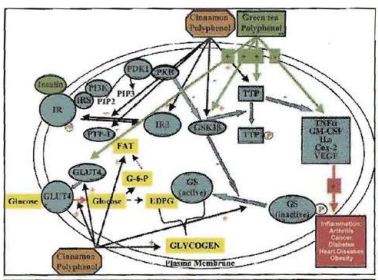
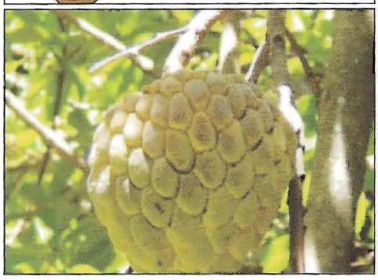
Comprehensive

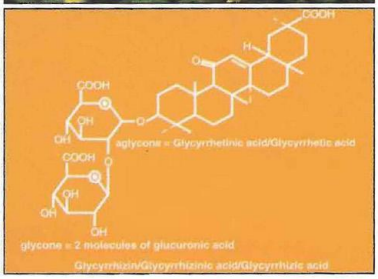
Bioactive Natural Products

Vol 2

Efficacy, Safety & Clinical Evaluation I







V K Gupta

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Bioactive Natural Products

Volume 2
Efficacy, Safety & Clinical Evaluation I

V.K. GUPTA

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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

- An Institution of Ritnand Balved Education Foundation - Thiruvananthapuram

Prof. (Dr.) P. PUSHPANGADAN,
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(UN Equator Initiative Laureate)
Director General & Senior Vice President, RBEF
(Former Director, NBRI, Lucknow)

08-06-2009

Foreword to the Series

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

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

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

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

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

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

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

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

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

(P. Pushpangadan)

About the Editor

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

Preface

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

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

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

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

At present, there is limited data on safety, efficacy and clinical trials to prescribe natural products in therapy. Realizing the need to document this knowledge gained through recent researchers, the present volume "***Efficacy, Safety & Clinical Evaluation-I***" of the book series entitled, "**Comprehensive Bioactive Natural Products**" presents 23 chapters discussing therapeutic potential of a large number of bioactive natural products of plant/animal origin investigated worldwide. Some of the interesting chapters included in this volume are: Perspectives in efficacy, safety and clinical evaluation of bioactive natural products; The modern application of traditional Chinese medicine: indigo naturalis as an alternative therapy in psoriasis vulgaris; Development of anti-angiogenesis therapies for treating cancer from Chinese medicinal herbs; Inflammation and mediators; Recommendation for reporting randomized controlled trials of herbal interventions: CONSORT for herbal medicine trials; Anxiolytic activity screening studies on extracts of a few medicinal plants; Larvicidal and antimicrobial activities of seeds of *Annona cornifolia;* Antidepressant activity of the extracts of three *Hypericum* species native to China*;* Cytolytic and toxic effects of ostreolysin, a protein from the oyster mushroom (*Pleurotus ostreatus*); Antioxidant, antisecretory and gastroprotective activities from *Leiothrix flavescens;* Evaluation of the immunity potency and security of inactivated oil-adjuvant vaccine against chlamydiosis in dairy cattle; Pharmacokinetics of active constituents of *Rhodiola rosea* SHR-5 extract; Metabolism and pharmacokinetic studies of coumarins; Naringin and its aglycone, research and managements; Biotransformation of drugs; Green tea: molecular targets in glucose uptake, inflammation, and insulin signaling pathways; Euphorbious plants as molluscicides and piscicides.

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

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

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Perspectives in Efficacy, Safety and Clinical Evaluation of Bioactive Natural Products

YOGENDRA NAYAK[1], VEERESH P. VEERAPUR[2], ANANTHA NAIK NAGAPPA[3] AND M.K. UNNIKRISHNAN[1]*

ABSTRACT

Natural products (NPs) include crude drugs, extracts, botanicals and pure compounds obtained from nature. NPs offer a rich source of diverse biologically active compounds with high molecular diversity, varying structural complexity and unparalleled novelty. A significant portion of the currently prescribed drugs owe their origin either directly or indirectly to NPs. The bioactivity of NPs not only builds the foundation for ethnic medicine systems of the world but also provides an impetus to modern drug discovery. Isolation of active component / molecule, structure elucidation, biological evaluation, dereplication, semisynthesis, biosynthesis, as well as optimization of downstream processing have undergone revolutionary changes in recent times, thereby enhancing the rate of drug discovery. The advent of high throughput screening and the availability of combinatorial compound libraries in natural products have been attracting major innovators towards natural products. Translational research has brought the drug discovery team closer to the clinical setting and has simultaneously increased the collective involvement of clinicians and pharmaceutical scientists. Complexity of natural products is fitly matched by the complexity

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of several pathological conditions, for instance diabetes, where multiple drug targets work in unison for producing a therapeutic outcome. Reverse pharmacology makes use of clinical evidences from traditional medicines, and then working backwards towards validation of claims in the laboratory. All the above themes are discussed in detail.

Key words : Combinatorial compound library, drug discovery, high throughput screening, multiple drug targets, natural products, reverse pharmacology, translational research

IMPACT OF NATURAL PRODUCTS ON DRUG DISCOVERY: HISTORICAL PERSPECTIVES

Natural products are rich sources of biologically active compounds with tremendous molecular diversity, possessing great promise in drug discovery and development (Butler, 2004; Newman *et al.*, 2003a; Koehn *et al.*, 2005; 2004b; Chin *et al.*, 2006; Cragg *et al.*, 2006; Blunt *et al.*, 2007; Gordaliza, 2007; Butler *et al.*, 2008). There is no dearth of illustrative examples to prove this paradigm. One of the oldest drugs, aspirin has had its origin from a plant (bark of willow), making it a historical land mark (Vane, 2000; Robert-II *et al.*, 2001). Penicillin was first isolated from a microorganism *Penicillum notatum* (Wainwright, 1990). Morphine obtained from opium poppy is still an important drug in pain management. Bergmann's discovery and subsequent identification of spongothymidine and spongouridine in the early 1950s from the Caribbean sponge *Tethya crypta* led to the development of a nucleoside with biological activity (Bergmann, 1950). An explosive growth in synthetic chemistry further led to the discovery of a close analogue, cytosine arabinoside, a potent antileukemic agent (Beránek, 1986; Suckling, 1991). This compound was subsequently commercialized by Upjohn (now Pharmacia) as Ara-C. A closely related compound adenine arabinoside (Ara-A) was later synthesized and commercialized by Burroughs Wellcome (now Glaxo Smith Kline). Similarly, insulin was first isolated from the pancreatic extracts of animals such as cows, pigs and sheep, till recombinant DNA technology replaced the old technology. Eli Lilly (Indianapolis, USA) later demonstrated the commercial feasibility of recombinant DNA technology with the introduction of human insulin in 1982, ushering in a paradigm shift in drug discovery and development, leading to the start of the 'biotech era' (Pillai, 2001). The discovery of cyclosporine from microbial sources revolutionized immunosuppressive therapy for organ transplant patients (Kahan, 1999). Indeed, it has been estimated that at least 60% of the roughly 1184 new therapeutic agents introduced world-wide over the last twenty five years (1981-2006) have had their origins from natural products in one way or the other (Newman *et al.*, 2007). The changing strategies and innovations in NP research have greatly influenced drug discovery programmes, in which

sample selection and collection, processing, isolation of active component/ molecule, structure elucidation, biological evaluation, dereplication, semisynthesis, biosynthesis, as well as optimization of downstream processing has become the norm. Such strategies have substantially enhanced the rate of discovery of novel NP with renewed interest in recent years (Gullo *et al.*, 2005; Lawrence, 2005a, 2007b). With the advance of high throughput screening (HTS) and new instrumentation, the discovery of the new molecules with biological activity has hastened further. Newer guidelines and statutes implemented by various regulatory authorities have enhanced the time frame for the assessment of safety and efficacy of drugs in human populations. It can take up to fifteen years for the development of a molecule before it is commercialized into a marketed drug. Apart from the passage of time, a great amount of money is being spent in the process of developing a product into a clinically useful drug (Frank, 2003; Schmid *et al.*, 2007). It is therefore important to examine the various events in drug discovery from natural sources and the associated regulatory rigors in assessment of quality, efficacy and safety of the drug. It is also essential to look into the translational aspects of NPs from preclinical to clinical studies (Dickson *et al.*, 2004).

