetylates much easier using less drastic conditions. β-chitin is less stable than the α form [21]. On dissolution or extensive swelling, β-chitin converts to α-chitin. The third type the γ is less common and it is simply a mixture of the two previously mentioned types. Chitin has not been exploited enough due to its insolubility in most solvents. The deacetylated form Chitosan has found many applications. Chitosan has applications in food preservation, pharmaceutics, water treatment due to its strong chelating ability, it has many biomedical applications as antitumor and wound healing agent. The antibacterial

activity of chitosan has been established and was found that chitosan kills bacteria through cell membrane damage. Extraction of chitin and its deacetylation into chitosan have attracted the attention of many researchers allover the world. Chitin has been extracted from several marine sources shrimps, crab, crayfish, lobster, the pens of squid and cuttlefish.

The kinetics of deacetylation of α and β chitin has been investigated; optimization of the extraction and deacetylation has been established. The second source of chitin is insects. Chitosan can be extracted from the larva of housefly, *Musca domestica* and from the beetle larva cuticle and silkworm (*Bombyx mori*) pupa exuvia. The extraction procedure is basically similar to that from crustacean except for the absence of minerals which renders insects a better source. The chitin and chitosan obtained from insects were found to be almost identical to those from crustaceans, as indicated by spectral analysis and X-ray diffraction studies. The chrysalides of silkworm are the adult form of the larva responsible for the production of silk threads, and the chrysalides itself constitute a byproduct from the silk industry. Insect chitin was also prepared from the bumblebee corpses by treatment with HCl and NaOH.

Chitin is a common constituent of fungal cell walls. Advances in fermentation technologies suggest that the cultivation of selected fungi can provide an alternative source of chitin and chitosan. The amount of these polysaccharides depends on the fungi species and culture conditions Filamentous fungi have been considered as an attractive source of chitin and chitosan for industrial applications because their specific products can be manufactured under standardized conditions Usually, the Zygomycetes Class has higher amounts of chitin and chitosan in their cell walls when compared to other classes of fungi. The use of biomass from fungi has demonstrated great advantages, such as: independence of seasonal factor, wide scale production, simultaneous extraction of chitin and chitosan, extraction process is simple resulting in reduction in time and cost required for production, and also absence of proteins contamination, mainly the proteins that could cause allergy reactions in individuals with shellfish allergies. Efforts have been made to obtain economic culture media that promote the growth of fungi and stimulate the production of the polymers. The mycelia of various fungi have been used for the production of chitosan.: examples are *Aspergillus niger*, *Cunninghamella elegans*, using the economic yam bean medium, *Mucor rouxii*, *Rhizopus aryzae, Absidia coerulea, Gongronella butleri, Absidia glauca* var. *paradoaxa*, and others. It is essential to free the chitin/chitosan from its anchorage in the membrane and the β-glucan. High concentration of NaOH is required in this first step, alpha-amylase enzyme has to be applied to separate the chitosan from the glucan fraction. Using this treatments a better quality of chitosan can be produced. This fungal chitosan will find its way in the pharmaceutical industry. Enzymes have been used for improvement of the extraction of chitin even from the

crustacean sources. The deacetylation of chitin using various enzymes has been attempted. Chitin deacetylase is an enzyme present mainly in the mycelium of fungi, can be used for the enzymatic deacetylation of chitin or chitosan. This trend will gain further importance in the future.

Microwave assisted deacetylation of chitin has been studied and it was found that the deacetylation time can be dramatically reduced when using microwave heating. Only preliminary data on using microwave heating are available in the literature till now.

By mixing polymers and inorganic materials (ceramics or their precursors) at the molecular level, it is possible to create hybrid materials known as ceramers. They can exhibit useful properties of both components: heat resistance, retention of mechanical properties at high temperature and low thermal expansion coefficient from ceramics and toughness, ductility and ease of processing from the high polymers. In an attempt to improve the mechanical properties of chitosan, hybrid material using silica and chitosan was prepared by the sol-gel method. The chitosan-silica hybrid materials obtained showed improved stability in water to elevate the potential of chitosan as a biomaterial and as separation material. A successful approach for preparing such hybrid materials has been the *in situ* polymerization of metal alkoxides in organic polymer matrices via the sol-gel process. The hydrolysis and polycondensation of a metal alkoxides $M(OR)_n$, where M can be Si, Ti, Zr, Ce etc. can produce rigid inorganic networks. The sol-gel process involves hydrolysis and condensation processes. Partially deacetylated chitin/ silica hybrid via a sol-gel process using tetra- ethoxysilane as a precursor has been prepared. The resulting polymer hybrids were compatible with each other over a wide range of composition. γ Glycidoxypropyl-trimethoxysilane, 3-isocyanatopropyl-triethoxysilane and 3-aminopropylt-riethoxysilane have been used as compatibilizers. Developing covalent bonds between the silica domain and chitosan chains increases mutual compatibility and moderates the rate of silanol condensation reaction to prevent phase separation during film formation. The chitosan-siloxane hybrids also show interesting photoluminescent features. The photoluminescence spectra show the presence of a new band with higher energy and longer lifetime, relative to the characteristic emission of pure low molecular weight chitosan. These hybrids are bioactive materials, the apatite formation was shown to depend on the amount and arrangement of silanol groups. Due to the interesting features of these chitosan hybrid materials more investigations will be devoted for preparation and characterization of various architecture and metal polymer combination.

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Chemical Composition of the Mango Stem Bark Extract (*Mangifera indica* L)

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ABSTRACT

An aqueous decoction of mango (Mangifera indica L.) stem bark has been developed in Cuba on an industrial scale to be used as a nutritional supplement, cosmetic, and phytomedicine. The mango stem bark extract (MSBE) has been reported to have antioxidant, anti-inflammatory, analgesic, immunomodulatory, anti-angiogenic and induced-apoptosis inhibition activities. A full phytochemical investigation of the MSBE has been conducted in order to characterize its volatile, non-volatile and inorganic components leading to the identification and quantification of 80 organic components and 9 inorganic elements. Isolation methods have included solvent extraction (batch and Sohxlet), simultaneous vacuum steam distillation / solvent extraction, preparative reversed-phase high performance liquid chromatography (RP-HPLC) and humid digestion. Identification and quantification were conducted by UV/VIS, FT-IR, GC/MS, HRGC, HPLC, ^{1}H-, and ^{13}C-NMR as well as 2D NMR experiments and AE-ICPS techniques. Mangiferin, a glycosylated xanthone (1,3,6,7-tetrahydroxyxanthone-C2-β-D-glucoside) was identified as the major component of the MSBE (10 %). Other relevant components were β-elemene, aromandrene, α-guaiene, β-selinene, hinesol, β-eudesmol, β-sitosterol, and β-campesterol in the volatile fraction; palmitic, oleic and linoleic acids in the fatty acid fraction; phenolics (gallic acid, 3,4-dihydroxy benzoic acid, gallic acid methyl ester, gallic acid propyl ester, benzoic acid and benzoic acid propyl ester), flavonoids (catechin and epicatechin); free sugars (galactose, glucose, and arabinose); polyols (sorbitol,

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myoinositol, and xylitol) in the non-volatile fraction, and K, Ca, Mg, Fe, Se, Cu and Zn in the inorganic fraction. Possible correlations of MSBE chemical components with the reported pharmacological actions are discussed.

Key words : *Mangifera indica* L., mango stem bark, nutritional supplement, phytomedicine, solvent extraction, simultaneous steam distillation/solvent extraction, Soxhlet extraction, humid digestion, volatile, non-volatile and inorganic components, 1D and 2D NMR spectroscopy

INTRODUCTION

Mango *(Mangifera indica* L.) extracts from leaves, fruit seed kernel, fruit pulp, roots, bark, and stem bark for medicinal purposes in many countries have been extensively reported in the *Napralert* Data Base (Napralert, accesed on March, 2008). Specifically, the mango stem bark (hereafter MSB) has been traditionally used for the treatment of menorrhagia, scabies, diarrhea, syphilis, diabetes, cutaneous infections, and anemia, using an aqueous extract obtained by decoction. The ethnomedical use of the MSB in Cuba has been documented widely (Guevara-Garcia *et al.*, 2004) and it has been extensively used in cancer, diabetes, bronchial asthma, infertility, lupus, prostatitis, benign prostatic hyperplasia, gastric disorders, arthralgies, mouth sores, and tooth pain, as the more frequent diseases. Non-documented verbal references from old Cuban people told about uses of the MSB by African slaves in Cuba since the XIX century. Cuba has developed a relatively sophisticated pharmaceutical sector, originally to provide medicinal products for her own population and, more recently, to earn hard currency through exports. The importance of this sector is particularly seen with respect to the strong changes that have taken place in Cuba in recent decades and which still carry on. For this reason, an extract of this plant has been developed in Cuba on an industrial scale to be used as a nutritional supplement, cosmetic, and phytomedicine. The industrial extract obtained by decoction and drying of mango stem bark is a homogeneous brown powder which melts with decomposition from 215 to 218°C and has a particle size of 30–60 μm. The Ministry of Agriculture in Cuba has an inventory of 273 mango varieties which were introduced into the country from more than 1,200 reported worldwide. Sixty-eight mango varieties are distributed along the island for extensive cultivation and fruit collection but only 16 of them have proven to be effective for the production of MSB Extract (hereafter MSBE) according to the polyphenols content by phytochemical screening and *in vitro* preliminary toxicological tests.

MSBE has been extensively tested both *in vitro* and *in vivo* in order to demonstrate its antioxidant, analgesic, anti-inflammatory and immunomodulatory activities, and its pharmaceutical formulations (*Vimang*)

–tablet, 300 mg, and cream, 1.2%- have been used in clinical trials for geriatrics, skin care and HIV/AIDS treatment successfully as recently reported (Gil del Valle *et al.*, 2002). Its use within the Cuban Primary Health Care system is a recommended practice for physicians as anti-inflammatory and analgesic (Nuñez-Selles *et al.,* 2007a).

Documented data from more than 7,000 patients with 84 diseases have been compiled in Cuba during the past 10 years. Focal studies have been done on specific diseases of relevant importance like cancer (Tamayo *et al.*, 2001) to evaluate disease progress and the improvement of the patient's quality of life in a 6 months field trial: 84.8% of patients experienced decreasing level of depression, 82.2% of patients had a better integral evaluation, and 89.7% of them were able to have a normal life with an average dose of 20 mg/kg body weight (b.w.) after oral administration three times a day of an aqueous decoction of MSB (30 mL). Initial *in vitro* tests demonstrated that MSB had no cytotoxic effects on tumor cells. However, more than 95% of cancer patients treated with MSB (2,286 patients) evidenced an improvement in terms of their quality of life (appetite, body weight, self-independence for the daily life, etc.); inflammation and/or pain were significantly reduced and several biochemical markers were improved in time (*i.e.* haemoglobin and transaminase, being the most significant) (Nuñez-Selles, 2005). It was relevant that more than 60% of patients with diabetes mellitus (408 patients) reduced the insulin dose by 20 IU after 6 months of MSB oral administration; ca. 80% of patients with benign prostate hyperplasia (826 patients) improved the urine retention after 3 months of MSB administration (oral and rectal); and 95% of patients with different types of dermatitis (1,297 patients) were improved after one-week treatment with topical MSB. Also significant was that 87% of patients with Lupus erythematosus (675 patients) improved their quality of life after the first month of MSB treatment (oral and topical administration).

The above-described results were considered as a basis for the development of a new natural product from the MSB with the hypothesis that so many successful applications would be sustained from its antioxidant effect better than a specific medical application. Therefore, the MSB extraction was developed up to the industrial scale in order to obtain a standardized MSBE, and formulated to be used as an antioxidant nutritional supplement, a cosmeceutical product, and phytomedicine strongly related to oxidative stress, pain, inflammation and immunomodulation (Nuñez-Selles, 1999) with adequate protection of the intellectual property by a patent and registered brand name (*Vimang*) (Nuñez-Selles *et al.*, 2002a). The present chapter summarizes all the work which has been performed in order to characterize fully the MSBE chemical components and their possible correlation with the observed pharmacological results.

MATERIALS AND METHODS

Plant Material (MSB)

MSB was collected from plants grown in a fruit farm (Alquizar, Havana), cultivated on a red-ferralytic soil type, without affecting the ecosystem. Thus, the bark was carefully cut along the mango tree stem, without affecting the inner part, from the top (25 cm below the lowest branch) to the bottom (25 cm above the highest root). Cut width was not larger than 20 cm. MSB was collected free of microbial contamination and subsequently dried and milled to obtain particles of around 5 cm with water content around 10%. The standardized industrial MSBE was provided by the Center of Pharmaceutical Chemistry, Havana, Cuba. Sixteen varieties, which were grown in two cultivars, with the same type of soil (red ferralytic), were selected for the study of inorganic elements. Plants were classified into two groups according to the tree age and stored at room temperature (25 ± 2°C) in vacuum-sealed PVC bags until sample preparation. Voucher specimens of the MSB were deposited at the Natural Products Archive, Center of Pharmaceutical Chemistry, Havana, Cuba (Code: 41722).

Mango Stem Bark Extract (MSBE)

MSBE was obtained by decoction of MSB for 2 h and concentrated on a double-effect thin-film evaporator under vacuum. The concentrate was preserved from fungal contamination with 0.5% sodium benzoate and stored at 8 ± 2°C until drying. The concentrate was spray-dried on a Niro Spray Dryer, Model P-6.3-R (Denmark), and the powder was homogenized in a V-shape homogenizer. MSBE was packaged in PVC bags under vacuum and poured into HD-PTFE flasks. MSBE thus obtained was a homogeneous brown powder which melts with decomposition from 215 to 218°C and has a particle size of 30-60 μm, partially soluble in water, soluble in dimethyl sulphoxide (DMSO), pH = 6.8 ± 0.2, polyphenols content = 45 ± 5%, and water content = 4.0 ± 2.0%.

Chemicals

Pure standards were purchased from Sigma Chemicals (Milan, Italy) or BDH (United Kingdom). Organic solvents and reagents (Pure for Analysis Grade) from Carlo Erba, Milan (Italy) or E. Merck, Darmstadt (Germany). Acids (Suprapur Grade) for humid digestion from BDH (United Kingdom). Water was purified by a Milli-Qplus system from Millipore (Milford, MA) for all experiments.

Sample Preparation

Volatile Components (Terpenoids and Sterols)

Soxhlet Extraction

MSB (200 g) was extracted with 1.5 L of petroleum ether (b.p. 40–60°C) for 12 h (Extract A = EA). The EA was vacuum dried and extracted thereafter with 1.5 L of chloroform, then concentrated to dryness by rotary evaporation (Büchi, Switzerland) and 1 g was dissolved in acetone, filtered and further fractionated by semi-preparative high pressure liquid chromatography (Merck-Hitachi, Darmstadt, Germany) on silica gel. Three fractions (Q1, Q2 and Q3) were obtained by successive elution with hexane, hexane/ethyl acetate (1:1, v/v) and ethyl acetate, respectively. Fractions were concentrated on a Kuderna-Danish apparatus down to 1 mL, dried overnight (6 ± 2°C) with anhydrous sodium sulfate, brought into a stoppered vial, and kept at 8 ± 2°C until chromatographic analysis.