The recent increase in the development-costs of new chemical entities (NCEs) has forced many innovators to search for active drug molecules from nature. The vast reservoir of diverse sources and the infinite variety in nature's molecular repertoire offers a fascinating arena for the drug discovery team.

NATURAL PRODUCTS FROM PLANT ORIGIN

Medicines from plant sources have been used since ancient times for a variety of ailments. Herbal preparations have been used in the art of healing since time immemorial. Primitive man, through trial and error, gained knowledge of herbals and passed it on to their progeny. It appears that for thousands of years herbs were perhaps used for both their magical powers as well as medicinal values (Oumeish, 1998). Ancient civilization flourished in 3000 BC onward in Egypt, Middle East, India and China, with a parallel growth in the refinement of herbal medicine. The Egyptian *Ebers Papyrus* (1500 BC) is one of the earliest records providing information on herbs (Frey, 1986; El-Gammal, 1994). In India, *circa* 1500 BC, *Vedas* included the *Ayurveda* system of medicine along with its vastly sophisticated information-based on herbs, with about 350 herbals cited in this compilation (Patwardhan *et al.*, 2004). Chinese traditional pharmacopoeia lists over 5700 traditional medicines which are mostly of herbal origin (Qiu, 2007). Chinese traditional medicine now enjoys credibility that is comparable to Western medicine in many parts of the world. Many universities in China teach and practice herbal medicine. Over the last 10 years Europe has witnessed a growth in the practice of Traditional Chinese Herbal Medicine.

The development of organic chemistry has shown that the ability of herbal medicine to treat the body depended upon its chemical constituents. Chemists first began extracting and isolating chemicals from plants in the 18th century. Encouraging results accelerated their enterprise and NP chemists have given to the world some of its most useful drugs such as morphine, digoxin, d-tubocurarine, ephedrine, quinine, vincristine, paclitaxel etc. A detailed list is presented in Table 1.

Table 1. Prescription drugs derived from natural sources

Use	Drug name and the source
Amino glycoside antibiotic	Streptomycin (Bacteria; *Streptomyces griseus*) Gentamicin (*Micromonospora*) Kanamycin (*Streptomyces kanamyceticus*) Neomycin (*Streptomyces fradiae*) Netilimicin sulfate (*Micromonospora*)
Analgesic	Morphine (Fruit exudate; *Papaver somniferum*)
Non-opioid analgesic	Epibatidine (Alkaloid that originally is found in the skin of a neotropical poisonous frog, *Epipedobates tricolor*); 200 times stronger than morphine; derivatives are under clinical investigation
Ansamycin antibiotic;	Rifamycin (*Amycolatopsis rifamycinica*); Anti-TB
Anti-Alzheimer's	Galantamine hydrobromide (An alkaloid obtained from *Galanthus nivalis*); Selective acetylcholinesterase inhibitor
Antibiotic	Mupirocin (Mixture of several pseudomonic acids obtained from *Pseudomonas fluorescens*); active against methicillin-resistant *Staphylococcus* aureus (MRSA) Chloramphenicol (bacterium; *Streptomyces venezuelae*) Tetracycline (polyketide antibiotic from *Streptomyces*) Daptomycin (*Streptomyces roseosporus*); Cyclic lipopeptide antibacterial agent for skin infections Teicoplanin (*Actinoplanes teichomyceticus*); Glycopeptide antiobiotic active against MRSA Vancomycin (*Amycolatopsis orientalis*); Glycopeptide antibiotic active against penicillin resistant *Staphylococcus aureus* Lincomycin (*Streptomyces lincolnensis*); Lincosamide antibiotic Erythromycin (*actinomycete, Saccharopolyspora erythraea*); Macrolide antibiotic Aztreonam (*Chromobacterium violaceum*); Monobactam antibiotic Cephalosporin (fungus; *Cephalosporium acremonium*); Beta lactum antibiotic Penicillin (Fungus; *Penicillum notatum*); Beta lactum antibiotic Colistin (polymyxin E) (*Bacillus polymyxa* var. *colistinus*); Polymyxin antibiotic Bacitracin (*Bacillus subtilis* var *Tracy*); Polypeptide antibiotic Fusidic acid (fungus: *Fusidium coccineum*); Urinary antibiotic

Table 1. *Contd.*

Use	Drug name and the source
Anticancer	Arglabin (from a species of wormwood *Artemisia glabella* endemic to the Karaganda region of Kazakstan) Bleomycin (Glycopeptide antibiotic from bacterium: *Streptomyces verticillus*) Etoposide (Herb: *podophylum*) Masoprocol (Nordihydroguaiaretic acid; NDGA; is a potent antioxidant compound found in the long-lived creosote bush: *Larrea tridentata*) Paclitaxel (plant; *Taxus brevifolia*) Solamargines (plant; *Solanum marginatum*) Vinblastin, vincristine (herb; *Vinca rosea*)
Anticancer antibiotic	Daunorubicin (*Streptomyces peucetius*) Pentostatin (*Streptomyces antibioticus*) Daunomycin (from bacterium: *Streptomyces peucetius*) Doxorubicin (Adriamycin; related to daunomycin from new strain of *Streptomyces peucetius*) Mithramycin (Plicamycin; *Streptomyces plicatus*) Mitomycin C (*Streptomyces lavendulae*) Sarkomycin (*Streptomyces erythrochromogenes*).
Anticoagulant	Heparin (sheep liver)
Antidiabetic	Insulin (pig and sheep) Voglibose (*Aspergillus awamori*)
Antifertility	Diosgenin (rhizome; *Dioscorea deltoideae*)
Antihypertensive	Reserpine (*Rauwolfia serpentina*)
Antimalaria	Quinine (*Cinchona* bark) Artemisinin (herb, *Artemisia Annua*)
Antiplatelet	Bivalirudin a new, genetically engineered form of hirudin, the substance in the saliva of the leech (*Haementeria officinalis*); used to reduce the risk of blood clotting in unstable angina who are undergoing a procedure to open blocked arteries in the heart.
Anti-spasmodic	Ephedrine (herb: *Ephedra*)
Cardiotonic	Digoxin (Fox glove; *Digitalis* species)
Hypolipidemic	Mevastatin (fungus; *Penicillium citrinum*)
Immunosuppressant for organ transplant	Sirolimus (Rapamycin; from bacterium *Streptomyces hygroscopicus*) Tacrolimus (bacterium *Streptomyces tsukubaensis*) Cyclosprin (fungus; *Tolypocladium inflatum*)
Local anaesthetic	Cocaine (plant; *Erythroxylum coca*)
Muscle relaxant	d-tubocurarine (plant; *Chondrodendron tomentosum*)
Mydriatic	Atropine (herb; *Atropa belladonna*)