Simultaneous Steam Distillation-Solvent Extraction

MSBE (5 g) was poured in 90 mL of a saturated solution of sodium chloride and brougth into a 150 mL flask. The sample was extracted on a Likens-Nickerson apparatus with 10 mL of diethyl ether by heating at 140°C for 1 h. Cooling water in the condenser was fixed at 0°C. The extract was concentrated to 1 mL in a Kuderna-Danish apparatus, dried overnight (6 ± 2°C) with anhydrous sodium sulfate, brought into a stoppered vial, and kept at 8 ± 2°C until chromatographic analysis (Extract B = EB).

Fatty and Organic Acids

Fatty Acids

MSBE (10 g) was extracted on a Soxhlet apparatus with a mixture (1:1) of chloroform and methanol. The extract was evaporated under vacuum on a rotary evaporator (Büchi, Switzerland) to 1 mL, followed by saponification with 6% KOH and methylation with diazomethane as described elsewhere. The fatty acids extract (I) was dried over anhydrous sodium sulphate, brought into a PTFE stoppered vial and kept at 8 ± 2°C until chromatographic analysis.

Unsaturated Fatty Acids

MSBE (3 g) was extracted according to the Folch´s method (Folch *et al.*, 1957) on mild conditions. Such extract was subsequently purified by column chromatography (Sephadex G-25, Pharmacia) under vacuum with a mixture of ethyl acetate and n-hexane (9:1). Fraction containing unsaturated fatty acids (II) was kept as described above until chromatographic analysis.

Organic acids

1 mL of extract II was derivatized with a mixture of hydrochloric acid and methanol (1:1) and subsequently extracted with chloroform to render methyl esters of organic acids. Fraction containing organic acids (III) was kept as described above until chromatographic analysis.

Non-Volatile Components (Phenolics, Flavonoids, Free Sugars and Polyols)

Preparative Reversed-Phase (RP) HPLC

Separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector, a U6K injector, and a Waters μ-Bondapak C-18 column (30 cm × 7.8 mm i.d., 4 mm) using MeOH:H_2O (30:70 v/v, flow rate = 2.5 mL/min).

Sample Preparation

MSBE (500 mg) was partitioned twice with 80 mL of *n*-BuOH/H_2O (1:1, v/v) at room temperature. The aqueous extract containing only free sugars and polyols was analyzed by GC-MS. A portion (3 mg) of the dried BuOH-soluble material (87 mg) was diluted to a volume of 3 mL (1 mg/mL) in a volumetric flask. Standard solutions containing 1 mg/mL of gallic acid, (-)-epicatechin, and benzoic acid in methanol were also prepared.

Quantitation

Quantitation was performed by reporting the measured integration area in the calibration equation of the corresponding standard. Phenolics were assayed as gallic acid equivalent; benzoates as benzoic acid equivalents; xanthones and flavonoids as (-)–epicathechin equivalent, respectively.

Total Polyphenols Assay

Estimation of the global polyphenol content in extracts was performed by the Folin-Ciocalteu method. MSBE (1.15 mg) was dissolved in MeOH (2 mL), and the extract was diluted 10-fold with water. Folin-Ciocalteu reagent (0.5 mL; Merck) was added to the diluted solutions (0.5 mL), then 0.5 mL of a 100 g/L solution of Na_2CO_3 was added. The absorbance was measured at 720 nm with a blank sample (water plus reagents) in the reference cell. Quantification was obtained by reporting the absorbances in the calibration curve of gallic acid used as standard phenol.

Free Sugars and Polyols

The aqueous extract, obtained as described above, was dried, and 10 mg of residue was diluted with 40 μL of dry pyridine. The diluted residue was then directly silylated at 80°C for 30 min with 50 μL of *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane as catalyst. The TMS derivatives were kept in isooctane and analyzed by GC-MS (Shimadzu, Japan). Individual sugars and polyols were identified by comparison of MS spectra and retention times of peaks with those of TMS derivatives of authentic compounds. Results represented the mean ± SD of 10 determinations. The concentrations of each compound in the extract were calculated from the experimental peak areas by interpolation to standard calibration curves.

Elements

MSB samples were dried at 105 ± 2°C for 6 h, subsequently milled (Hammer mill 705, UEMI, Havana) and sized to obtain pieces around 3 cm large, and then stored in vacuum-sealed PVC bags at room temperature (25 ± 2°C) until processing. Elements from the MSB were extracted by humid digestion according to standard procedures (Bock, 1979). The glassware was cleaned prior to use by soaking overnight with 10% v/v nitric acid. Dried MSB (1 kg) was poured into an Erlenmeyer flask (2.5 L) and heated up to 70°C; 4 mL of nitric acid (65%) was added twice until full organic combustion. Thereafter, 4 mL of acids mixture (65% nitric acid, 98% sulfuric acid, and 70% perchloric acid; 10:1:3) were added, and the heating temperature was increased up to 130°C until a transparent and colorless solution was obtained. The same procedure with the acids mixture was repeated and the heating was continued until the solution was reduced to 1 mL. The sample was poured into a 50 mL volumetric flask and filled with distilled water. Samples were stored at 8 ± 2°C until spectrometric analysis. A blank sample was prepared in parallel for substraction in the spectrometric determinations. Each sample had three replicates.

Standard Preparation

Five solutions of pure standards for quantitative analyses were prepared by successive dilutions in distilled water with concentrations of 25, 5, 2.5, 0.25, and 0.025 $\mu g.mL^{-1}$ for As, Ca, Fe, K, and Pb; 10, 2, 1, 0.1, and 0.01 $\mu g.mL^{-1}$ for Cd, Cu, Mg, and Zn; and seven solutions of 50, 10, 5, 1, 0.5, 0.1, and 0.01 $\mu g.mL^{-1}$ for Se. The technique of cold vapor was used for the determination of Hg.

Quantitation

Calibration curves and equations were obtained for each standard in the concentration ranges as described above. Absorbance values of each element were expressed as the mean value of six determinations ± SD. Concentration of each element was determined by interpolation and calculation from calibration curves and equations, respectively.

Equipment

Volatiles

Capillary high resolution gas chromatography was performed on a Carlo Erba (Italy), model MEGA 2, coupled to a VG (UK) mass detector, model TRIO 1000, with splitless injection and a cross-linked fused silica capillary column SPB-1 (Supelco, USA, 30 m × 0.32 mm i.d.). Carrier gas (He) flow rate was 1 mL.min^{-1}. The volume injected was 1 µL with 30 or 60 s as splitless time and injector temperature was 260-290°C. Oven heating was programmed from 30–60°C to 250–300°C at 4–10°C.min^{-1}. The column was connected through a direct inlet interface (280°C) to the quadrupole ionic source (EI+) fixed at 70 eV and 230 or 270°C. Mass spectra were recorded from 10 to 600 amu at a scan rate of 0,8 s and stored on hard disk until data processing. Experimental data was processed with a Lab-Base™ software (Fisons, UK) and chromatographic peak identification was done for direct comparison with a library search program and/or spectra from standard compounds whenever available.

Fatty and Organic Acids

Capillary high resolution gas chromatography was performed on a Fisons, Trio 1000, as described above but using a OV-1701 cross-linked fused silica capillary column (25 m × 0.25 mm), splitless injection (100:1) and He as carrier gas (1 mL.min^{-1}). Column temperature was programmed from 150°C (1 min) to 270°C (10 min) at 10°C.min^{-1}; injector and direct inlet interface were set at 270°C. Mass detector (EI+ mode) was operated at 70 eV, and 280°C. Mass spectra were recorded and stored as described above. Experimental retention times and fragmentation patterns were compared to those of pure standards from a laboratory data base.

Non-Volatiles

Mass spectra of phenolics and flavonoids were recorded on a Fisons Platform spectrometer both in the positive (90 V) and negative (100 V) modes. The sample was dissolved in MeOH and injected directly. UV spectra were measured with a HP 8472-A spectrometer in MeOH, (c=1) and IR spectra with a Nicolet Impact 400, in KBr. A Bruker DRX-600 spectrometer, operating at 599.19 MHz for ^{1}H and 150.858 for ^{13}C, with the UX-NMR software package, was used for NMR experiments measured in CD_3OD. The DEPT experiments were performed using a transfer pulse of 135° to obtain positive signals for CH and CH_3 and negative ones for CH_2. Polarization transfer delays were adjusted to an average CH coupling of 135 Hz. 1H-1H DQFCOSY (Rance *et al.*, 1983), ^{1}H-^{13}C HSQC, and HMBC (Homans, 1990; Martin *et al.*, 1991) experiments were obtained using the conventional pulse sequences as described in the literature, and 1D TOCSY (Davis *et al.*, 1985) was acquired using waveform generator-based GAUSS shaped pulse, mixing time ranging from 80 to 100 ms, and a MLEV-17 spin-lock field of 10 kHz was preceded by a 2 ms trim pulse. CD measurement was performed on a Jasco J-7140 spectropolarimeter. UV spectra were recorded on a Shimadzu UV-2101 spectrophotometer.

Mass spectra of free sugars and polyols were recorded on a Shimadzu MS-QP5050 system; column, DB1 J&W, 25 m × 0.2 mm i.d., 0.33 μm; injection, 1 μL; split ratio, 2; oven temperature, 100°C for 1 min, to 180°C at 4°C.min^{-1}, then to 290°C at 10°C.min^{-1}, and held for 20 min; injector temperature, 290°C; detector EMV 1.35-1.5 kV; carrier gas, helium; flow, 1 mL.min^{-1}.

HPLC analyses were performed on a Waters 600 E-Multisolvent Delivery System liquid chromatograph, equipped with a U6-K injector (fitted with a 20 μL loop), a Waters 486 tunable UV-V/s spectrophotometric detector, and a Waters 746-data module integrator. The column was a C18 μ-Bondapak (300 × 4.0 mm i.d.). Chromatographic separation was carried out using isocratic elution with two solvents [A = acetonitrile; B = water/2% acetic acid (98:2 v/v)] in the ratio 15:85 (v/v). Detection wavelength was 278 nm. Flow rate was 0.7 mL.min^{-1}, and the injection volume was 10 μL. Phenolics and flavonoids, for which standards available, were identified by chromatograms according to their retention times. If standards were not available, they were identified by comparing the retention times of the peaks in the extracts with those of the same compounds previously isolated and characterized by NMR analysis.

Elements

Samples, both blank and standards, were analyzed by ICP-AES (Model Spectroflame, Spectro, Germany) with the following conditions: Paschen-Runge mode; 128 analytical channels; 5 optic systems; Rowland circle diameter, 750 mm; Zerodur holographic lattice; plasma flux, 13 L.min^{-1}; auxiliary flux, 4 L.min^{-1}; nebulizator flux, 1 L.min^{-1}; sample aspiration velocity, 3 mL.min^{-1}; radio frequency, 27.12 MHz; power, 2.5 kW (maximum). The absorbance data for each element were recorded at the following wavelengths: Pb (λ = 168.2 nm), As (λ = 193.1 nm), Se (λ = 196.0 nm), Zn (λ = 213.9 nm), Fe (λ = 259.9 nm), Mg (λ = 285.2 nm), Cu (λ = 324.7 nm), Cd (λ = 327.4 nm), Ca (λ = 422.7 nm), and K (λ = 766.5 nm). The monochromator was direct-access type with 4 inlets and 6 outlets and a photomultiplier. Each sample was analyzed twice.

RESULTS AND DISCUSSION

Volatiles

The identification of volatile components showed the presence of sesquiterpene hydrocarbons as the main fraction. The largest components were β-elemene, α-guaiene, aromandrene and hinesol. Composition and relative abundances (RAs) of volatile components are shown in Table 15.1. The RAs of compounds were calculated as percentage of their chromatographic peaks areas (internal normalization) as referred to an internal standard. Industrial processing of MSB did have a significant influence on the volatiles chemical composition of the MSBE, overall in terms of sesquiterpene hydrocarbons, which were reduced by 50% approximately.

Table 15.1. MSB volatile components extracted by Soxhlet (1), simultaneous steam distillation/solvent extraction (2), and preparative high resolution liquid chromatography with n-hexane (3), n-hexane:ethyl acetate, 1:1 (4) and ethyl acetate (5). Components were isolated and identified by gas chromatography/mass spectrometry through a library search data base, and by direct comparison of mass spectra with pure standards when available

No.	Name	Identified in extracts	No.	Name	Identified in extracts
High Relative Abundance (> 80%, Internal Normalization Method)					
01	β-Elemene	(1) (2) (3)	06	1-Octadecene	(3)
02	β-Selinene	(1) (2) (4)	07	Squalene	(3)
03	Palmitic acid	(1) (2) (4)	08	6-Methyl-3-heptanol	(4)
04	α-Guaiene	(3)	09	β-Eudesmol	(4) (5)
05	Aromandrene	(3)	10	Methyl linoleate	(4)
Medium Relative Abundance (50–80%, Internal Normalization Method)					
11	β-Chamigrene	(1) (4)	18	3-Eicosene	(3)
12	n-Hexadecane	(1)	19	N-Phenyl-1-naphtalenamine	(3) (4)
13	Bulnesol	(1) (3)	20	β-Sitosterol	(3) (4)
14	9-Octadecenoic acid glycerid	(1)	21	β-Amyrin	(3)
15	γ-Selinene	(3) (4)	22	Cycloartane-3β, 25-diol	(3)
16	Ledol	(3)	23	2,5-Dihydroxy-α-methylphenethyl alcohol	(5)
17	Methyl-13-methylpentadecanoate	(3)			

Table 15.1. *Contd.*

No.	Name	Identified in extracts	No.	Name	Identified in extracts
Low Relative Abundance (<50%, Internal Normalization Method)					
24	*Trans*-Caryophyllene	(1)	43	3-β-Campesterol	(3)
25	α-Humulene	(1)	44	3β,5α-4,4-dimethylcholesta-8, 14-dien-3-ol acetate	(3)
26	n-Heptadecane	(1)	45	D:C-Friedoolean-3-one (Multifluorenone)	(3)
27	Guaiol	(2)	46	4-Stigmasten-3-one	(3)
28	α-Eudesmol	(2)	47	24-Methylencycloartanol	(3)
29	Juniper camphor	(2)	48	Phenol	(4)
30	Myristic acid	(2)	49	1,2-Benzenediol	(4)
31	Heptadecanenitrile	(2)	50	1-(2-hydroxy-5-methylphenyl)ethanone	(4)
32	Octanal	(3)	51	4-Hydroxymethyl benzoate	(4)
33	Dodecanal	(3)	52	α-Selinene	(4)(5)
34	2-Butyloctanol	(3)	53	3,4,5-Trimethoxyphenol	(4)
35	α-Elemene	(3)	54	3,4,5-Trimethoxybenzenemethanol	(4)
36	1-Ethyldecylbenzene	(3)	55	4-Hydroxy-3,5,6-trimethyl-4-(3-oxo-1-butenyl)-2-cyclohexen-1-one	(4)

Table 15.1. *Contd.*

No.	Name	Identified in	No.	Name extracts	Identified in extracts
Low Relative Abundance (<50%, Internal Normalization Method)					
37	Spathulenol	(3)	56	9-Octadecenamide	(4)
38	Nootkatone	(3)	57	3-Octylphenol	(4)
39	3,8-Dimethyl-4-(1-methylidene) -(8S-cis)-2,4,6,7,8,8α-hexahydro -5(1H)-azulenone	(3)	58	3-Pentadecylphenol	(3) (4)
40	3-Methyldibenzothiophene	(3)	59	9-Methyl-(3β,5α)-androstan-3-ol	(4)
41	Methyl 13-methylpentadecanoate	(3)	60	Hexanoic acid	(5)
42	Methyl-2-oxohexadecanoate	(3)	61	1,1-Ethoxypropoxyethane	(5)

The presence of sesquiterpene hydrocarbons (β-elemene and α-guaiene) and hinesol were significant on Q1 in terms of RAs. β-sitosterol (Q1) was found at medium RA. Alkyl-substituted phenols with five or more carbon atoms in the alkyl radical (3-octyl- and 3-pentadecylphenols) were significant in fraction Q2. The large RA of β-eudesmol in Q2 was also significant. Polar oxygenated components were considerably lower as compared with non-polar components, with only one polyalcohol being significant. Quantification by GC-Internal Normalization showed that major volatile MSBE components of possible biological relevance were (in decreasing order): β-elemene (**1**), aromandrene (**2**), α-guaiene (**3**), β-selinene (**4**), hinesol (**5**), β-eudesmol (**6**), β-sitosterol (**7**) and β-camphesterol (**8**) (Fig 15.1). Described findings may be of importance in order to explain some reported results from ethnomedicine as follows.

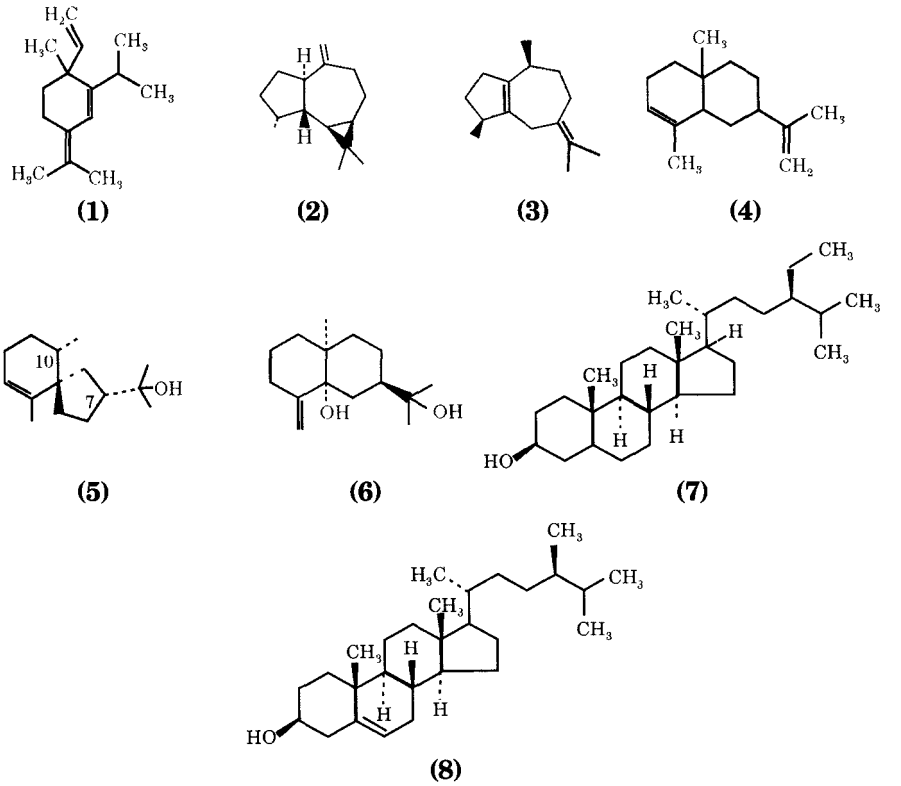


Fig 15.1. Chemical structures of MSB and MSBE main volatile components

(a) ***β-Elemene*** (**1**). It has shown to exhibit anti-tumor activity on several types of leukemia (*in vitro* and *in vivo*) by inducing the apoptosis of tumor cells (Zheng *et al.*, 1997). A recent report indicated its high anti-proliferative activity in glioma cells, and also *in vitro* inducer of apoptosis in these cell lines (Zhou *et al.*, 2003).

(b) ***Hinesol* (5)**. It has inhibited H^+, K^+-ATPase by interaction with the enzyme in the E1 state (Satoh *et al.*, 2000), and such result might explain the ethnomedical observed benefitial effect on gastric disorders. Moreover, hinesol has enhanced the inhibitory effect of omeprazole on the hydrogen-pump.

(c) ***β-Eudesmol* (6)**. It has been reported to modify neuronal functions by inducing neurite extension, significant increase of intracellular [Ca^{2+}], and promoting phosphorylation of both mytogen-activated protein kinase (MAPK), and constitutive Activated Mytogen Protein (cAMP)-responsive element binding protein (Obara *et al.*, 2002). Both, hinesol and β-eudesmol, have correlated by dose-response effect to the inhibition of the *in vitro* binding of [3H]arginine vasopressin and [3H]angiotensin II to kidney membrane receptors (Kimura, 2006).

(d) ***β-Sitosterol* (7)**. It must been considered in terms of its nutraceutical and pharmacological relevance. It has been reported to reduce levels of lipids at low concentrations and act as support of fat metabolism by reducing plasma cholesterol levels (Jones *et al.*, 2000). It has been claimed as an essential component within a healthy diet in foods like soja and fish. Its importance on chemoprevention and chemotherapy for benign prostate hyperplasia has been also reported (Klippel *et al.*, 1997).

(e) ***α-Guaiene* (3) *and* *β-Selinene* (4)**. These major components of MSBE have similar reports in the literature (Miller *et al.*, 1997; O´Brien *et al.*, 1996) as food or cosmetic additive, and anti-parasitary against *P. falciparum*, respectively.

(f) ***Aromandrene* (2)**. This MSBE component has no reports of biological relevance.

Summarizing, 93% of isolated volatile components could be identified (52 compounds), but only six of them (11.5%) could be related to some of the ethnomedical findings, as described above, according to the literature. Therefore the main contribution of MSBE chemical components to the reported nutritional and pharmacological effects might be found in the non-volatile and inorganic fractions. Nevertheless the synergic contribution of these volatile MSBE components to its biological properties should not be neglected in further assessments.

Fatty and Organic Acids

Tables 2 shows the composition of all extracts (Sohxlet, Folch´s and trans-methylation) representing the composition of fatty, unsaturated, and organic acids in MSBE with a detection limit calculated at 8.56 $ng.mL^{-1}$ (methyl stearate), which afforded the identification of organic acids up to the trace level. MSBE total organic acids fraction ranged from 0.1 (Sohxlet) to 1.5 % (trans-Me), according to the extraction method. Identified saturated fatty acids were $C_{14:0}$, myristic; $C_{16:0}$, palmitic; and $C_{18:0}$, stearic. Unsaturated fatty acids were $C_{18:1}$, oleic; $C_{18:2}$, linoleic, and $C_{20:3}$, eicosatrienoic. Five unsaturated di- and tri-carboxylic acids were identified as hydroxyfumaric, fumaric, hydroxyl-β-carboxyglutaric, succinic, and malonic, being the first report of fatty and organic acids composition of MSBE.

Table 2. MSBE fatty and organic acids composition

Acids	g/kg MSBE	%
(a) **Fatty acids**		
Myristic ($C_{14\,0}$)	0.01 ± 0.003	0.4
Palmitic ($C_{16\,0}$)	1.25 ± 0.12	41.6
Stearic ($C_{18\,0}$)	0.77 ± 0.20	25.8
Oleic ($C_{18\,1}$)	0.92 ± 0.30	30.8
Linoleic ($C_{18\,2}$)	0.02 ± 0.01	0.6
Eicosatrienoic ($C_{20\,3}$)	0.05 ± 0.02	1.7
(b) **Organic acids**		
Benzoic Hydroxyfumaric Hydroxy-β-carboxyglutaric Succinic Malonic Cholesterol Derivative	Traces	

The importance of free fatty acids (FFAs) or their esters derivatives have attracted increasingly the attention of the scientific community according to their role in living organisms (Mostofsky *et al.*, 2001). Whereas saturated FFAs are important molecules for energy storage, polyunsaturated FFAs have been reported as a nutritional food source in order to improve cardiovascular and brain functions, and also for the chemoprevention of cancer (de Deckere, 1999; Kris-Etherton *et al.*, 2002; Stoll, 1999).

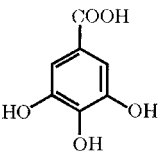
Although the presence of FFAs within the MSBE composition (around 1.5%, 11 compounds) might be considered as negligible, it is important to notice that around 70% of the FFAs fraction is polyunsaturated mainly due to $C_{18:1}$ (oleic) and $C_{18\,2}$ (linoleic). This means that within a recommended daily dose of 3 *Vimang* tablets (300 mg MSBE each), about 10 mg of polyunsaturated FFAs are supplemented to the organism. That amount would be considerably low, but the possibility of its synergic effect should not be discarded *a priori*.

Non-Volatiles

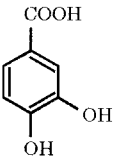
A thorough discussion of the chemical characterization of non-volatile MSBE components was published recently (Nuñez-Selles *et al.*, 2002b). Results of importance to assess MSBE biological effects within the non-volatile fraction are highlighted as follows.

Preparative RP-HPLC separation yield compounds **9** to **17,** which were identified as gallic acid (**9**), 3,4-dihydroxy benzoic acid (**10**), benzoic acid (**11**), methyl gallate (**12**), propyl gallate (**13**), mangiferin (**14**), (+)-catechin (**15**), (-)-epicatechin (**16**), and propyl benzoate (**17**) (Fig 15.2). The structures and molecular formulas of compounds **9-17**were determined from their ES-MS spectra, as well as from 1D and 2D ^{1}H- and ^{13}C-NMR data. The most abundant phenolic of the non-volatile fraction (**14**) showed 19 carbon atom signals from its ^{13}C- NMR spectrum. The presence of a glycosyl moiety was

clearly suggested from the analysis of the 2D COSY, HSQC, and HMBC experiments. The sugar was identified as a β-D-glucopyranose on the basis of its ^{1}H- and ^{13}C-NMR data (Agrawal, 1989). The mass spectra did not show the usual fragmentation pattern for *O*-glycoside derivatives, and these data together with the chemical shifts of H-1 (δ=4.86) and C-1 (δ=73.4) unambiguously showed the C-linkage of the sugar. ^{1}H- and ^{13}C-NMR chemical shifts of the aglycon were in accordance with those reported in the literature for tetrahydroxyxanthones (Frahm *et al.*, 1979). The glycosidic linkage of **14** was determined to be at the C-4 position based on the cross-peaks due to ^{3}J long-range coupling between the anomeric-H (δ=4.86, H-1) and C-2 (δ=107.9), C-1 (δ=162.1), and C-3 (δ=164.1) in the HMBC spectrum. From these considerations the structure of mangiferin (Bathia *et al.*, 1967) was assigned to **14**. Further NMR studies about isomeric composition of mangiferin extracted from MSBE (90.6% purity) showed the presence of two dimethoxyl derivatives at positions 1,3 of the xanthone moiety (*homo*-mangiferin) and 1,5, which has not been reported before, that we have called *vi*-mangiferin (Nuevas, 2004).



(9)



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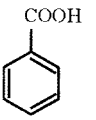

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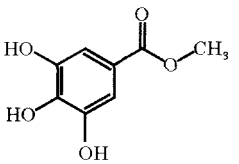

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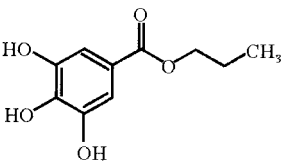

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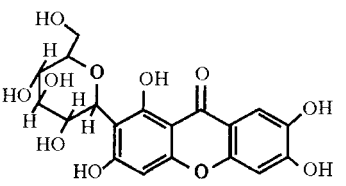

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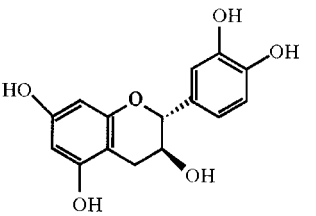

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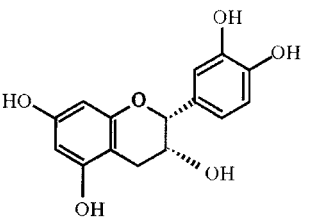

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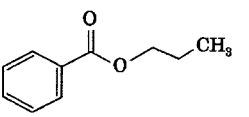

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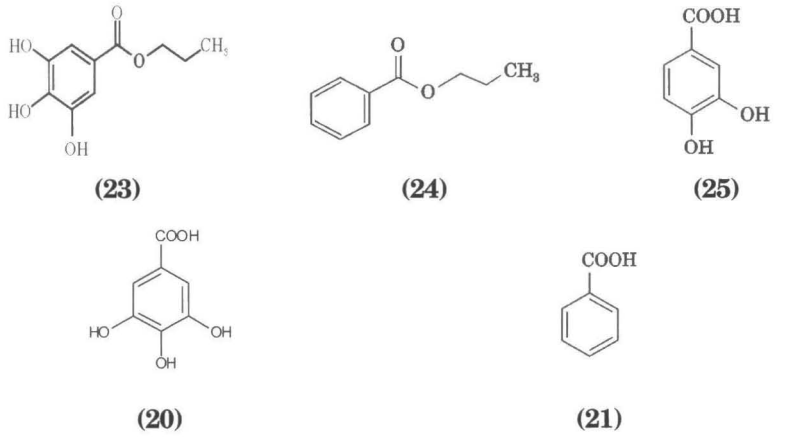

(23) **(24)** **(25)**

(20) **(21)**

Fig 2. Chemical structures of MSBE main non-volatile components

Also relevant was the identification of compounds **15** and **17**, which were identified as catechin and epicatechin, respectively, by HPLC comparison with authentic samples. Their identities were also apparent from their ^{13}C-NMR spectra which showed similar carbon signals for the phloroglucinol A-ring and catechol B-ring, but with slight differences in signals for the pyran ring. The absolute configuration of C-2 and C-3 for (+)-catechin and (-)-epicatechin were deduced as 2S from the CD spectroscopy measurement in comparison with the data reported in the literature (Barrett *et al.*, 1979).

The quantitative analysis of the phenolic compounds in MSBE was performed by HPLC. The concentrations of each compound in the extract, calculated from the experimental peak areas by interpolation to standard calibration curves were **9**=1.12%, **10**=1.30%, **11**=1.14%, **12**=2.56%, **13**=2.74%, **14**=41.06%, **15**=7.52%, **16**=4.64%, and **17**=2.29%. The sum of all phenolic compound concentrations obtained by quantitative HPLC (10.61 g/100 g dry weight) was compared with the results obtained from the Folin-Ciocalteu assays, generally considered as the method of choice to estimate total phenol contents in plant extracts (Scalbert, 1992). No significant difference was found between the two methods, as the total polyphenols in MSBE determined by the Folin-Ciocalteu method, and expressed as gallic acid equivalents, was 9.4 g/100 g dry weight.