In spite of the great advances in synthetic and combinatorial chemistry, medicinal plants still make an important contribution in pharmaceutical innovation. According to the World Health Organization (WHO), about 65-80% of the world's population living in developing countries, still depends essentially on plants for primary health care because of poverty and lack of access to modern medicine (Pal *et al.*, 2003). Some Western countries such as Germany, France, Italy and the United States have developed appropriate guidelines for registration of herbal medicines. Discovery of the blockbuster anticancer drug paclitaxel (Taxol) from the yew tree (*Taxus brevifolia*) once again helped to revive the interest towards herbal medicine (McChesney *et al.*, 2007). Nowadays, several major pharmaceutical companies demonstrate renewed interest in investigating plants for discovering new sources for new lead structures and also for the development of standardized phytotherapeutic agents with improved efficacy, safety and quality (Gordaliza, 2007; Potterat *et al.*, 2008).

NATURAL PRODUCTS FROM MARINE SOURCES

Marine NPs have yielded a considerable number of new drug candidates which are still in preclinical or early clinical development (Haefner, 2003; Marris, 2006; Saleem *et al.*, 2007). However a few are already in the market, such as cytarabine. Drugs from marine sources have a wide range of therapeutic benefits such as anticancer, antiviral, antiinfective, antimicrobial, analgesic, anti-inflammatory, anti-neurodegenerative in addition to many other therapeutic classes (Donia *et al.*, 2003; Rawat *et al.*, 2006; Bao *et al.*, 2007; Diers *et al.*, 2008; Uemura, 2006). Some of the NPs isolated from marine invertebrates have been shown to be, or are expected to be of microbial origin (Tan, 2007). Marine microorganisms, whose immense genetic and biochemical diversity is only beginning to be appreciated, appear to serve as a rich reservoir of NCEs. The prokaryotic marine cyanobacteria are a potential source of structurally bioactive secondary metabolites. A number of cytotoxic compounds such as hectochlorin, lyngbyabellins, apratoxins, and aurilides have been identified as potential lead compounds for the development of anticancer agents (Jimeno *et al.*, 2004; Braña *et al.*, 2006).

NATURAL PRODUCTS FROM MICROORGANISM

The seemingly endless potential of microorganisms dawned upon mankind in 1928 with Alexander Fleming's sensational discovery of an antibacterial filtrate "penicillin" from a commonplace mould *Penicillium notatum* (Ligon, 2004). Re-isolation and clinical studies pioneered by Chain, Florey, and co-workers in the early 1940s, and the subsequent scale-up towards commercially viable manufacture of synthetic penicillins revolutionized the foundation of antimicrobial drug discovery (Bentley, 2005). The

phenomenal success of penicillin encouraged drug companies and research groups to pool their resources together for a large scale culture of a diverse array of microorganism from which several newer antibiotics emerged (Knight *et al.*, 2003). Early years of this large scale hunt for antibiotics yielded a number of successful antibiotics, some of which are widely prescribed to this day. Prominent among these include pathbreaking molecules in the history of modern medicine, such as streptomycin, chloramphenicol, tetracycline, cephalosporin, erythromycin, and vancomycin. Each one of these compounds, either in its original form or in its synthetic derivative, can be found on the shelves of a pharmacy to this day. The impact of these drugs has even served to change the very attitude towards infective diseases from one of horror and despair to that of a routine ailment of trivial consequence. More lives have been saved in history by antibiotics derived from nature, than through any other class of prescribed drugs. In other words, NP derived drug molecules have contributed the most towards increasing the life expectancy of man. Antibiotics of natural origin also remain some of the most often prescribed drugs in the world.

Some of the earliest compounds discovered in the early 1970s using rational approaches based on mechanism of action, include the beta-lactamase inhibitor clavulanic acid from *Streptomyces clavuligerus* (Brown *et al.*, 1976) and the HMG-CoA reductase inhibitor mevastatin (earlier code-named as ML-236B) from *Penicillium citrinum* (Endo, 1981). A mixture of clavulanic acid and amoxicillin (the combination is called Augmentin) is being used today as a front line antibiotic in several critical infections, while mevastatin and lovastatin, have been the leads in the discovery of a series of drugs in the treatment of dyslipidemia. Today, these groups of statins are among the top revenue earners for some of the richest drug companies in the world (Manzoni *et al.*, 2002). The arrival of statins has also widened the scope of therapeutic possibilities in compounds isolated from microbes. Many biotech industries are now utilizing microorganisms as the raw material for the production of not only antibiotics, but also various enzymes, antibodies, vaccines and many genetic engineered hormones.

EXPLOITING NEW APPROACHES FOR NATURAL PRODUCT BY PHARMACEUTICAL COMPANIES

Just as the arrival of aspirin revolutionised the preparation of medicines from an individual enterprise of laboratory dispensing into a scale of industrial manufacture, the invention of penicillin and other antibiotics ushered in a new era of large-scale manufacture through biotechnology. Although 60% of the new drugs owe its origin to NPs, this enormous pool of resources is being steadily overlooked by several large pharmaceutical innovators for over a decade (Baker *et al.*, 2007). This strategy seems

inappropriate because several synthetic analogues of the NPs have often proved themselves to be more efficacious and less toxic. For instance, chloroquine is less toxic than quinine, and homatropine is shorter-acting and therefore produces less cycloplegia than atropine. Lignocaine, the more therapeutically useful product, was produced by modifying the cocaine molecule. Semisynthetic penicillins like methicillin expanded the range of cure. Similarly, second and third generation synthetic cephalosporins have extended the antibacterial spectrum of the prototype.

The advent of high throughput screening (HTS, see below) and the availability of combinatorial compound libraries have been attributed as major reasons for this change in attitude (Boldi, 2004). NP-based drug discovery has been finding it difficult in adapting itself to modern techniques such as HTS and rapid hit-identification process. This disadvantage is being felt most acutely by large pharmaceutical companies with enormous funds at their disposal. The long time-gap between drug discovery and its marketing tends to challenge the wisdom in this tendency to move away from natural products. However, the scenario will become clear only in the long term.