The major sugars within the non-volatile fraction were galactose, glucose, and arabinose. Fructose was detected at a significant lower concentration. Additionally, MSBE contained three polyols in appreciable quantities: sorbitol, -685.2-, myoinositol-303.2-, and xylitol-52.5 mg/100 g dry weight-, respectively.

Mangiferin (hereafter MF), as the main component of MSBE (around 10%), has several interesting chemical attributes of biological relevance and fulfill the four requisites described for a high bioavailability of a molecule for oral administration (Lipinski *et al.*, 1997):

1. Molecular weight less than 500 amu ($C_{19}H_{18}O_{12}$)
2. Less than 5 donor functions for hydrogen bonds (4)
3. Less than 10 aceptor functions for hydrogen bonds (2)
4. Calculated Log P (potential) less than + 5 (log P_{MF}=+2,73)

Thus, MF has the possibility to reach tissues and organs significantly because of its high bioavailability not only by oral administration but topical and parenteral as well. The aglycon obtained from MF hydrolisis after its metabolism (norathyriol) has been found in plasma, feces and urine (Sanugul *et al.*, 2005), and has been reported to have a potent scavenging effect of singlet oxygen (Hsu *et al.*, 1997).

The four hydroxyl groups in MF structure give it interesting properties from the acid-base equilibrium point of view and its possible influence on human physiological events according to the administration route. The UV spectra from MF through a pH range from 4.0 to 11.2 are shown in Fig 15.3, where four absorption bands may be seen. The first band (λ_{max}=240 nm) shows a medium energy transition of the aromatic ring (C_1), whereas the second (λ_{max}=230-260 nm) is associated to the OH group involved in the enolic equilibrium (C_3). The third band (λ_{max}=317 nm) was the less intense, associated to a high energy transition related to the OH group bonded to one of the aromatic carbons (C_6 or C_7); the last OH group is involved with an intramolecular charge transfer (λ_{max}=390 nm) (Gomez-Zaletta *et al.*, 2006).

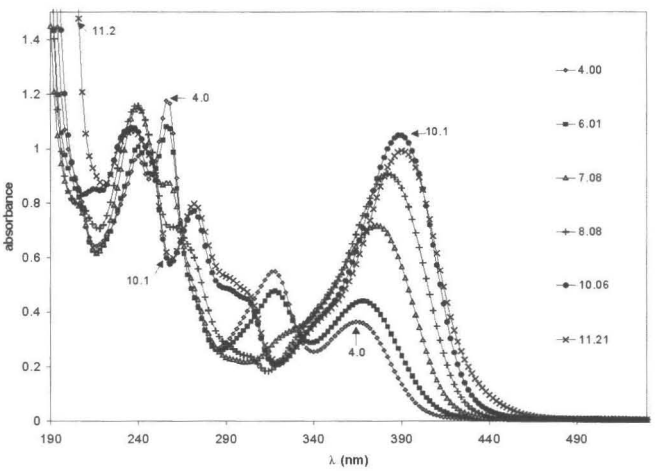

Fig 15.31. UV spectra of MF (4.0 x 10^{-5} M) by potentiometric titration at pH between 4.0 and 11.2. Displacement of absoption bands was associated to proton release from MF phenolic groups, which lead to the determination of MF acidic constants (pKa). It is possible to assume that polyphenols and flavonoids should have higher biological activity when they are released from pharmaceutical formulations at higher pH values (*i.e.* suppository)

As the pH was increased, the first band increased its absorption until pH = 7.6, which may be associated with the release of the first proton (C_1); the second band showed a bathocromic shift related, possibly, to the release of the second proton (C_3) at pH = 8.0; the third band disappeared a pH = 8.8

related to the release of the third proton (C_6), and the fourth band showed its highest intensity at pH = 9.7 owing to the release of the fourth proton (C_7). The acid-base titration of MF lead to the determination of acidity constants (pKa) as follows:

$$H_4(MF) \leftrightarrow H_3(MF)^- + H^+ \qquad pKa_1 = 10.21 \pm 0.07$$

$$H_3(MF)^- \leftrightarrow H_2(MF)^{2-} + H^+ \qquad pKa_2 = 8.54 \pm 0.36$$

$$H_2(MF)^{2-} \leftrightarrow H(MF)^{3-} + H^+ \qquad pKa_3 = 7.83 \pm 0.33$$

$$H(MF)^{3-} \leftrightarrow (MF)^{4-} + H^+ \qquad pKa_4 = 6.66 \pm 0.30$$

From these results, it may be assumed that MF, and therefore MSBE, could have higher biological potential within a basic or slight basic medium, *i.e.* through a rectal administration, and such potential might be decreased with the decrease of pH from neutral (*i.e.* topical administration) down to slight acidic (*i.e.* vaginal administration) or high acidic (*i.e.* oral administration) media. Therefore, the strategy of pharmaceutical formulation development involved these four administration routes, which was also according with the ethnomedical practice described elsewhere.

MF has been attracting increasingly the attention of the scientific and medical communities owing to its broad field of applications as antioxidant and analgesic (Dar *et al.*, 2005; Pauletti *et al.*, 2003; Garrido *et al.*, 2001; Hsu *et al.*, 1997), anti-hyperglicemic, anti-atherogenic, and anti–hyperlipidemic (Srijayanta *et al.*, 2001; Muruganadan *et al.*, 2002, 2005a; Yoshikawa *et al.*, 2002; Huang *et al.*, 2006; Moreno *et al.*, 2006), anti-inflamatory and immunomodulator (Muruganadan *et al.*, 2005b; Leiro *et al.*, 2003, 2004; Guha *et al.*, 1996; Sarkar *et al.*, 2004), antiapoptopic (Tang *et al.*, 2004), anti-tumoral (Yoshimi *et al.*, 2001), radioprotector (Jagetia *et al.*, 2005a; Sarkar *et al.*, 2004), cardiovascular (Jagetia *et al.*, 2005b; Prahbu *et al.*, 2006a, b, c), neuroprotector (Nair *et al.*, 2006; Amazzal *et al.*, 2007; Gottlieb *et al.*, 2006; Ibarretxe *et al.*, 2006).

MF as compared to other antioxidants like vitamins C, E and β-carotene doubled GSH (Reduced Glutathion) concentrations, a NF-κB modulator. At the same time, it reduced GSSG (Oxidized Glutathion) and increased catalase activity. MF, therefore MSBE, should be considered within the alternatives for antioxidant and anti-inflammatory therapies according to its high capacity to inhibit NF-κB-activation and increase GSH intra-cellular concentration. Last but not least, MF and MSBE, have shown to modulate gene-expression on viral replication, apoptosis regulation, and carcinogenesis, which support the criteria to use them as therapeutic agents on HIV and cancer in future clinical trials.

Flavonoids was the second largest group besides polyphenols in MSBE (around 25% of (+) catechine and (-) epicatechine). Bioflavonoids have a high antioxidant activity through the inhibition of lipid peroxidation, both

in isolated cell lines and membranes, and their activity depend not only from the chemical structure but the medium where the chain reaction of ROS generation takes place, and the characteristics of the reaction initiator (Cook *et al.*, 1996). Their relevance as antioxidants have been discussed elsewhere (Bagchi *et al.*, 1997; Chen *et al.*, 1996; Rice-Evans *et al.*, 1996). Also, they have a high diversity of chemical structures, and are distributed widely in fruits, vegetables, roots, seeds, and barks of several species (Hotta *et al.*, 2001). Specifically, (+) catechine and (-) epicatechine, MSBE main components besides MF, have been reported as components of *Ginkgo biloba* and *Vitis vinifera* extracts too (Bombardelli *et al.*, 1995), which have had a certain impact in medical practice. These flavonoids are the monomeric structures of high-molecular weight natural polymers named as **proanthocyanidines** or **procyanidines**, to which have been attributed therapeutical effects for the treatment of vein insufficiency, varicoceles, and microvascular disorders, including capillary fragility and retinopathies (Murray *et al.*, 1999).

(-) Epicatechine has been reported as one of the natural cacao components, with the attribute to improve the cardiovascular system through several mechanisms including the induction of NO-production, vascular tissue relaxation, and a significant improvement of blood flow. These mechanisms may have a positive effect on the treatment of arterial hypertension (Zhou *et al.*, 2005).

The contribution of free sugars and polyalcohols (around 10% and 3% of the non-volatile fraction, respectively) to MSBE bioactivity was considered non-significant, except sorbitol. This MSBE component, used normally in medical practice as laxative, might explain the observed effects in the ethnomedical practice (increase of daily fecal deposition and/or improvement of constipation).

Summarizing, 88% of the non-volatile organic MSBE components (17 compounds) could be identified and quantified. Present work is focused on saponins and other relevant organic families in order to identified fully MSBE organic chemical composition.

Elements

MSB samples from 16 mango varieties from two cultivars grown on the same soil –red ferralytic- (A=12, and B=26 years old) were studied in order to assess statistical significant differences within element concentrations. These selected varieties are the source for the production of standardized industrial MSBE, and therefore coded (Table 15.2) for trade secrecy protection. All MSB element-extracts had a mineral ion content below 1% (dry weight). ICP-AES was a useful technique for analyzing the mineral ion content of the MSB and results are reported in Table 15.4 for Group A

and Table 15.5 for Group B cultivars, respectively. A comprehensive discussion of these results has been published recently (Nuñez-Selles *et al.*, 2007b).

Table 15.3. Variety codes from two cultivars for element analysis

Variety code (Group A - 12 years old)	Variety code (Group B - 26 years old)
H01	M11
C20	R12
M13	N13
CH4	S14
CA5	D15
B69	O16
F77	-
SH8	-
BL9	-
A10	-

The most important findings regarding the biological relevance of elements present in MSBE may be summarized as follows:

(a) The detection of Se in all varieties, without a significant difference, and its concentration was not dependent on plant age or the variety. The mean concentration of Se for all varieties (1.49 μg/g) was within the Daily Recommended Allowance (DRA) as dietary supplement for both Groups A and B, when considering its content in *Vimang* formulations. The importance of Se as a chemoprevention factor for several types of cancer is a fact widely accepted by the medical and scientific communities (Combs *et al.*, 1998; Salonen *et al.*, 1995; Rikiishi, 2007). Also, Se has been reported as a cofactor of GSX (Schulz *et al.*, 2000), which may contribute to explain the antioxidant effect of MSBE. The relative constant concentration of Se for all varieties, therefore, should not affect the stem bark collection for the production of MSBE antioxidant commercial formulations.

(b) The presence of Ca as the main component in all varieties (mean value = 460.1 μg/g) followed by K (mean value = 254.2 μg/g). Ca concentration was within the DRA for nutritional purposes when considering its content in *Vimang* formulations (Porrata *et al.*, 1996). Considering the osmotic importance of K for the human organism and the relevance of Ca supplementation for the human body, it was interesting to note that plants

Table 15.4. MSB element concentrations in 10 mango varieties (Group A = 12 years old) determined by ICP-AES

Element ($\mu g.g^{-1}$)	Variety									
	Group A (12 years old)									
	H01	**C20**	**M13**	**CH4**	**CA5**	**B69**	**F77**	**SH8**	**BL9**	**A10**
Ca	309.3 ± 10.	9387.5 ± 6.5	372.5 ± 13.6	358.8 ± 12.3	436.3 ± 11.6	448.8 ± 28.8	651.5 ± 11.1	446.8± 13.8	377.3 ± 19.3	432.2± 27.3
K	183.5 ± 8.8	220.1 ± 10.4	328.8 ± 7.9	378.3 ± 11.4	319.7 ± 6.3	215.3 ± 12.7	256.1 ± 1.5	434.7± 8.8	379.4 ± 11.3	215.4± 8.1
Mg	32.4 ± 1.6	44.5 ± 1.9	27.8 ± 6.8	32.1 ± 2.1	35.3 ± 0.3	42.5 ± 3.3	13.0 ± 1.3	21.4± 0.4	36.4 ± 0.1	16.2± 0.2
Fe	7.0 ± 0.2	19.9 ± 7.0	9.3 ± 0.6	9.5 ± 1.0	9.5 ± 2.6	6.1 ± 0.4	2.9 ± 0.5	5.8± 0.6	5.9 ± 0.4	5.8± 0.1
Cu	0.8 ± 0.04	2.6 ± 0.2	1.6 ± 0.1	2.8 ± 0.5	1.7 ± 0.02	1.0 ± 0.04	0.2 ± 0.05	1.2± 0.1	0.3 ± 0.01	0.6± 0.01
Zn	0.6 ± 0.2	0.9 ± 0.02	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5± 0.1	0.4 ± 0.1	0.5± 0.04
Se	2.0 ± 0.1	2.0 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2± 0.1	1.2 ± 0.1	1.1± 0.1
Pb	0.1 ± 0.01	0.2± 0.01	0.3 ± 0.03	0.1 ± 0.04	0.1 ± 0.03	0.5 ± 0.5	0.1 ± 0.1	0.1± 0.01	0.1 ± 0.05	0.1± 0.01
Total	535.8	677.7	741.9	783.1	804.3	715.8	925.3	911.8	797.8	671.7

of Group B had higher concentrations of Ca associated with a minor content of K, when compared to Group A.

(c) Although Fe concentrations were below the DRA for nutritional purposes (mean value = 6.42 $\mu g.g^{-1}$) and its intra-variety differences were not significant, overall considering the soil type, its biological relevance should be highlighted. Iron excess in organism may lead to the formation of extremely toxic ROS, which ultimately cause peroxidative damage to vital cell structures, particularly mitochondria (Briton, 1996). MSBE has shown its capability to bind Fe and it has been related to its anti-lipoperoxidative mechanism (Martínez *et al.*, 2000). Moreover, a recent report has discussed thoroughly the increase of MSBE cytoprotecting and antioxidant capacities through the formation of MF-Fe complex(es) (Pardo-Andreu *et al.*, 2008), with a high protective capacity of mitochondria, a fact that should be considered for MSBE future applications on the treatment of iron-overload related diseases as sicklemia, β-talasemia and Friedrich´s ataxia.

(d) The mean values of Cu and Zn, when considering the 16 varieties, were 0.84 and 0.48 $\mu g.g^{-1}$, respectively, with non-significant differences from the biological point of view. Cu and Zn play significant roles in the activation of SOD (Jobe, 2003; Salvemini *et al.*, 2003), and therefore may contribute to the MSBE anti-inflammatory effect. Cu and Zn deficiencies in human plasma have been correlated with several inflammatory diseases like ophthalmic and peripheral neuritis (Beck, 2000). Recent works about Zn relevance in several physiological systems have been published elsewhere (Prasad, 1996; Valee *et al.*, 1993).

(e) Only Pb was detected as toxic element in all varieties with a mean value=0.11 $\mu g.g^{-1}$, which was considerably below the toxic dose for human consumption (< 2 mg/kg b.w.) (Sax *et al.*, 1989). Cd, As, and Hg were not detected in all MSB samples analyzed by ICP-AES at detection limits of 0.005, 0.02, and 0.01 $\mu g.g^{-1}$, respectively. Those results were in accordance with acute and chronic toxic evaluations of MSB for its use in *Vimang* formulations, leading to classify *Vimang* formulations as non-toxic for oral and topical administration before health authorities (Gonzalez *et al.*, 2007; Rodeiro *et al.*, 2006).

Research work will in due course evaluate ionic complexes of Fe, Se, Cu, and Zn with MSB polyphenols, which seem to be of biological relevance to increase the antioxidant and cytoprotecting effects of *Vimang* formulations.

Table 15.5. MSB element concentrations in 6 mango varieties (Group B = 26 years old) determined by ICP-AES

Element ($\mu g.g^{-1}$)	Variety					
	Group B (26 years old)					
	M11	R12	N13	S14	D15	O16
Ca	523.8 ± 14.4	487.5 ± 23.6	554.8 ± 18.2	358.8 ± 0.8	576.2 ± 13.5	639.2 ± 14.2
K	198.9 ± 4.3	231.5 ± 1.7	154.4 ± 8.2	207.7 ± 15.7	217.5 ± 1.9	126.1 ± 1.5
Mg	4.6 ± 0.9	21.8 ± 0.6	24.8 ± 0.9	9.9 ± 1.6	37.1 ± 1.3	31.5 ± 0.9
Fe	2.9 ± 0.4	4.4 ± 0.6	3.5 ± 0.2	3.4 ± 0.4	4.8 ± 0.9	2.1 ± 0.2
Cu	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.2 ± 0.01	0.1 ± 0.01
Zn	0.2 ± 0.05	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.03	0.3 ± 0.1
Se	1.1 ± 0.1	1.1 ± 0.1	1.01 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
Pb	0.01 ± 0.0	0.05 ± 0.03	0.04 ± 0.01	0.02 ± 0.02	0.05 ± 0.03	0.04 ± 0.02
Total	741.5	746.9	739.2	581.3	837.3	800.3

SUMMARY

Table 15.5 summarizes the results of the chemical composition of the Mango Stem Bark Extract *(Mangifera indica* L.) –MSBE- being the first report of an integrated chemical-biological discussion of its antioxidant, analgesic, anti-inflammatory, and immunomodulatory effects as reported elsewhere. Even when mangiferin MF as MSBE's, main component (10%), has shown to have a significant contribution to MSBE biological effects, more than 70% of the experimented biomodels have demonstrated that MSBE had a significant higher effect than pure MF.

That synergism effects more than the addition of single component, seems to be the rationale in order to explain the experimental evidences of MSBE in oxidative stress-related conditions. It could be the basis to assess the therapeutic effects which have been observed in clinical trials with *Vimang* formulations. Perhaps the future applications on different fields of medical practice of *Vimang* formulations will rely on its capacity to restore the homeostasis of the human beings to increase the quality of life.

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Appendix

Table of Contents of Other Volumes (1 to 5 & 7 to 8)

ISBN : 1-933699-51-5

VOLUME 1: POTENTIAL & CHALLENGES

1. Biological Activity of Peptides Derived from Marine Organisms 1-22
SE-KWON KIM AND Y. DOMINIC RAVICHANDRAN (REPUBLIC OF KOREA, INDIA)

2. Bioactive Natural Products as Anti-Staphylococcal Infections 23-74
SUPAYANG PIYAWAN VORAVUTHIKUNCHAI, BEATRICE OLAWUMI IFESAN, SASITORN CHUSRI AND JONGKON SAISING (THAILAND)

3. Pharmacological Activities, Phytochemical Investigations and *In vitro* Studies of *Gymnema sylvestre* R.Br. – A Historical Review 75-99
A. BAKRUDEEN ALI AHMED, N. KOMALAVALLI, M. MUTHUKUMAR, J.H.F. BENJAMIN, A.S. RAO, SE-KWON KIM AND M.V. RAO (INDIA, SOUTH KOREA)

4. Andirobas of the State of Acre (Brazil): Chemical and Biological Aspects Associated with the Use and Management 101-129
ANA CLAUDIA F. AMARAL, JOSÉ LUIZ P. FERREIRA, SILVIA L. BASSO AND JEFFERSON ROCHA DE A. SILVA (BRASIL)

5. Natural Bioresource of the North Himalayan Region as a Source of Promising Radiation Countermeasure Agents: Lessons from *Podophyllum hexandrum* 131-156
RAJESH ARORA, A.S. DHAKER, J. SHARMA, M. ADHIKARI, D. GUPTA, R. CHAWLA, R. KUMAR, A. SHARMA, R.K. SHARMA, S. SULTANA, G.N. QAZI, P.S. AHUJA AND R.P. TRIPATHI (INDIA)

6. Red Ginseng as a Potential Anti-Obesity Agent 157-174
JI HYUN KIM AND INSOP SHIM (SOUTH KOREA)

7. Carotenoids in Commercially Important Crustaceans from Indian Waters 175-189
SACHINDRA, N.M., BHASKAR, N. AND MAHENDRAKAR, N.S. (INDIA)

8. Therapeutic Uses of Venoms 191-209
RUMI GHOSH AND VAIDEHI THANAWALA (INDIA)

9. Potentials of Bryophytes for Biotechnological Use 211-233
MARKO SABOVLJEVIC AND ANETA SABOVLJEVIC (SERBIA)

10. Review: Molecular Biology Approach to Viper Venoms 235-256
PONLAPAT ROJNUCKARIN, CHUANCHOM MUANPASITPORN, PON SINGHAMATR AND JARADPONG ARPIJUNTARANGKOON (THAILAND)

11. Bee-Pollen Therapeutical Value 257-277
MARIA GRAÇA R. CAMPOS, CHRISTIAN FRIGERIO AND FRANCISCO FERREIRA (PORTUGAL)

12. Herbal Products in Healthcare: Challenges and Potential 279-306
MOHAMMED MOSIHUZZAMAN AND LEIV K SYDNES (PAKISTAN, NORWAY)

13. Anticancer Properties of Plant-Derived Food, Phytochemicals and Plant–Expressed Recombinant Pharmaceuticals 307-321
KUMAR V.L. AND KUMAR V. (INDIA)

14. Global Medicinal Plants with Anti-HIV Activities 323-331
THET THET HTAR AND GABRIEL A. AKOWUAH (MALAYSIA)

15. New Fungal Metabolites, as Antifungal, Herbicides and Insecticides for Biocontrol of Agrarian Plants Pests 333-389
A. EVIDENTE (ITALY)

16. On the Potential of Some Natural Colouring Agents: An Overview 391-407
ANNIE SHIRWAIKAR, ARUN SHIRWAIKAR, RICHARD LOBO AND KIRTI S. PRABHU (INDIA, U.A.E.)

Index 423-444

ISBN : 1-933699-52-3

VOLUME 2: EFFICACY, SAFETY & CLINICAL EVALUATION I

1. Perspectives Efficacy, Safety and Clinical Evaluation of Bioactive Natural Products 1-29
YOGENDRA NAYAK, VEERESH P. VEERAPUR, ANANTHA NAIK NAGAPPA AND M.K. UNNIKRISHNAN (INDIA)

2. The Modern Application of Traditional Chinese Medicine: Indigo Naturalis as an Alternative Therapy in *Psoriasis vulgaris* 31-43
YIN-KU LIN AND JONG-HWEI SU PANG (TAIWAN)

3. Development of Anti-Angiogenesis Therapies for Treating Cancer from Chinese Medicinal Herbs 45-65
STEPHEN M. SAGAR, RAIMOND K. WONG AND DONALD R. YANCE JR. (CANADA, OREGON)

4. An Anthology of the Studies on the Anti-inflammatory, Antinociceptive and Febrifuge Effects of Medicinal Plants in Turkey 67-119
ESRA KÜPELI AKKOL AND ERDEM YESILADA (TURKEY)

5. Comparative Study of the Antigenotoxic Effects of Some Selected Natural Plant Products Against Methylmethane Sulphonate Induced Genotoxic Damage in Cultured Mammalian Cells 121-131
YASIR HASAN SIDDIQUE AND MOHAMMAD AFZAL (INDIA)

6. Inflammation and Mediators 133-178
S. SINGH, A. KOUL, R. SHARMA, G.D. SINGH, P. KOUL, A. KHAJURIA AND V.K. GUPTA (INDIA)

7. Recommendations for Reporting Randomized Controlled Trials of Herbal Interventions: CONSORT for Herbal Medicine Trials 179-209
JOEL J. GAGNIER N.D., HEATHER BOON, PAULA ROCHON, DAVID MOHER, JOANNE BARNES AND CLAIRE BOMBARDIER FOR THE CONSORT GROUP (CANADA)

8. Anxiolytic Activity Screening Studies on Extracts of a Few Medicinal Plants 211-218
RICHA SHRI, MANJEET SINGH AND ANUPAM SHARMA (INDIA)

9. Study of *Eugenia jambolana* and *Momordica charantia* in Respect to Safety and Antidiabetic Effect *In vivo* and *In vitro* 219-230
YELE, SANTOSH AND VEERANJANEYULU, A. (INDIA)

10. Larvicidal and Antimicrobial Activities of Seeds of *Annona cornifolia* A. St. -Hil. (Annonaceae) 231-237
LUCIANA ALVES R.S. LIMA, MARIA AMÉLIA D. BOAVENTURA, JACQUELINE A. TAKAHASHI AND LÚCIA P.S. PIMENTA(BRASIL)

11. Antidepressant Activity of the Extracts of Three *Hypericum* species Native to China 239-249
DEPO YANG, JIE BAI, ZHEN LI, GEORGE Q LI AND DONGMEI WANG (CHINA, AUSTRALIA)

12. Cytolytic and Toxic Effects of Ostreolysin, a Protein from the Oyster Mushroom (*Pleurotus ostreatus*) 251-264
KRISTINA SEPČIĆ AND ROBERT FRANGEŽ (REPUBLIC OF SLOVENIA)

13. Antioxidant, Antisecretory and Gastroprotective Activities from *Leiothrix flavescens* 265-279
THIAGO DE MELLO MORAES, MARCELO APARECIDO SILVA, CLENILSON MARTINS RODRIGUES, LOURDES CAMPANER DOS SANTOS, MIRIAM SANNOMIYA, LUCIA REGINA MACHADO DA ROCHA, ALBA REGINA MONTEIRO SOUZA BRITO, TAIS M. BAUAB, WAGNER VILEGAS AND CLÉLIA AKIKO HIRUMA-LIMA (BRAZIL)

14. Evaluation of the Immunity Potency and Security of Inactivated Oil-Adjuvant Vaccine against Chlamydiosis in Dairy Cattle 281-291
QIU C., ZHOU J., CAO X. AND LIN G. (CHINA)

15. *Annona crassiflora* Wood Constituents: Antimalarial Efficacy, Larvicidal and Antimicrobial Activity 293-305
MARY ANE GONÇALVES, VANESSA CARLA FURTADO MOSQUEIRA AND LÚCIA PINHEIRO SANTOS PIMENTA (BRAZIL)

16. Pharmacokinetics of Active Constituents of *Rhodiola rosea* SHR-5 Extract 307-329
A. PANOSSIAN, A. HOVHANNISYAN, H. ABRAHAMYAN, E. GABRIELYAN AND G. WIKMAN (ARMENIA, SWEDEN)

17. Metabolism and Pharmacokinetic Studies of Coumarins 331-353
BEHERA D. AND ADDEPALLI V. (INDIA)

18. Naringin and its Aglycone, Research and Managements 355-387
RICKY W.K. WONG AND FANNY Y.F. YOUNG (HONG KONG)

19. Biotransformation of Drugs 389-398
M.G. TYAGI, S. PRABHAKARAN AND K.R. DHANASEKHAR (INDIA)

20. Pharmacokinetics and Drug Interactions of Glycyrrhizin/Glycyrrhiza 399-416
A.K. AGGARWAL AND D.K. MAJUMDAR (INDIA)

21. Determination of Total Antioxidant Status and Rosmarinic Acid from *Orthosiphon stamineus* in Rat Plasma 417-427
GABRIEL AKYIREM AKOWUAH AND ISMAIL ZHARI (MALAYSIA)

22. Green Tea: Molecular Targets in Glucose Uptake, Inflammation, and Insulin Signaling Pathways 429-448
HEPING CAO, ANNE M. ROUSSEL AND RICHARD A. ANDERSON (USA, FRANCE)

23. Euphorbious Plants as Molluscicides and Piscicides: A Review 449-460
RAM P. YADAV AND AJAY SINGH (INDIA)

Index 475-493

ISBN : 1-933699-53-1

VOLUME 3: EFFICACY, SAFETY & CLINICAL EVALUATION II

1. Allylsulfides as Bioactive Compounds with Chemotherapeutic and/or Chemopreventive Effects 1-33
C. SCHERER, C. JACOB, M. DICATO AND M. DIEDERICH (GERMANY, USA)

2. Targeting Tumor Angiogenesis for Preclinical Validation of Antiangiogenic Compounds from Medicinal Plants 35-93
BHARATHI P. SALIMATH, THIPPESWAMY G., SHEELA M.L., JOHN T. LUCAS JR. AND STEVEN A. ROSENZWEIG (INDIA, USA)

3. Effects of Hydroalcoholic Extracts of Four Chinese *Hypericum* species on Learning and Memory in Mice 95-104
DONGMEI WANG, JIE BAI, GEORGE Q LI AND DEPO YANG (CHINA, AUSTRALIA)

4. Chemistry and Pharmacology of *Shorea robusta* 105-124
K. PUNDARIKAKSHUDU, MD. YASEEN KHAN AND HEMANT SAGRAWAT (INDIA)

5. Safety, Efficacy and Preclinical Evaluation of Plant Products 125-148
VADDE RAMAKRISHNA AND ORUGANTI H SETTY (INDIA)

6. *In vitro* and *In vivo* Combined Anti-Influenza Virus Effects of a Plant Polyphenol-Rich Extract and Synthetic Antiviral Drugs 149-165
JULIA SERKEDJIEVA (BULGARIA)

7. Antihypertensive and Hypolipidemic Effects of Tuber of *Apios americana* Medikus in SHR 167-181
KUNIHISA IWAI, SHUSUKE KURAMOTO AND HAJIME MATSUE (JAPAN)

8. Anti-Ulcer Effects of Aqueous Extract of *Persea americana* Mill (Avocado) Leaves in Rats 183-188
BAMIDELE V. OWOYELE, ISMAILA K. ADEBAYO AND AYODELE O. SOLADOYE (NIGERIA)

9. Anti-Asthmatic Medicinal Plants 189-227
EZIKE A.C. AND AKAH P.A. (NIGERIA)

10. Hepatoprotective Effects of *Pimpinella anisum* Seed Extract in Rats 229-235
NUREDDIN CENGIZ, HANEFI ÖZBEK AND AYDIN HIM (TURKEY)

11. Safety and Efficacy Study of Polyherbal Formulations "Mersina", "Limit" and "Emuil" in Experimental Animals 237-249
SATEESH B. AND VEERANJANEYULU A. (INDIA)