Interestingly, smaller biotech companies are emerging with renewed strategies to focus on exploiting new approaches for NP-based drug discovery. These approaches include the application of genomic tools, identification of novel sources of organisms, newer technologies (listed in the Table 2) and improved processes of sample preparation for screening. In addition, the recent focus on the synthesis of diversity-oriented combinatorial libraries based on NP-like compounds is an attempt to enhance the productivity of synthetic chemical libraries (Marcaurelle *et al.*, 2008). One of the breakthroughs in drug discovery was the use of mechanism-based screening for bioassay-guided fractionation (Phillipson *et al.*, 2002). Efforts continue to improve screening formats, reagent production, robotics, and data management. Mechanism-based screening

Table 2. Technologies/Techniques applied in drug discovery from natural products

Name of the technology/ technique	Applications
Chromatography: High performance liquid chromatography (HPLC) High performance thin layer chromatography (HPTLC) Gas chromatography (GC) Liquid chromatography (LC)	Isolation of components from the natural sources. *In vitro* and *in vivo* drug metabolism and pharmacokinetic (DMPK) assays, HTS new molecule identification, Biomarker identification (Korfmacher, 2005; Castro-Perez, 2007)

Table 2. *Contd.*

Name of the technology/ technique	Applications
Supercritical fluid chromatography (SFC)	For the isolation of chiral compounds from natural sources (Zhang *et al.*, 2005)
Mass Spectroscopy (MS): GC-MS, HPLC-MS, HPLC–MS–MS, LC-MS, Fluorescence activated cell sorting (FACS)-MS, Liquid chromatography photodiode array mass spectrometry (LC-PDA-MS)	Molecular identification, and the structural elucidation of natural products usually combined with GC or HPLC or LC (Ma *et al.*, 2006; Koehn, 2008)
FTICR-MS: Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometry	Biomarker discovery and analysis.Metabonomics, proteomics, natural product analysis and noncovalent complexes (including thermodynamics) (Feng *et al.*, 2007; Zhang *et al.*, 2005)
Nuclear magnetic resonance spectroscopy (NMR)	Study on ligand binding to the protein, helps in the structure based drug design; Structural activity relationship (Robert, 2000; Coles *et al.*, 2003; Pauli *et al.*, 2005)
Saturation Transfer Difference (STD)-NMR	Molecular isolation and identification usually combined with the HTS analysis (Koehn, 2008)
LC–NMR–MS, On-line solid-phase extraction (LC–SPE–NMR)	For DMPK studies, HTS analysis of natural products (Corcoran *et al.*, 2003)
Fluorine magnetic resonance (19F MR)	Studies of psychoactive agents in brain, Drug accumulation and metabolism in cancer, Quantitation and metabolism in the brain, liver and other organs (Paulus *et al.*, 2007)
Functional NMR (fNMR), Functional annotation screening technology by NMR (FAST-NMR)	To assess the biological function for unannotated proteins expressed by different genes with the protein structure solved (Reid *et al.*, 2008; Powers *et al.*, 2008)
Positron emission tomography	Disease specific marker identification in nurological diseases (Wang *et al.*, 2005; Beckmann *et al.*, 2007; Hutchins *et al.*, 2008)
High-throughput X-ray crystallography Wide-angle X-ray scattering	For protein structure studies, screening functional ligands, and ligand binding (Senior, 2005)
High-content high-throughput fluorescent microscopy	For the study of protein localization, dynamics and interactions with the natural products (Auer *et al.*, 1998; Zemanová *et al.*, 2003; Starkuviene *et al.*, 2007)

has become the mainstay of HTS (Zheng *et al.*, 2008). By utilizing new approaches, these companies are increasing the chemical diversity of their collections.

SCREENING OF NATURAL PRODUCTS

Traditionally, crude extracts have been used routinely for screening. Aqueous or alcoholic extracts are most frequently used for the initial round of biological and phytochemical screening. Recently developed bioactivity-guided fractionation processes can now separate crude fractions into mixtures containing only a few compounds. Column chromatography, high-performance liquid chromatography (HPLC), countercurrent chromatography (CCC), or centrifugal partition chromatography (CPC) are some of the techniques used for fractionation (Maugh, 1983). Fractionation will allow active compounds present in amounts too small to detect, whose activity is masked in a crude extract. However, by screening at higher concentrations, interfering compounds will also be more frequently identified. The paradox is that the total number of fractionated extracts screened is inversely proportional to the biodiversity. A decision must be made whether the loss of biological diversity is adequately compensated by fractionation. The answer to this question will help determine the optimal number of fractions that should be generated for each extract. The advent of sophisticated separation by analytical instruments attached to the HTS enables scientists to assemble large libraries of pure NPs, which can then be screened in a manner analogous to pure compound libraries (Liu *et al.*, 2004). A recent review by McChesney *et al.* (2007) discusses latest methods and techniques involved in the natural product extraction, isolation and purification.

The identification of the compounds that are responsible for the activity of an extract is called dereplication process (Konishi *et al.*, 2007). This process prioritizes extracts for further study and can save considerable research time. The most generic procedure used today involves separation of an extract using reversed-phase HPLC and splitting of the eluent post column into a mass spectrometer (usually MS/MS to match fragmentation ions) and a fraction collector using micro-titer plates to test tubes, depending on the scale (Strege, 1998). The fractions are screened and the retention time, UV spectra, MS data, and activity of fractions are analyzed for common interfering compounds and known inhibitors using commercial and in-house databases (Schuffenhauer *et al.*, 2005). Other methods for dereplication that have been used include separation by solid-phase extraction cartridges and CCC. Alternative methods for analysis include affinity capillary electrophoresis, LC-NMR, and online biochemical assay (Bobzin *et al.*, 2000; Beger, 2006).

An increase in the speed of bioassay-guided fractionation has been facilitated by a marked improvement in HPLC automation, MS, and column technology, as well as rapid turnaround of screening results provided by HTS (Mishra *et al.*, 2008). The advent of new probe technology and higher magnetic fields has led to a significant shortening in acquisition time for

NMR data, and the structure elucidation of NPs can be achieved routinely on amounts less than 1 mg (Liu *et al.*, 2004; Xing *et al.*, 2007). Automated structure-solving algorithms have made the job simpler but no technique can replace the structure elucidation skills of an experienced chemist (Ross *et al.*, 2001). However, these programs add value to the tools that search for alternative structures that fit the same NMR data (Beger, 2006).

HIGH THROUGH PUT SCREENING OF NATURAL PRODUCTS

The discovery of truly novel NPs is a laborious and time consuming task when pursued through conventional programs such as extraction, fractionation, isolation and screening for biological activity. This has necessitated the adoption of high-throughput screening (HTS) by major players in the drug discovery team. HTS is defined as the automatic testing of potential drug candidates at a rate in excess of 10,000 compounds per week (Liu *et al.*, 2004; Pereira *et al.*, 2007; Koehn, 2008; Jenwitheesuk *et al.*, 2008). HTS has gained widespread popularity over the last few years. The aim of high throughput drug discovery is to test large compound collections for potentially active compounds ('hits') in order to allow further development of compounds for pre-clinical testing ('leads'). In this approach, the molecular diversity and range of biological properties displayed by natural products is a challenge to combinatorial strategies for NPs synthesis and derivatisation (Posner, 2005). Many large companies study a hundred targets or more each year, and in order to validate these targets, lead compounds must be found. HTS is the process of assaying a large number of potential effectors of biological activity (lead) against the targets (a biological event) by miniaturized *in vitro* assays. Thus HTS technology has emerged over the last few years as an important tool for drug discovery and lead optimization in NP based drug discovery (Posner, 2005).

HTS uses standard assay types known to most biological and biochemical scientists (*e.g.* ELISA, proliferation/cytotoxicity assays, reporter assays, and binding assays) (Inglese *et al.*, 2007). The biochemical assay will be selected depending on the mechanism of action or target specificity of the compound or the mixture of compounds (commonly the fractionated extract). The primary assay is either cell free biochemical assays or cell based assays. Cell based assays allow the HTS to address a specific target (Castel *et al.*, 2006).

In order to process thousands of assays per day, HTS uses a combination of multiple well microplates and robotic processing. For a number of years, HTS assays have been run in the standard 96-well microplate (working volume of up to 250 μL). The current goal of most companies is to move beyond this format to higher-density, lower-volume formats (*e.g.* 384 and 1536 well microplates). There are two primary advantages of these formats: increased throughput and lower volume, which

translates into lower cost. At screening rates of 500,000 compounds/week, even a cost of $1/ well becomes too big a burden on the company's weekly budget (Mishra *et al.*, 2008). The reduction of cost, rather than increase in throughput, is the primary driving force within most HTS groups to move towards higher-density, lower-volume microplates.