12. A Review of Biological Effects of Berberine and Croatian Barberry (*Berberis croatica* Horvat) as a New Source of Berberine 251-264
IVAN KOSALEC , MARIJANA ZOVKO, DARIO KREMER, NADA ORŠOLIC AND KSENIJA KARLOVIC (REPUBLIC OF CROATIA)

13. An Ethnopharmacological Review on *Calotropis* species 265-285
I.L. KOTHARI, M.H. PARABIA, J.H. PATEL, FARZIN M. PARABIA, SMITA S. PATHAK, FALGUNI SHETH, HIREN V. DOSHI AND M.A. PATEL (INDIA)

14. Antisickling Activity and Thermodegradation of an Anthocyanin Fraction from *Ocimum basilicum* L. (Lamiaceae) 287-295
P.T. MPIANA, V. MUDOGO, D.S.T. TSHIBANGU, K.N. NGBOLUA, P.K. MANGWALA, E.K. ATIBU, M.K. KAKULE, L.K. MAKELELE AND M.T. BOKOTA (RD CONGO)

15. Pilot Clinical Trial of KJJ® in Children with Uncomplicated Respiratory Tract Infections 297-307
S. BABAYAN, G. ASLANYAN, E. AMROYAN, E. GABRIELYAN AND A. PANOSSIAN (ARMENIA, SWEDEN)

16. Antidiabetic Effect of a Herbal Powder Consisting of *Curcuma longa*, *Emblica officinalis*, *Gymnema sylvestre* and *Tinospora cordifolia* 309-318
ANDALLU B., SUBHA V., VINAY KUMAR A.V. AND RAMA KUMAR K.S. (USA, INDIA)

17. Effect of *Aegle marmelos*, *Gymnema sylvester* and *Momordica charantia* on Blood Glucose and Lipid Profile in Alloxan Induced Diabetic Rabbits 319-328
G.B. JADHAV AND S.A. KATTI (INDIA)

18. Chemical Constituents from Anti-inflammatory Fraction of *Taraxacum mongolicum* and Their Inhibition of AChE 329-342
KEXIN HUANG, SHU YUN SHI, FENG LI, LIYAN SONG, MING YIN, ZEJIAN WANG, RONGMIN YU, YIHANG WU, QIAOFENG TAO, YU ZHAO, YAN XU, XIUMEI WU, XIAOKUN LI, JOACHIM STOCKIGT AND JIA QU (CHINA)

19. Inhibition of Hyaluronidase by Essential Oils and Other Natural Fragrant Extracts 343-349
EMILIE ULRICH AND BENOÎT AUFFRAY (FRANCE)

20. Chemical, Biological and Pharmacological Studies of the Essential Oil of *Croton nepetaefolius* Baill 351-362
JOSÉ HENRIQUE LEAL-CARDOSO, VÂNIA MARILANDE CECCATTO, SELENE MAIA DE MORAIS, ANDRELINA NORONHA COELHO-DE-SOUZA, ORIEL HERRERA BONILLA, SANDRA MARIA DIAS MORAIS, GLORIA ISOLINA PINTO DUARTE, SAAD LAHLOU AND PEDRO JORGE CALDAS MAGALHÃES (BRAZIL)

21. Pharmacokinetic Studies on Hepatic Uptake Mechanism of Tetrodotoxin in the Puffer Fish *Takifugu rubripes* 363-391
TAKUYA MATSUMOTO AND YUJI NAGASHIMA (JAPAN)

22. Herbal Drugs - Applications in the Post Genomic Era 393-402
ISHA DHANDE AND RUMI GHOSH (INDIA)

23. Antifungal Activity of Marine Bacterial Associates of Deep-Sea Origin Against Plant Pathogens 403-411
A. VIMALA, C.C. RATH AND A. SREE (INDIA)

Index 425-437

ISBN : 1-933699-54-X

VOLUME 4: ANTIOXIDANTS & NUTRACEUTICALS

1. The Challenges of Antioxidant Therapy with Natural Products 1-35
A.J. NÚÑEZ SELLÉS AND G. GARRIDO GARRIDO (CUBA)

2. Antioxidant Activity of the Phytochemicals 37-66
BRATATI DE AND ARCHANA BANERJEE (INDIA)

3. Determination of Antioxidant Capacities of Non Alcoholic Beverages Prepared from Three Wild Fruits of Zimbabwe 67-87
NHUKARUME L., CHIKWAMBI Z. AND MUCHUWETI M. (ZIMBABWE)

4. Spontaneous Short-Term Fermentation of Garlic Potentiates its Anti-Oxidative Activities 89-98
YOSHIMI NIWANO, EMIKO SATO AND MASAHIRO KOHNO (JAPAN)

5. Natural Antioxidant Phytochemicals in Fruits, Berries and Vegetables and their Degradation Status During Processing 99-131
SINGH DHEERAJ, BHUSHAN SASHI, LOBSANG WANGCHU, KAVITA A. AND MOOND S.K. (INDIA)

6. The Grape Fruit Flavanone, Naringin Reduces Ferric Ion Induced Oxidative Stress *In vitro* 133-155
GANESH CHANDRA JAGETIA (INDIA)

7. Scavenging Capacity of *Allium* Species 157-166
ŠTAJNER D., POPOVIC´ B.M. AND ŠTAJNER M. (SERBIA)

8. Effect of *Aquilegia vulgaris* on Liver Antioxidant Status in Rats Treated with Diethylmaleate 167-177
JADWIGA JODYNIS-LIEBERT, MALGORZATA KUJAWSKA, IRENA MATLAWSKA, WIESLAWA BYLKA AND MAREK MURIAS (POLAND)

9. Antioxidant Activity, Medicinal Plants and Nervous Disorders: A Review 179-215
SHARMA KOMAL, SHARMA DURGESH, JAIN AYUSHI, JOSHI CHETAN AND BHATNAGAR MAHEEP (INDIA)

10. Free Radical Scavenging and DNA Damage Preventing Properties of *Amaranthus tricolor* L. 217-227
ANANYA MUKHOPADHYAY AND BRATATI DE (INDIA)

11. Free Radical Scavenging Activity of Methanolic Bark Extract of *Limonia acidissima* 229-234
THET THET HTAR AND G.A. AKOWUAH (MALAYSIA)

12. *Sorghum arundinaceum*, A Wild Cereal Grain with Potential: Comparison of its Antioxidant Potential with that of Domestic Cereals in the Same Family 235-243

CHITINDINGU K., BENHURA M.A.N. AND MUCHUWETI M. (ZIMBABWE)

13. Antioxidant and Micronutrient Potential of Some Marketed Mango Varieties and Mango Products 245-257
AGTE V. VAISHALI AND TARWADI V. KIRTAN (INDIA)

14. Vimang: Experiences from the Antioxidant Therapy in Cuban Primary Health Care 259-271
GILBERTO L. PARDO ANDREU, ALBERTO J. NÚÑEZ SELLÉS, MARIELA GUEVARA GARCÍA AND ALINA ALVAREZ LEÓN (CUBA)

15. Novel Nutraceutical Properties of *Stevia rebaudiana* (Bertoni) Bertoni 273-281
SHARMILA CHATTOPADHYAY (INDIA)

16. Oligosaccharides and Total Polyphenols Contents in Italian Common Bean (*Phaseolus vulgaris* L.) Landraces: A Quality Evaluation 283-292
FIBIANI M. AND LO SCALZO R. (ITALY)

17. Biological Activities and Main Compounds of Fruits 293-313
PATRICIA DALLA SANTA SPADA, GUSTAVO SCOLA, JOÃO A.P. HENRIQUES AND MIRIAN SALVADOR (BRAZIL)

18. Grape Juice: Its Compounds and Health Benefits 315-326
DANI CAROLINE, OLIBONI LIVIA S., HENRIQUES JOÃO A.P. AND SALVADOR MIRIAN (BRAZIL)

19. Nutraceutical Potential of Commonly Consumed Fruits and Vegetables 327-349
VAISHALI V. AGTE AND KIRTAN V. TARWADI (INDIA)

20. Bioactive Compounds and Functional Foods of Pseudocereals 351-371
TAMER H. GAMEL (EGYPT)

INDEX 387-399

ISBN : 1-933699-55-8

VOLUME 5: IMMUNE-MODULATION & VACCINE ADJUVANTS

1. Herbal Therapies as Immune Modulators for Anti-Cancer Therapeutics 1-19
STEPHEN M. SAGAR, DANIEL M-Y. SZE AND RAIMOND K. WONG (CANADA, AUSTRALIA)

2. Neem Leaf Glycoprotein as a New Vaccine Adjuvant for Cancer Immunotherapy 21-45
BARAL RATHINDRANATH, SARKAR KOUSTAV, MANDAL-GHOSH ISHITA AND BOSE ANAMIKA (INDIA)

3. Honey Bee Products: Immunomodulation and Antitumor Activity 47-89
NADA ORŠOLIĆ, DAMIR SIROVINA, IVAN KOSALEC AND IVAN BAŠIĆ (CROATIA)

4. Mitogenic and Anticoagulant Activity of a Fucoidan Isolated from Brown and Red Seaweed 91-104
LYDICE C.M. CARVALHO, HUGO A.O. ROCHA, FERNANDA W. OLIVEIRA, SUELY F. CHAVANTE, SELMA M.B. JERÔNIMO AND EDDA L. LEITE (BRAZIL)

5. Development of Plant Based Adjuvants for Vaccine Antigens 105-122
GUPTA AMIT, KHAJURIA A., SINGH J., TABASUM SIDIQI, SINGH S., GUPTA V.K., SURI K.A., SATTI N.K., SRINIVAS V.K., ELLA KRISHNA AND QAZI G.N. (INDIA)

6. Immunomodulation and Vaccine Adjuvants 123-162
TEENA MOHAN, BHAT A. AJAZ AND RAO D.N. (INDIA)

7. Recent Advances in the Use of Medicinal Plants as Immunostimulants 163-202
NAYAK SMITA AND MENGI SUSHMA (INDIA)

8. Balance of Pro-/Anti-Inflammatory Cytokines Release in Spleen Cells from Mice Treated with *Crotalus durissus terrificus* Venom 203-218
GARDUÑO-RAMÍREZ M.L., HERNÁNDEZ CRUZ A., ZUCATELLI MENDONÇA R. AND PETRICEVICH V.L. (MEXICO, BRAZIL)

9. Bioactive Agents from Herbal Sources with Immunopharmacological Properties Abating Inflammation and Malignancy 219-266
CHAKRAVARTY ASHIM K., MAZUMDER TAMAL AND YASMIN HADIDA (INDIA)

10. Differential Effects of Subchronic and Chronic Oral Treatments with *Orbignya phalerata* Mart. Mesocarp on the Inflammatory Response 267-281

FLÁVIA R.F. NASCIMENTO, ELIZABETH S.B. BARROQUEIRO, ANA PAULA S. AZEVEDO, MÁRCIA C.G. MACIEL, WANDERSON S. PEREIRA, SUSANNE C. FERREIRA, JARDEL C. FARIAS, MAYARA T. PINHEIRO, LUCILENE A. SILVA AND ROSANE N.M. GUERRA (BRAZIL)

11. A Mistletoe Lectin-Containing Preparation for Oral Use Provokes an Immune Response and Induces an Increase in the Population of Activated Natural Killer Cells 283-299

WINGE I., DALE T.M., TILREM J.P.P. AND PRYME I.F. (NORWAY)

12. Flavonoids from Complementary and Alternative Medicine: Mechanism of Imunomodulation of Macrophages 301-319

PANDIMA DEVI K., KIRUTHIGA P.V. AND KARUTHA PANDIAN S. (INDIA)

13. Herbal Immunomodulators 321-355

CHAUHAN SINGH NAGENDRA, SHARMA VIKAS AND DIXIT V.K. (INDIA)

14. Immunomodulatory Activity of Botanicals 357-383

KAUL A., KHAJURIA A., SINGH S., SINGH G.D., CHANDAN B.K. AND GUPTA V.K. (INDIA)

15. The Potentials of Immunomodulatory Substances of Natural Origin in Contemporary Medical Practice 385-403

CHUKWUEMEKA SYLVESTER NWORU AND PETER ACHUNIKE AKAH (NIGERIA)

16. Study of Immunomodulatory Activity of *Sphaeranthus indicus* 405-415

VIKANI K.V., LAHIRI S.K., SANTANI D.D., KAPADIA N.S. AND SHAH M.B. (INDIA)

Index 431-441

ISBN : 1-933699-57-4

VOLUME 7: STRUCTURAL MODIFICATIONS & DRUG DEVELOPMENT

1. Synthetic and Clinical Status of Marine Derived Anticancer Peptides† 1-28

DIWAN S. RAWAT, RAM SINGH, NITIN KUMAR, MUKUL SHARMA AND M.S.M. RAWAT (INDIA)

2. Microwave-Assisted Non-Conventional Esterification of Polysaccharides 29-43

SROKOVÁ, I. AND EBRINGEROVÁ, A. (SLOVAKIA)

3. Structural Modifications of Parthenin: A Sesquiterpene Lactone of Biological Potential 45-76

BHAHWAL ALI SHAH AND SUBHASH CHANDRA TANEJA (INDIA)

4. Prospects for the Development of Polyphenolic Acetate as the Potential Drug Candidate: A Review 77-95

HANUMANTHARAO G. RAJ, AJIT KUMAR, RANJU KUMARI, SEEMA BANSAL, PRIJA PONNAN, PRABHJOT SINGH, NIVEDITA PRIYA, GARIMA GUPTA, SHVETAMBRI ARORA, ANIL S. BAGHEL, SANJAY GOEL, RUCHIKA GULATI, USHA SINGH, RASHMI TANDON, DAMAN SALUJA, BILEKERE S. DWARAKANATH, ANANT N. BHAT, TAPESH K. TYAGI, AMIT VERMA, VISHWAJEET ROHIL, ASHOK K. PRASAD, EKTA KOHLI, ANJANA VIJ, SURINDER K. BANSAL, VANNAN K. VIJAYAN, SUBASH C. JAIN, RAMESH C. RASTOGI, AND VIRINDER S. PARMAR (INDIA)

5. Mining Bioactive Conformations: A Novel Methodology for Computing Predictive 3D-QSAR Models 97-119

JAYENDRA B. BHONSLE AND DONALD P. HUDDLER (USA)

6. Synthesis and Structure-Activity Relationships of Some Taxoids as Multidrug Resistance Modulator in MDR Cancer Cells 121-155

LIMING BAI, TOSHIAKI HASEGAWA, JIAO BAI, MUTUMI KITABATAKE, JINLAN WANG, JUNICHI MATUBARA, JUN-ICHI SAKAI, JUNGUI DAI, SHUJUN ZHANG, WANXIA TANG, LIYAN WANG, YUHUA BAI, MINJIE LI, KATUTOSHI HIROSE AND MASAYOSHI ANDO (JAPAN, PEOPLE'S REPUBLIC OF CHINA)

7. Antioxidant and Neuroprotective Effects of Synthetic Curcumin Analogues and Natural Phenolics 157-181

YU ZHAO, LEIXIANG YANG, KEXIN HUANG, LIHONG HU, XIUMEI WU, XIAOYU WANG, XIAOKUN LI, XIAOJIANG HAO, JOACHIM STÖCKIGT AND JIA QU (CHINA)