Fluorescence polarization (FP) detector technology is widely acceptable in the plate reader system (Burke *et al.*, 2003; Rogers, 1997). For example screening assay for detection of apoptosis through activated caspase is assessed as liberation of free rhodamine following incubation of treated cells with rhodamine-labeled (quenched) substrate solution. Compounds showing a five fold or greater increase in fluorescence are considered as hits, being inducers of caspase activity. However, intrinsic fluorescence of natural products can interfere with such detection. Use of special reagents such as Europium-labeled reagents and time resolved fluorescence detection, avoids this problem (McMahon *et al.*, 2000).

COMPOUND DEVELOPMENT: TOTAL SYNTHESIS, SEMI-SYNTHESIS OR COMBINATORIAL SYNTHESIS

Perhaps the biggest obstacle to NP chemistry is the supply of large amounts of NPs. For instance, a steady supply of paclitaxel from nature would require cutting down a large number of whole yew trees followed by tedious extraction of the active molecule (McChesney *et al.*, 2007). A sustainable source of the NP should be identified whenever total synthesis is not possible. However, a microbial source does not face such a problem on account of the brief life cycle of the organism and efficient culturing methods sustainable in lab conditions. This is precisely the reason why most companies prefer screening of microbial products. Rapid advances in microbial combinatorial biosynthesis have hastened the process in recent times (Dougherty *et al.*, 2006; Adrio *et al.*, 2006).

There has also been progress with organisms usually considered to be problematic. For example, the steady supply of compounds from plants can be enhanced with minimal environmental damage through tissue culture and genomic methods (Wilkinson *et al.*, 2005; Chaturvedi *et al.*, 2007; Gómez-Galera *et al.*, 2007). The study is now being extended to cover aquaculture of marine invertebrates and the identification, cloning, and expression of symbiotic microbial genes (Munro *et al.*, 1999). The molecular complexity of NP derived NCEs can also impede the lead optimization process.

A very important trend in recent years has been a significant effort devoted to the semisynthesis and synthesis of complex NPs (Ortholand *et al.*, 2004; Shang *et al.*, 2005; Paul *et al.*, 2007; Bulger *et al.*, 2008) (Listed Table 3). The success of this strategy is evident from history. Literature

shows that a total of 322 NP-derived drugs have been launched from 1981 to 2006. More than 70% of these drugs (232 compounds) are manufactured by semisynthetic processes. Further, about 15% (47NCEs) have been produced by total synthesis (Newman *et al.*, 2007; Butler, 2008). Paclitaxel, an anticancer drug manufactured by total synthesis, artemether, a semisynthetic drug from artemisinin, have emerged as synthetic breakthrough while vinblastine and vincristine (approved for cancer treatment in 1963 and 1965) templates are extremely difficult to synthesize economically.

Table 3. Synthetic/semisynthetic analogues of natural products

Name of drug	Source/Indication
Acarbose	Hypoglycemic drug derivative of natural product from *Aspergillus awamori*
Alitretinoin	Anticancer; a first generation retinoid
Amrubicin	Derivative of doxorubicin from *Streptomyces peucetius* var *caesius*. Used in breast, lung, and gastric cancer
Anidulafungin	Antifungal agent; semi-synthetic lipopeptide from a fermentation product of *Aspergillus nidulans*.
Apomorphine hydrochloride	Synthetic analogue of morphine in Parkinson's disease
Arteether, artemether, artenusate	Semisynthetic derivative of artemesinin (from the plant *Artemisia annua*) to treat malaria
Azithromycin	Macrolide antibiotic; Azane-substituent of erythromycin obtained from actinomycete *Saccharopolyspora erythraea*
Captopril, enalapril and cilazapril	Antihypertensive, developed from teprotide, isolated from Brazilian viper venom (*Bothrops jararaca*)
Carbepenams: Biapenem, Imipenam, Doripenem, Meropenam, Ertapenem	Derived from a compound called thienamycin, produced by *Streptomyces cattleya*
Carumonam	Monobactam antibiotic, very resistant to β-lactamase; derivative of aztreonam obtained from *Chromobacterium violaceum*
Caspofungin acetate	Semisynthetic lipopeptide derived from pneumocandin B0, a fermentation product of *Glarea lozoyensis*; antifungal
Caspofungin acetate	Semisynthetic derivative of pneumocandin produced by fungi: *Zalerion arboricola*
Cefditoren pivoxil	An oral prodrug of cefditoren, a derivative of cephalosporin isolated from *Cephalosporium* species
Cladribine	Anticancer; related to nucleoside adenoside
Clarithromycin, Telithromycin, Dirithromycin	Macrolide antibiotic; Semi-synthetic erythromycin

Table 3. *Contd.*

Name of drug	Source/Indication
Clindamycin	Semisynthetic derivative of lincomycin, produced by the actinobacterium *Streptomyces lincolnensis*.
Cytarabine ocfosfate	Derivative of spongothymidine obtained from Caribbean sponge
Dicoumarol	Anticoagulant, derivative of plant coumarine isolated from fungal infected hay
Docetaxel	Anticancer; paclitaxel derivative
Ellipticine, Celiptium	Semisynthetic derivative from plant *Ochrosia elliptica*
Epirubicin	4′-epimer of doxorubicin, an anticancer drug
Epirubicin HCI	Anticancer antibiotic related to doxorubicin
Etoposide phosphate	Anticancer; semisynthetic derivative from podophyllotoxin, found in the American Mayapple (*Podophylum peltatum*)
Everolimus	Orally active 40-O-(2-hydroxyethyl) derivative of rapamycin, originally produced from *Streptomyces hygroscopicus*.
Exemestane, Formestane	Anticancer; aromatase inhibitor structurally related to the natural substrate androstenedione
Exenatide	Antidiabetic: a synthetic analog of exenadin-4, which was originally isolated as a 39 amino acid peptide from the saliva of the Gila monster (*Heloderma suspectum*)
Fosfomycin trometamol	Antibacterial; analogue of fosfomycin obtained from *Streptomyces fradiae*.
Idarubicin hydrochloride (4-demethoxydaunorubicin)	Anticancer antibiotic; derivative of daunourubicin
Irinotecan, topotecan	Camptothecin derivatives for cancer
Isepamicin	Aminoglycoside antibiotic; derivative of gentamicin B from Micromonospora, a genus of Gram-positive bacteria widely available in water and soil
Ivermectin	Anti-parasitic; derivative of avermectin obtained from microbe *Streptomyces avermitilis*
Micafungin sodium	Antifungal agent of the echinocandin type obtained from the culture broth of the fungus *Coleophoma empetri* , and inhibits β-(1, 3)-D-glucan synthase of fungi.
Micronomicin sulfate	Aminoglycoside antibiotic; 6′-N-methyl derivative of gentamicin C
Miglitol	Antidiabetic; Acarbose related drug
Miglustat	For type I Gaucher disease. An analogue of nojirimycin isolated from the broth filtrate of *Streptomyces lavendulae*