8. Action of Plant Proteinase Inhibitors Using Biological Models 183-192

MARIA LUIZA V. OLIVA AND MISAKO SAMPAIO (BRAZIL)

9. Plant Hormone Conjugates 193-245

ANDRZEJ BAJGUZ AND ALICJA PIOTROWSKA (POLAND)

10. Biological Prospective of Pinitol and its Structurally Modified Products 247-266
BHAHWAL ALI SHAH AND SUBHASH CHANDRA TANEJA (INDIA)

11. Drug Sensitivity of Curcumin Analogues and Bioconjugates 267-298
ARCHANA PANDEY, VISHNU DWIVEDI AND KRISHNA MISRA (INDIA)

12. Structural Based Drug Design of Estrogen Receptor Beta Selective Ligands 299-314
B. BALAJI, C. SABARINATH AND M. RAMANATHAN (INDIA)

Index 329-336

ISBN : 1-933699-58-2

VOLUME 8: QUALITY CONTROL & STANDARDIZATION

1. Chromatographic Techniques Applied to the Natural Products Chemistry 1-40
MARIA APARECIDA M. MACIEL, VALDIR F. VEIGA JR., ANGELO DA CUNHA PINTO, FABIANO E.S. GOMES AND TEREZA N.C. DANTAS (BRAZIL)

2. Quality Control of Natural Medicines by Immunological Assay System 41-56
WARAPORN PUTALUN, OSAMU MORINAGA, HIROYUKI TANAKA AND YUKIHIRO SHOYAMA (THAILAND, JAPAN)

3. Comparison of HPLC and HPTLC Methods for the Determination of Rosmarinic Acid from *Orthosiphon stamineus* 57-65
GABRIEL AKYIREM AKOWUAH AND ISMAIL ZHARI (MALAYSIA)

4. Quality Control and Standardization of Medicinal and Aromatic Plants 67-87
ANJAN BHATTACHARYYA, RAJLAXMI POI, MD. WASIM AKTAR AND N. SANYAL (INDIA)

5. Development of a Thin-Layer Chromatography-Densitometric Method for the Quantification of Podophyllotoxin in *Podophyllum hexandrum* 89-96
ARCHANA PESHIN RAINA, S.K. PAREEK AND K.S. NEGI (INDIA)

6. Standardisation of Polyherbal Formulations Using Marker Compounds 97-120

B.D. GUPTA, N.K. SATTI, V.K. GUPTA, PRABHU DUTT AND K.A. SURI (INDIA)

7. Characteristics Variation of Lavender Oil Produced by Different Hydrodistillation Techniques 121-135
G. D. KIRAN BABU AND BIKRAM SINGH (INDIA)

8. Quality Control Methods for Herbal Medicines 137-179
GEORGE Q. LI, VALENTINA RAZMOVSKI-NAUMOVSKI, ESHAIFOL OMAR, AIK WEI TEOH, BENJAMIN KIMBLE, VINCENT L. QIAO, MIN-KYONG SONG, WANNIT TONGKAO-ON, SRINIVAS NAMMI, SUILIN MO, NANTIGA VIRGONA AND KONG M. LI (AUSTRALIA, CHINA)

9. Evaluation of Biological Activity in Quality Control of Herbal Medicines 181-215
GEORGE Q. LI, VALENTINA RAZMOVSKI-NAUMOVSKI, ESHAIFOL OMAR, AIK WEI TEOH, MIN-KYONG SONG, WANNIT TONGKAO-ON, SRINIVAS NAMMI, SUILIN MO, NANTIGA VIRGONA AND KONG M. LI (AUSTRALIA, CHINA)

10. Chemical Analysis and Quality Control of Essential Oils 217-236
LAHLOU M. (MOROCCO)

11. Development and Validation of Ultraviolet Spectrophotometric Assay Method for the Powdered Leaves, Extracts and Formulations of *Loranthus micranthus* 237-249
UZOCHUKWU I.C. AND OSADEBE P.O. (NIGERIA)

12. Exploring Pharmacovigilance for Traditional Herbal Medicines 251-283
PULOK K. MUKHERJEE AND S. PONNUSANKAR (INDIA)

13. HPLC as a Tool for Qualitative and Quantitative Evaluation of Herbal Drugs and Formulations 285-305
R. GOVINDARAJAN, D.P. SINGH AND A.K.S. RAWAT (INDIA)

14. Consumer Protection and Regulatory Requirements for Herbal Drugs 307-321
N. SREEVIDYA AND S. MEHROTRA (INDIA)

Index 337-344

Index

(-)-epicatechin 397
(–)-oplopanone 307
(+)-catechin 397
1,2-benzenediol 393
1,2-dioleoylglycerol 307
1,2-naphthoquinone 97
1,4-β-D-xylan-4-xylanohydrolase 77
1,4-naphthoquinone 97
1,7,8-trimethyl plumbagate 62
12-O-methyljaborosalactone 23
13C-NMR 79
13C-NMR spectra 350
15α-hydroxymansumbinone 305
1-acidic-glycoprotein (AAG) 129
1-ethyldecylbenzene 393
1H-NMR analysis 79
2,3-di-O-methyl-L-arabinose 310
2,4,6-tri-O-methyl-D-galactose 309
24-methylencycloartanol 393
2-acetoxyfuranodienone 301
2-butyloctanol 393
2D NMR spectroscopy 384
2-decanol 316
2-methoxyfuranodienone 301
2-nonanone 316
2-octanone 316
2-undecanone 316
3,3',4-tri-O-methylellagic acid 157
3,3'-di-O-methylellagic acid 157
3,4-dihydroxy benzoic acid 397
3,4-seco-manumbinoic acid 307
3α,20(S)-dihydroxydammar-24-ene 154
3-α-campesterol 393
3α-hydroxy-T-cadinol 307
3-aminopropyl triethoxysilane (APTEOS) 367
3β,24-dihydroxyciloart-25-ene 154
3β,24-dihydroxyurs-12-ene 149
3β-acetoxydammar-24-ene 302
3-β-amyrinacetate 301
3β-hydroxydammar-24-ene 302
3β-hydroxy-mansumbin-13(17)-en-16-one 303
3-eicosene 392
3-hydroxy-4-methoxybenzoic acid 61
3-isocyanatopropyl-triethoxysilane (ICPTES) 367
3-oxo-T-cadinol 307
4-hydroxy-3,5-dimethoxybenzoic acid 61
4 O Me a D GlcA 79
4-O-methyl glucuronoxylan 72
4-O-methyl-glucuronic acid 76, 308
5-methoxypropaicin 155
6,7-dedihydroxyl plumbagic acid 62
6-methyl-3-heptanol 392
7-hydroxy-2,5-dimethyl-chromen-4-one 62
9-octadecenoic acid glycerid 392
α-amyradienol 149
α-amyrin 145, 300, 392
α-bisabolene 306
α-camphesterol 395
α-camphorene 310
α-chamigrene 392
α-chitin 343
α-cobratoxin 172, 177
α-copaene 299
α-elemene 392, 393, 395
α-elemolic acid 149
α-eudesmol 392, 393, 395
α-guaiene 392, 395
α-humulene 159, 393
α-hydroxynitrile 236
α-linolenic acid 229
α-neurotoxin 177
α-oxobisabolene 300
α-pinene 298
α-santalene 306
α-selinene 392, 393, 395

α-sitosterol 392, 395
α-spinasterol 313
α-terpineol 158
α-thujene 158, 299
β2-glycoprotein I (ß2GPI) 130
β-allyl lapachone 102
β-amyradienol 149
β-amyrin 145, 301
β-bisabolene 303
β-bourbonene 299
β-caryophyllene 159
β-chitin 343
β-cyanoalanine 237
β-elemene 298
β-farnesene 306
β-glucan 355
β-glucosidases 236
β-piene 303
β-sitosterol 298
δ-elemene 298
δ-lactone 23
γ-bisabolene 300
γ-cadinene 299
γ-chitins 343
γ-glycidoxypropyltrimethoxysilane (GPTMS) 367
γ-lactona 25
μ-cresol 298
ν-Heptadecane 393
ν-Hexadecane 392
π-cymene 299

A

Absidia butleri 360
Absidia coerulea 360
Accessory enzymes 77
Acetic acid 298
Acetoxyfuranodiene 309
Acetylcholinesterase 159, 169
Acnistus arborescens 4
A. ramiflorum 4
Adipocytes 204, 221
Adipokines 204, 205
Affinity chromatography 175
Aggregation 170
Agrobacterium rhizogenes 335
Ajuga bracteosa 4
A. parviflora 4
Alcaligenes faecalis 276
Aldobiouronic acid 309
Alkaloids 125
Allylcembrol 310
Alpha helical 269
Alpha-fetoprotein 120
Althea officinalis 82
Alzheimer's disease 124
Amygdalin 232
Analgesic 144, 384, 401
Anemia 384
Angiogenesis 1, 41, 84, 343
Angiogenic 123
Angiosperms 72
ANOVA 207
Anthelmintic 324, 325
Anthracyclines 125
Anthraquinone 98
Antiapoptopic 133, 401
Antiarthritic 36
Anti-atherogenic 126, 401
Anti-bacterial 1, 97, 268
Anti-biotics 125
Anti-cancer 97, 268
Anti-carcinogenic 126
Anti-cholinesterase 36
Anti-coagulants 168
Anti-complementary 72
Antidiarrhoeal 186, 325
Antifungal 97
Antigens 266
Anti-hyperlipidemic 401
Anti-inflammation 122
Anti-inflammatory 1, 36, 97, 158, 324, 384
Antileishmanial 1
Antimalarial 325
Antimicrobial 72, 171, 324, 343
Antimicrobial peptides (AMPs) 266
Antineoplastic 97
Anti-nocipetive activities 158
Antioxidant 126, 185,232, 384, 401
Anti-oxidative effect 119
Antiphlogistic 324
Antipyretic 36, 324
Antistress 1
Anti-stress effects 36
Antitrypanosomal 1
Anti-tumor 1, 36, 72, 97
Anti-ulcerogenic 205
Anti-viral 97, 268

ApoD 124
Apolipoprotein D 124
Apoptosis 42, 57, 65, 401
Arenicola marina 280
Argania spinosa 86
Ariesin 151
Aromandrene 392
Artemia salina 159
Arthralgies 384
Asergillus alliaceus 362
Asparagine 237
Aspergillus nidulans 362
A. niger 360
Aurelia aurita 272
Autohydrolysis 88
Autoimmune disorders 219
Avicennia alba 101
A. rumphiana 101

B

Bacillus subtilis 77, 160, 276
Bacterial flagellin 267
Bacterial lysis assays 266
Balsamodendron pubescens 296
Benign prostatic hyperplasia 384
Benzoic acid 397
Benzoquinone 97
Betulin 151
Binaphthaquinone 98
Bioactivity 87, 119
Bioavailability 399
Bio-guided assay 57
Biomedicines 168
Biosensors 370
Bombax malabaricum 105
B. mori 352
Bothrops insularis 176
B. jararacussu 175
B. moogeni 176
Brachistus stramonifolius 4
Bradykinin-potentiating peptides 169
Breast carcinoma 82
Bronchial asthma 384
Bulnesol 392
Burselignan 153
Burseneolignan 153
Bursera delpechiana 148
B. graveolens 147
B. permollis 152
B. tokinensis 152, 153, 154
Burseraceae 143, 287

C

Calceolaria andina 108
Calnexin 127
Calreticulin 127
Campesterol 313
Campestoside 330
Cancer 384
Car-3-ene 299
Carbraleadiol 302
Carbraleadiol-3-acetate 300
Carbraleone 302
Carcinoembryonic antigen (CEA) 122
Carcinogenesis 401
Carcinomas 123
Carcinosarcoma 41
Cardiovascular 186, 406
Carica papaya 362
Carotenoids 347
Caryophyllene 299
Cassia siamea 4
Castanea sativa 72
Catalase 132
Catalpa ovata 109
Catechin (C) 126
Catechin gallate (GC) 126
Catechins 126
CD spectra 59
CD spectroscopy 399
Cedrelone 316
Cell-cell adhesion 125
Cell-cycle inhibitory proteins 123
Cellular immunity 266
Cembrene 310
Centaurium erythraea 331
C. pulchellum 331
Ceruloplasmin 120
Characterization 185
Chemokines 267
Chemopreventive 126
Chemotaxonomic markers 30
Chemotaxonomy 324
Chestnut tree 72
Chiral HPLC 59
Chitin 267, 341
Chitosan 341
Chlorella vulgaris 131
Cholest-5-en-3b-ol 298
Cholinesterase 1

Chorionic gonadotropin 120
Chromatography 168, 172
Chromobacterium violaceusm 276
Chromostatin 123
Cinnamaldehyde 298
Cinnamomum burmannii 205
Cinnamon polyphenol extract 204
C-NMR 59
C-NMR spectrum 397
Coagulopathy 168
Cobra 169
Cobra venom factor (CVF) 180
Colletotrichum lindemuthianum 362
Comb jellyfish 272
Comic acid A 305
Commiphora 287
C. abyssinica 288
C. africana 289
C. confusa 289
C. cyclophylla 289
C. dalzielli 290
C. erlangeriana 290
C. erythraea 290
C. glandulosa 291
C. guidotti 292
C. guillaumini 292
C. holtziana 292
C. incisa 293
C. kua 290
C. madagascarensis 288
C. merkeri 293
C. molmol 293
C. mukul 294
C. myrrha 296
C. opobalsamum 296
C. rostrata 296
C. sphaerocarpa 297
C. tenuis 297
C. terebinthina 297
C. wightii 297
Comparative immunology 266
Contraceptive 144
Corymbiferin 329
Counter current extraction 185, 190
COX-2 210
COX-2 enzyme 40
Crepidospermum rhoifolium 154, 155
Cuminaldehyde 298
Cunninghamella echinulata 361
C. elegans 356
Curzernone 299
Cutaneous infections 384
Cyanocobalamin 239
Cyanogenic glycosides 230
Cyanohydrins 231, 235
Cyanomethemoglobin 240
Cyclic nucleotide gated channels 178
Cyclooxygenase 2 209
Cyclooxygenase 1
Cytokines 125, 204, 267
Cytotoxic 1, 72, 97
Cytotoxicity 57, 65, 170

D

D5-campestan-3b-ol 298
Dacryodes hopkinsii 148, 156
Datura fastuosa 4
D. ferox 4
D. innoxia 4
D. metel 4
D. quercifolia 4
D. tatula 4
Daturalicin 29
DBD glycoprotein 133
d-cadinene 299
De novo 237
Degree of polymerization 91
Demethyleustomin 331
Deoxypodophyllotoxin 151
Deprea orinocensis 4
D. subtriflora 4
Detoxification pathway 239
Dhurrin 235
Diabetes 204, 384
Diabetes mellitus 385
Diabetic retinopathy 219
Diarrhea 384
Didehydroguggulsterone-M 318
Diglucosides 233
Dimethylterphthlene 300
Dionacea muscipula 98, 101
Dioscorea batatas 133
Diospyros maritime 98
D. morrisiana 103
Diphtheria 186
Diterpen 287
Dodecanal 393
DPPH 133
Drosera rotundifolia 99
Drosophila 281