Table 3. *Contd.*

Name of drug	Source/Indication
Miokamycin	Derivative of macrolide antibiotic miodecamycin A1 ; 9,3′-diacetylmiodecamycin; has wide antimicrobial spectrum
Mitobronitol (1, 6-dibromo-1, 6-dideoxy-d-mannitol)	Alkylating anticancer; derivative of mannitol
Mycophenolate sodium	Mycophenolic acid, originally purified from *Penicillium brevicompactum*, used for prophylaxis in renal transplant
Nandrolone phenylpropionate	Anticancer; Analogue of naturally occurring nandrolone in human
Nitisinone	Derivative of leptospermone, an important new class of herbicides from the bottlebrush plant (*Callistemon citrinus*)
Orlistatin	Antiobesity; Semisynthetic product from lipstatin (a natural product)
Peplomycin	Semisynthetic analogue of bleomycin from bacterium *Streptomyces verticillus*
Pimecrolimus	A novel analog of ascomycin, isolated as a fermentation product of *Streptomyces hygroscopicus* var *ascomyceticus*, for allergic contact dermatitis and atopic dermatitis
Pirarubicin	Anticancer antibiotic similar to daunorubicin
Rifampicin	Semisynthetic compound derived from rifamycin which is obtained from *Amycolatopsis rifamycinica*
Rosuvastatin calcium	Hypolipidemic drug; derivative of mevastatin isolated from *Penicillium citrinum* and *P. brevicompactum*
Telithromycin	Semi-synthetic derivative of the 14-membered macrolide, erythromycin-A, isolated from *Saccharopolyspora erythraea*
Testolactone	Anticancer; derivative of progesterone
Tigecycline	9-tert-butyl-glycylamido derivative of minocycline, which is a semi-synthetic product of chlortetracycline isolated from *Streptomyces aureofaciens*.
Tiotropium bromide	Synthetic derivative of atropine from *Atropa belladonna* (Solanaceae) to treat COPD
Triproamylin acetate	Antidiabetic; amylin analog
Valrubicin	Anticancer antibiotic similar to doxorubicin
Vapreotide acetate	Anticancer; Somatostatin analogue
Vindesine and vinorelbine	Anticancer; semisynthetic derivatives of vinblastine from herb *Vinca rosea*
Ziconotide	Non opioid analgesic; synthetic form of one of the ω-conotoxins, obtained from cone snail (*Conus magus*) venom

Synthetic effort has been particularly critical for NP-derived anticancer drugs where total synthesis has been crucial in lead optimization and for obtaining enough material for clinical trials. Synthetic chemists are actively engaged in such ventures where there are 15 derivatives of paclitaxel, 15 of camptothecin, 4 of combretastatin, 4 of vinca alkaloids and 2 of podophyllotoxin in the clinical trial process (Saklani *et al.*, 2008).

With the advent of combinatorial chemistry, a large number of compounds can be produced from a single lead discovered from the NPs (Lee *et al.*, 2001; Nielsen, 2002; Boldi, 2004; Ganesan, 2004; Tan, 2004). Combinatorial libraries based on natural product scaffolds, analogs and derivatives are being currently pursued. For example, combinatorial chemistry techniques applied to scaffolds of tubulin inhibitors, have yielded second-generation paclitaxel analogs as anticancer drug candidates (Ladame *et al.*, 2008). Further, compounds active against vancomycin-resistant bacteria have emerged from combinatorial libraries of vancomycin- dimers (Griffin *et al.*, 2003). The combinatorial libraries of anticancer molecules designed to target receptor tyrosine kinase, rest on the evidences obtained from a natural product genistein (Kissau, 2003). Thousands of camptothecin derivatives have been synthesized with the combinatorial approach to target topoisomerase I. Topotecan and irinotecan are derivatives obtained in this manner (Li *et al.*, 2006).

In spite of all the sophistication, combinatorial chemistry has failed to deliver the high numbers of drug leads as was predicted. This is mainly because of the complexity and diversity of the NP scaffold. Limited success has paved way for "diversity oriented combinatorial synthesis" which proposes to produce molecules with greater structural complexity including enhanced stereo-chemical variations (Rouhi, 2003; Liao, 2003; Tan, 2005; Marcaurelle *et al.*, 2008), but these approaches may have limited prospects in future compared to the natural product based combinatorial library (Koppitz *et al.*, 2006).

TRANSLATIONAL RESEARCH IN ASSESSING SAFETY AND EFFICACY OF BIOACTIVE NATURAL PRODUCTS: FROM BENCH TO BED SIDE

In drug discovery and development programs, a vast majority of tested compounds do not progress from laboratories to patients' bedsides. Historical evidence indicates that only 14% of the tested products entering phase I trials eventually obtain clearance from the FDA and enter the market (DiMasi *et al.*, 2003). In recent times, chances of success have reduced to just 8% (Graul, 2007). This high attrition rate is being attributed to the knowledge gaps between *in vitro* data, preclinical data and the final clinical outcome (Eisenstein *et al.*, 2008). The basic knowledge about the investigational molecule or the natural product is not being translated

appropriately to the clinical setting. The failures are not being analysed effectively together in a concerted manner by the drug discovery team and the clinical team. A two-way dialogue between scientists operating in preclinical drug discovery and clinical developers who understand the pathology and its alleviation, are required if novel leads and biologicals are to become useful drugs. Translational research, which focuses on such themes, has emerged as a new discipline in this context. The recent surge in translational research is expected to reduce the gap between the initial stages of drug discovery and subsequent clinical development (O'Connell *et al.*, 2006).

The American Physiological Society (APS) has defined translational research as 'the transfer of knowledge gained from basic research to new and improved methods of preventing, diagnosing or treating disease, as well as the transfer of clinical insights into hypotheses that can be tested and validated in the basic research laboratory (O'Connell *et al.*, 2006). It is a kind of iterative learning experience from experimentation in basic and clinical fields. Thus in NP drug discovery, translational research interlinks preclinical and clinical research, where experiments progress steadily from lead generation towards proof of concept (early-stage clinical studies) (Sultana *et al.*, 2007). Further, translational research assumes a broad perspective, embracing diverse areas such as clinical demands, public health policies and economic compulsions (Trusheim *et al.*, 2007).

The preclinical studies carried out in animals can predict efficacy (confidence in rationale) and safety (confidence in safety) of compounds or the natural product extracts in early development. The animal models and biomarkers (see below) can only give a preliminary idea of clinical correlation. Further, this may be poorly correlated to clinical outcomes in man. The pharmacokinetics and pharmacodynamics (PK-PD) of the compound in humans, which becomes clear only after the first dose in healthy volunteers, will help in determining the dose and route of administration. Research in normal human volunteers and patients enables a clearer understanding of the biology and pathophysiology, and can therefore trigger innovations in *in vitro* and animal experiments that better mimic the clinical condition. This strategy is therefore likely to enhance confidence in the appropriateness of a given target and help in dose selection for subsequent trials (Sartor, 2003).