Dunalia australis 5
D. brachyacantha 5
D. solanacea 5

E

Efficacy 187
Elemol 299
Ellagic acid 157, 312
Enterobactor aerogenes 276
Enzymatic extraction 341
Enzymatic hydrolysis 259
Epi-β-santalene 306
Epicatechin (EC) 126
Epigallocatechin (EGC) 126
Epigallocatechin gallate (EGCG) 126
Epi-lupeol 148, 302
Erlangerins 302
Escherichia coli 131, 160, 276
ES-MS spectra 397
Ethnopharmacology 144
Etoposides 125
Eudesme 307
Eugenol 298
Eustomin 331
Exodeconus maritimus 5
Exons 169

F

Febrifuge 325
Fibrigenolysis 181
Fibrillin-1 129
Fibrinogenolytic activity 181
Fibronectin 125
Fibrosarcoma 82
Flavanols 126
Flavonoids 323, 388
Flax 230
Flow cytometry analysis 66
Folin-ciocalteu method 399
Folk medicine 144, 325
Follicle-stimulating hormone 120
Formic acid 298
Free sugars 388
Friedelin 149, 154
Fucose 120
Furanodiene 299
Furanodiene-6-one 308
Furanodienone 299
Furanoeudesmane 308
Furanogermacrene-3 309

G

Galactoproteins 355
Galactose 120
Galactoseamine 120
Gallic acid 397
Gardenia jasminoides 132
Gas chromatography (GC) 390, 241
Gastric disorders 384
GC-MS 76
Gel-filtration 172
Gene expression 204
Gentiacaulein 332
Gentiakochianin 333
Gentiana kochiana 332
G. lutea 324
G. pannonica 324
G. punctata 324
G. purpurea 324
Gentianella 328
G. albanica 328
G. austriaca 328
G. bulgarica 328
G. crispata 328
Gentiopicrin 325, 327, 335
Germacrene D 159, 299
Ginkgo biloba 402
GJE glycoprotein 132
Glucocorticoid 41
Glucose 120, 204
Glucose transporter 204
Glucoseamine 120
Glucuronoproteins 355
Glucuronoxylan 72
GLUT4 mRNA 209
Glutathione peroxidase 132
Glutathione S-transferase 205
Glycoprotein hormone receptors 126
GM-CSF genes 216
Golgi apparatus 121
Golgi complexes 121
Gongronella butleri 361
Goniothalamus 114
Gram negative bacteria 280
Granulocyte-macrophage colony-stimulating factor 209
Green chemistry 359
Guaiane 306
Guaiol 393

Guggulsterol Y 318
Guggulsterol-1, II 311
Guggulsterone 287

H

Haemoglobin 385
Haemostatic 144
Hannahpep 169
Hemachatus haemachatus 179
Hemolymph 267
Hepatocarcinoma 82
Heptadecanenitrile 393
Herbal medicine 186
Herbivores 232
Hexanoic acid 394
High performance liquid chromatography (HPLC) 241
High speed countercurrent chromatography (HSCCC) 188
Hinesol 395
H-NMR spectrum analysis 60
Host defense 266
HPLC analyses 391
Humid digestion 384
Humoral immunity 266
Humulene 299
Hyaluronidases 169
Hydrophobic interaction chromatography 175
Hydroxyl radicals 133
Hydryxocobalamin 239
Hyoscyamus niger 5
Hyperalgesia 171
Hypolipidemic 135
Hypotensive 325

I

Ichthyotoxic 97
IFNγ 219
Illex argentinus 350
Immunoblotting 205
Immunoglobulin gene 122
Immunomodulatory 36, 384, 401
Immunostimulating effects 1
Immunosuppressive 36
Impatiens balsamina 103
Imunoglobulins 120
In situ 367
In vitro 42, 82, 131, 204, 247, 335, 351, 384
In vivo 42, 131, 204, 384
Infertility 384
Inflammation 204
Innate immunity 267
Insulin 219, 221
Insulin signaling pathway 204
Integrin chains beta-1 127
Introns 169
Invertebrate 266
Iochroma australe 5
I. coccineum 5
I. fuchsioides 5
I. gesnerioides 5
Ion chromatography 259
Ion-exchange chromatography 172
Ionic channel blockers 171
IR spectrometry 359
Isofouquierone 302
Isofuranogermacrene 299
Isonicotinic acid-barbituric acid 257
Isoorientin 328
Isoorientin-3'-O-glucoside 328
Isoorientin-4'-O-glucoside 327
Isophysalin B 30
Isovitexin 328
Ixocarpalactones 2, 34

J

Jaborolactone 25
Jaborolone 35
Jaborosa araucana 5
J. bergii 5
J. caulescens 5
J. integrifolia 5
J. kurtzii 5
J. laciniata 5
J. leucotricha 5
J. magellanica 5
J. odonelliana 5
J. rotacea 5
J. runcinata 5
J. sativa 5
Jaborosalactone 22
Jaborosotetrol 35
Jatropha mulfidita 230
Juniper camphor 393

K

K-bungarotoxin 179

Keratinocytes 83
Kigelia pinnata 110
Kurita's process 349

L

Lachesis muta rhombeata 175
Lactobacillus paracasei 364
L. pentosus 365
Lantana 102
Lantana involucrata 102
L-arabinose 308
Lautostralin 230
Laxative 144
LDL cholesterol 204
Ledol 392
Leishmania (V) *panamensi* 38
L. major 38
Leishmanicidal 97
Lignin 71
Limonene 158, 159, 298
Linalool 158
Linamarin 230
Lindestrene 299
Linum usitatissimum 229, 232
Linustatin 230
Lipopolysaccharide (LPS) 206, 267
Lipoproteins 267
L-isoleucine 234
Listeria monocytogenes 131
Lithospermum erythrorhizon 102
Long chain tetrols 314
L-tyrosine 232
Lupane 144
Lupeol 302
Lupus 384
Lupus erythematosus 385
Luteinizing hormone 120
L valine 234
Lysosomes 121
Lysozyme 266, 270

M

Macrobrachium rosenbergii 351
Macrophages 207
Magainin 269
Mahonia aquifolium 82
MALDI mass-spectrometry 181
MALDI-MS analysis 88
Malignant cells 83
Mangifera indica L. 384
Mangiferin 328, 397
Mango stem bark 384
Manihot esculenta 232
Mannoproteins 355
Mannose 120
Mansumbin-13(17)-en-3,16-dione 303
Mansumbinoic acid 304
Mansumbinol 304
Mansumbinone 304
Matrix metalloproteases 84
Medicinal plants 186
MeGA 91
Menorrhagia 384
Metabolic syndrome 219
Metalloproteinases 180
Methemoglobin 240
Methoxyfuranodiene 309
Methyl gallate 397
Methyl plumbagate 57, 61
Methyl-13-methylpentadecanoate 392
Methyl-2-oxohexadecanoate 394
Micrococcus luteus 274
Microwave assisted extraction 341
Mitochondrial transmembrane potential 67
Mnemiopsis leidyi 272
Molluscicidal 36
Monoglucosides 233
Moon jellyfish 272
Morelensin 151
Mucor rouxii 357
Muramidase 271, 282
Musca domestica 351
Muscanone 318
Myccobacterium bovis 332
M. tuberculosis 39
Myeloma kidney 128
Myrcene 299
Myricyl alcohol 310
Myristic acid 393
Myrrhanone A 315
Myrrhone 152, 316

N

N-acetylglucosamine 271
N-acetylmuramic acid 271
Naja kaouthia 172, 177

N. oxiana 175
Naphthaquinones 97
Naphthoquinone 97
Narcotic 186
NARP-HPLC 185
Natural killer cells 267
Nduce platelet aggregation 171
Necrosis 42
Nematodes 232
Neolinustatin 230
Neotropic region 144
Nephropathy 128
Neuroendocrine marker 123
Neuropeptides 123
Neuroprotector 401
Neurotoxins 168
Neurotrophin 128
Neutraceutical 229
Nicandra physaloides 5
Nicotiana tobacum 185
Nicotine 186
Non-volatile 384
Nootkatone 394
Norswertianin-1-O-glucoside 327
Nucleases 169
Nucleoplasmic glycoprotein 120
Nucleosides 169
Nutritional supplement 384

O

Obesity 204
Ocimine 303
Octanal 393
OFI Glycoprotein 132
O-GlcNAc cycling 121
Oleane 144
Oleoresin 144
Oligopeptides 169
Oncogene 125
Ophiophagus hannah 179
Opioids 125
Opuntia ficus-indica 132
Orosomucoids 120

P

Pachyrhizus erosus 356
Paepalathus latipes 105
Pain reliever 186
Palmitic acid 298, 392
PAMPs 267
Pancreastatin 123
Parastatin 123
Parkinsonism 186
Pathogen recognition receptors (PRRs) 267
Pathogenicity 362
Pectin 73
Pelargonidin-3, 5-di-O-glucoside 312
Penaeus azetecus 346
P. durarum 346
P. indicus 347
P. monodon 347
Pentas longiflora 113
Peptidoglycan 267
Perulactones 2
P-glycoprotein 125
Phagocytes 266
Phaseolus lunatus 232
Phellamurin 300
Phenolics 388
Physalins 2, 29, 30
Physalis angulata 6
P. chenopodifolia 6
P. cinerascens 6
P. coztomatl 6
P. divericata 6
P. minima 6
P. peruviana 6
P. philadelphica 6
P. pruinosa 6
P. solanaceus 6
P. viscosa 6
Phyto-chemicals 144, 186, 187
Phytomedicine 384
Picropolygamain 150, 304
Pinene 287
Plant glycoprotein 119
Plasminogen 120
Platelet 170
Plumbagic acid 57, 61
Plumbagin 57, 62, 65, 97
Plumbago scandens 98
P. zeylanica 57, 98
Podisus maculiventris 269
Podocarpusflavone A 157
Podophyllontoxin 153
Polygamain 307
Polyols 388

Polypeptides 167
Polyphenols 204
Polysaccharide 72
Proanthocyanidines 402
Procathepsin D 125
Procyanidines 402
Pro-inflammatory cytokines 205
Propaicin 155
Prophenoloxidase 268
Propyl benzoate 397
Propyl gallate 397
Prostate carcinoma 82
Prostatitis 384
Proteoglycan 119
Proteus mirabilis 160, 276
P. vulgaris 276
Prothrombin 120
Protium apiculatum 156
P. araconchini 159
P. hebetatum 154
P. heptaphyllum 145, 147, 148, 157
P. icicariba 147
Pseudomonas aeruginosa 160, 221, 365
Purification strategy 168
Pyridine-barbituric acid 257
Pyridine-pyrazolone 257

Q

Quality 187
Quercetin-3-O-α-L-arabinoside 312
Quercetin-3-O-β-D-glucuronide 312

R

Radioprotective effects 333
Radioprotector 401
Real-time PCR Analysis 207
Retinopathies 402
Reversed-phase chromatography 172
Rheumatic pain 58
Rhiacanthus 115
Rhizomucor pusillus 356
Rhizopus oryzae 359
Rosiglitazone 219
RP-HPLC 189, 280
Rudbeckia fulgida 82
RVS Glycoprotein 133

S

Sabinene 299
Safety 187
Salpichroa origanifolia 6
Salvia officinalis 74
Saracha viscosa 7
Scabies 58, 384
Scopulariopsis brevicaulis 356
SDS-electrophoresis 181
SDS-PAGE analysis 273
Sec-isobrionoic acid 154
Secoiridoid 323
Secondary metabolites 97, 323
Secretory granules 121
Serratia marcescens 364
Sesamum indicum 104
Sialic acid 120
Sideritis taurica 108
Sitosterol 287
Slow rotary counter current chromatography (SRCCC) 188
Snake venom 167
SNL glycoprotein 131
Solanaceae 2
Solanesol 185
Solanum cilistum 7
S. nigrum 131
S. sisymbiifolium 7
Solid-phase extraction 273
Solvent extraction 384
Soxhlet extraction 191, 384
Spathulenol 394
Squalene 392
Squamous cell carcinoma 82
Staphylococcus auresus 160, 276
S. epidermis 276
Steroidal lactones 2
Steroids 125
Stigmasterol 313
Stomachic 325
Streptocarpus dunnii 105
Streptococcus pyogenes 332
Streptomycies rishirienes 115
Stricto sensu 73
Student-newman-keuls method 207
Styela plicata 280
Subtrifloralactone 25
Sudden death syndrome 239

Superoxide anion 133
Superoxide dismutase 205
Sweroside 325, 327
Swertia chirata 325
Swertiamarin 325, 327, 335
Syphilis 384

T

Tabebuia avellanedae 101
Tacca chantrieri 7, 28
T. plantaginea 7
Taccalonolide 34
Tachypleus tridentatus 269
Taraxerol 154
Taxol 186
T-cadinol 305
T-cell receptor complex 127
Tenascin 125
Terpenes 287
Terpenoid 98
Terpinene-4-ol 158, 304
Tetragastris altíssima 155, 156
T. panamensis 160
Thaicobrin 178
The könig reacton 249
The picrate method 251
Therapeutic agents 401
Therapeutical potential 1
Thermogravimetry 359
Thin layer chromatography (TLC) 189, 253
Thiobarbituric acid-reactive substances (TBARS) 132
Thyroid stimulating hormone 120
Tirucalol 154
Titrimetric methods 251
T-lymphocytes 266
TNF-α 131, 219
Toll-like receptors (TLRs) 267
Topoisomerase 97
Transaminase 385
Trans-caryophyllene 393
Transcription factors 42
Trattinnickia glaziovii 157
T. peruviana 154
T. burserifolia 151
Trechonolide 34
Triglycerides 204
Trimethylsilyl ether 253
Trisesquiterpenoid 186
Tristetraprolin 204
Triterpenes 144
Troglitazone 219
Trypanocidal 36, 97
Trypanossoma cruzi 39
Tubocapsicum anomalum 7
Tumor necrosis factor (TNF) 209
Tumorigenesis 122
Typhus 186

U

UDN glycoprotein 131
Ulmus davidiana 105, 131
U. pumila 105
Ultra sonication 187
Uronic acid 87
Ursane 144

V

Vassobia lorentzii 7
VEGFA 210
Venom 169
Ventilago leiocarpa 112
Vimang 384
Vinblastine 186
Vincristine 186
Vipera nikolskii 181
Viscosity 366
Vitis vinifera 402
Volatile 384
Voltage-gated calcium channels 178

W

W-3 fatty acid 229
Withametelinol 29
Withametelinone 29
Withania adpressa 7
W. coagulans 7
W. hunzikeri 7
W. somnifera 7, 8
Withanolides 2
Withaphysalins 2, 32
Withasteroids 1
Witheringia hunzikeri 8
W. solanacea 8

X

Xanthones 324

Xanthorrhizol 304
Xenopus laevis 269
Xylan acetylesterase 77
Xylan-α-D-1,2-glucuronohydrolase 77
Xylanases 77
Xylans 71
Xylomannoproteins 355
Xylosyl-oligosaccharides 74

Z

Zanthoxylum piperitum 133
Zona pellucida 120
ZPDC glycoprotein 133