Biomarkers are quantitative measures of biological effects that provide informative links between mechanism of action and clinical effectiveness (Wehling, 2006). Assessment of biomarkers can reduce the uncertainty around key risks in the drug development cycle (pharmacology, PK-PD, toxicity, safety etc.). Further, biomarkers reflect biological effects induced by disease/ test-compound and are the main tools to predict and describe its efficacy and safety. Specific markers like serum enzymes (ALT,

AST, Catalase, Alkaline phosphatase etc) and more general markers of disease-severity like C-reactive protein in inflammatory diseases are well known biomarkers. Histological parameters, imaging of tumors and the morphology of atherosclerosis, liver fat (safety biomarker), positron emission tomography (brain disorders) scans etc. are some disease-specific biomarkers (Colburn, 2003). In preclinical studies, death could be a biomarker in models of myocardial infarction, cancer, acute toxicity etc., being correlatable measures of efficacy. These biomarkers are used to achieve predictive power and this can vary from almost useless (*e.g.* serial brain slices etc., which can never be a human biomarker) to surrogate (*e.g.* LDL cholesterol, one of the few surrogates accepted by FDA), with most cases falling somewhere in between.

Translational research initially was said to be bidirectional but it has got multidirectional approaches (Sonntag, 2005). It has an important role in identifying new indications for established therapies – a process termed by some authors as 'indications discovery'. New drug target hypotheses can be generated through preclinical experiments on novel target organs. It can also result from clinical observations such as side effects or additional pharmacological effects beyond those expected from efficacy in the main indication (O'Connell *et al.*, 2006). Sildenafil, a selective Phosphodiesterase-5 inhibitor, is an example of a drug initially assessed for angina pectoris but was subsequently licensed for a completely different pathology; male erectile dysfunction and pulmonary artery hypertension (Ghofrani *et al.*, 2006).

NATURAL PRODUCTS FOR TYPE 2 DIABETES: A CASE STUDY

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (Wild *et al.*, 2004). Type 2 diabetes (T2D) is prevalent in 90% of the patients with diabetes. By 2025, more than three-quarters of all persons with diabetes will reside in developing countries. India and China are leading this surge in diabetes, and sub-Saharan Africa is currently at a lower prevalence rate (Dagogo-Jack, 2006). Poorly managed diabetes leads to several complications (*e.g.* end-stage renal failure, blindness, amputation and heart disease) that many developing countries are ill equipped to tackle (Home, 2003).

Diabetes is a heterogeneous metabolic disorder characterized by a decline in insulin action (insulin resistance), followed by pancreatic beta cell dysfunction (Chiasson *et al.*, 2004). Insulin resistance will be associated with common conditions such as metabolic syndrome, dislipidaemia, obesity,

endocrine disorders and hypertension. Abnormal obesity, increased lipid profiles, atherosclerosis and hypertension are symptoms of the metabolic syndrome (Wild *et al.*, 2007). Prescription drugs available today reduce blood glucose level either by increasing the tissue uptake or by increasing the insulin secretion. There is no successful drug to treat the metabolic complexes associated with diabetes (Matfin, 2008).

A wide range of natural products are prescribed to the diabetic patient as nutraceuticals or dietary supplements for the intervention of disease, treatment and/or prevention (for more details http://www.naturaldatabase.com). Compounds of chromium, selenium, vanadium, herbal preparations of garlic, ginseng, α-lipoic acid and various mixed-herbal products are among the dietary supplements that are currently being used by many diabetics (Table 4). A recent review by Hays *et al.* (2008) discusses the rationale behind prescribing these natural products. Despite compelling evidence supporting an important role for dietary chromium in the restoration of normal glucose tolerance among chromium- deficient individuals, the ability of chromium to improve insulin action remains controversial (Balk *et al.*, 2007). While a number of animal models have generally demonstrated anti-diabetic properties for ginseng, relatively few clinical studies in humans have been reported. Taken together, research data suggest that ginseng is likely to show a beneficial effect on certain metabolic parameters in T2D (Xie *et al.*, 2005). However, it is difficult to make standardized clinical recommendations on account of the large reported variability in metabolic responses to different ginseng species, different batches of the same species, and different parts of the ginseng plant. On the whole, standardization of natural products remains a major bottleneck in the search for clinical evidence.

Table 4. Commonly used conventional and natural products for diabetes

Prescription drugs	Natural products
Hypoglycemic agents	
Chlorpropamid	Banaba (*Lagerstroemia speciosa*)
Glimepiride	Bitter melon (*Momordica charantia*)
Glipizide	Fenugreek (*Trigonella foenum-graecum*)
Glyburide	Gymnema (*Gymnema sylvestre*)
Nateglinide	
Repaglinide	
Tolazamide	
Tolbutamide	

Table 4. *Contd.*

Prescription drugs	Natural Products
Insulin sensitizers	
Metformin	American ginseng (*Panax quinquefolius*)
Pioglitazone	Banaba (*Lagerstroemia speciosa*)
Rosiglitazone	Cassia cinnamon (*Cinnamomum aromaticum*)
	Chromium
	Ginseng (*Panax ginseng*)
	Prickly pear cactus (*Opuntia ficus-indica*)
	Soya (*Glycine max*)
	Vanadium
Carbohydrate absorption inhibitors	
Acarbose	Blond psyllium (*Plantago ovata*)
Miglitol	Fenugreek (*Trigonella foenum-graecum*)
	Glucomannan (*Amorphophallus konjac*)
	Guar gum (*Cyamopsis tetragonoloba*)
	Oat bran (*Avena sativa*)
	Prickly pear cactus (*Opuntia ficus-indica*)
	Soy (*Glycine max*)
Miscellaneous	
Exenatide	α-lipoic acid
Pramlintide	Selenium
Sitaglitpin	Stevia (*Stevia rebaudiana*)

Numerous cell culture and *in vivo* animal studies have demonstrated that treatment with the water soluble antioxidant α-lipoic acid is associated with improved skeletal muscle glucose transport activity, reduced whole-body insulin resistance, and reduced oxidative stress. Although relatively few clinical trials have been reported, the combination of compelling *in vitro* and *in vivo* animal data in the presence of limited clinical data indicate the need for further research in human subjects while only supporting a general recommendation for α-lipoic acid supplementation (Bilska *et al.*, 2005).

Epidemiological studies on various populations living on a diet consisting of a high proportion of fatty fish (a rich source of omega-3 fatty acids) demonstrate a lower incidence of T2D compared to matched population groups. Positive changes in lipid profile and blood pressure demonstrated by epidemiological and other studies suggest that omega-3 fatty acid supplementation may reduce cardiovascular disease risk in T2D patients, even if no direct improvement in insulin or glucose homeostasis has been conclusively demonstrated (Lombardo *et al.*, 2006).

Many Indian medicinal plants have shown marked efficacy in the treatment of diabetes (Saxena *et al.*, 2004; Modak *et al.*, 2007). A recent review by Mukherjee *et al.* (2006) has discussed the Indian medicinal plants

with antidiabetic activity. However, detailed clinical investigations on the simultaneous use of prescription drugs along with natural products and dietary supplements are required for ensuring clinical safety and efficacy. A thorough preclinical and clinical investigation is necessary for establishing evidence.

Diabetes presents a singularly complex condition where multiple targets operate in conjunction with the very complex physiology of nutrition and energy homeostasis. The number of druggable targets in diabetes is steadily increasing, with more and more successful drug classes entering the market. For instance, the DPP-IV (Dipeptidyl peptidase IV) inhibitor sitagliptin has recently been launched. While synthetic compounds address these targets individually, natural products probably tackle them in a collective and perhaps more holistic fashion. Restoring the equilibrium in the disturbed scenario of metabolic syndrome is probably linked to the ability to manage multiple targets taken together. While the effect on each individual target is sub-clinical, the collective pharmacodynamic picture is likely to play a role in restoring the complex metabolic equilibrium. For instance, we have demonstrated in our labs that extracts of *Dodonaea*

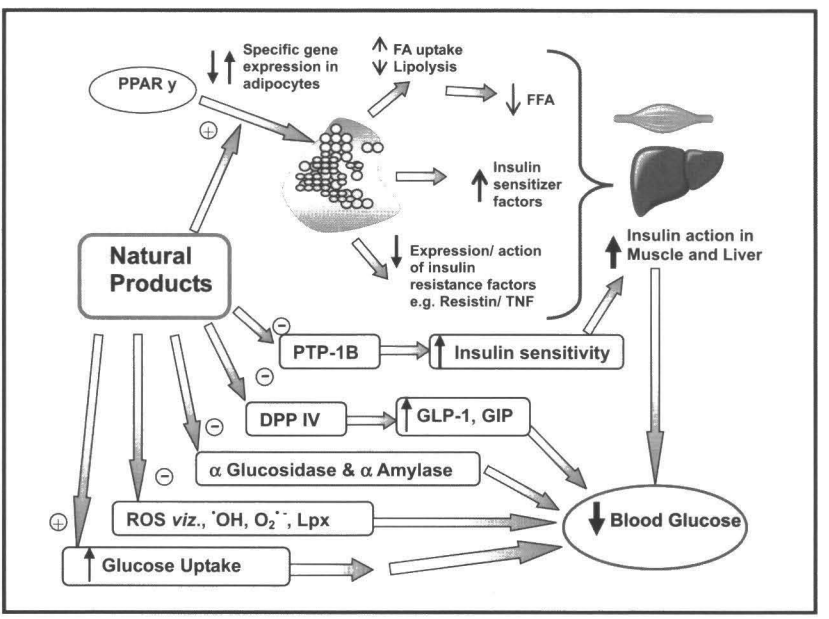

Fig 1. Schematic representation of the possible mechanisms by which a typical NP affords antidiabetic activity (PPAR γ: Peroxisome Proliferator-Activated Receptor-*Gamma*; FA: Fatty Acids; FFA: Free Fatty Acids; TNF: Tumour Necrosis Factor; PTP-1B: Protein Tyrosine Phosphatase 1B; GLP-1: Glucagon-like peptide-1B; GIP: Gastric inhibitory polypeptide); DPP IV: Dipeptidyl peptidase IV; ROS: Reactive Oxygen Species; ·OH: Hydroxyl Radical; $O_2^{\bullet -}$: Superoxide Radical; Lpx: Lipoxide Radicals)

viscosa and *Ficus racemosa* stem bark show antidiabetic activity through multimodal mechanisms in animal models. The possible mechanisms suggested are increase in binding of PPAR-γ (Peroxisome Proliferator-Activated Receptor-gamma), inhibition of PTP-1B (Protein tyrosine phosphatase 1B), DPP-IV (Dipeptidyl peptidase IV), α-amylase, α-glucosidase, tissue glucose uptake and reduction in the oxidative stress (Unpublished data, See Fig 1). The pharmacodynamic complexity is fitly matched by the diversity of targets addressing euglycemia in the body. While restoring or correcting these anomalies individually, one at a time, is the objective of conventional antidiabetic therapy, herbal products appear to tackle many of these targets in a concerted manner, providing a more sustainable method with perhaps a lower incidence of adverse events (Veerapur, 2007).

REVERSE PHARMACOLOGY: AN EFFECTIVE STRATEGY

The beaten track of drug discovery rests on the paradigm that the course of innovation begins in the lab and ends in the clinic. Reverse pharmacology is a complete turnaround in strategy, where clinical evidence works at the root of drug discovery (Patwardhan *et al.*, 2004). It is essentially a clinic to lab strategy, working backwards, where existing claims by traditional systems of medicine, such as *Ayurveda*, give clues to possible clinical value of existing formulations in traditional medicine. In such situations, clinical safety and proof of concept are the premises upon which the program is built, thus saving millions of dollars that are customarily spent on ensuring clinical safety and meeting regulatory rigours. Consequently, efficacy becomes a matter of validation, with the help of scientific evidence gathered from laboratory research. It is therefore a confluence of traditional wisdom, modern medicine and science. If exploited intelligently, it becomes a viable mode of drug discovery where holistic systems and traditional wisdom are integrated with laboratory evidence and modern science. The major advantage of reverse pharmacology is that it helps in reducing three major bottlenecks that plague the drug discovery programmes *viz.,* time, money and toxicity. These bottlenecks are major hurdles in drug discovery impeding not only the pace of its progress but also increasing the costs. Increase in regulatory rigour has become one of the major reasons for the ever diminishing entry of NCEs. In this context, reverse pharmacology appears to be a viable alternate strategy. Several industries have realized the need for reinventing the drug discovery strategies for innovating with value added products.

Ayurveda and such other traditional systems of alternate medicine have a rich knowledge base with a vast materia medica consisting of thousands of plants, minerals, animal products used singly or in combinations, with various processes for the formulation of medicines. Most

of these formulations and therapeutic strategies have survived on the basis of evidence that have sustained centuries of clinical experience. However, lack of standardization and validation continues to undermine the pharmacopoeias of alternate medicine. It is in this context that modern medicine and science can act as a complementary support for establishing proof of concept and validation of claims.

CONCLUSIONS

The last three decades have witnessed an unprecedented worldwide growth in the interest in NP- based medicines, both in developed and developing countries. Historical evidence shows that NPs offer a resource-pool for lead identification in drug discovery. NPs have a reasonable likelihood of biological activity, and isolation of a novel molecule is easier than *de novo* synthesis, especially when the molecular structure of the compound is very complex. NP-derived molecules are amenable to semisynthetic modifications and derivatisation. Such compounds are also significantly more complex and novel than most synthetic compounds, in addition to being chirally pure. Chiral specificity is very much in tune with the physiology of the body, giving the drug candidates a far greater level of specificity than non-chiral molecules. On account of such advantages, pharmaceutical companies are opting for NP based drug-leads. At present there is limited data on safety, efficacy, and clinical trials to prescribe NPs in therapy. However, the NP market has been continually growing at an increased rate during the last 30 years, mainly as nutraceutical, dietary supplements, cosmoceuticals and a few of them as chemopreventives. NP based therapy should emphasise on well-controlled and randomized clinical trials to prove safety and efficacy. International regulatory guidelines have to be followed in order to harmonise the use of NPs. In some countries, the newer strategies and guidelines have been tried for the NPs (Briggs, 2002; Barnes, 2003; Vaidya *et al.*, 2007; Calapai, 2008). Reverse pharmacology, which is driven by clinical evidence drawn from ancient wisdom, rather than preclinical data, also offers a viable time and cost saving strategy.

Emphasis should also be given to the production and genetic improvement (whenever possible) of medicinal plants and other NPs. Recombinant DNA technology offers opportunities to synthesise the cryptic molecules of nature in a simple environmental-friendly way. Further, the emphasis must shift towards discovering leads from unexplored realms of biodiversity (Harvey, 2000; Harvey, 2007). Alternative sources for natural products as also the techniques to save the extinction of biodiversity should be given due importance for sustaining the program (Kennedy, 2008). Finally, but not any less important, is the need for a more detailed legislation on intellectual property rights related to the marketing of NPs. All these strategies are urgently needed to avoid both business conflicts and territorial conflicts on biodiversity.

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The Modern Application of Traditional Chinese Medicine: Indigo Naturalis as an Alternative Therapy in Psoriasis vulgaris

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ABSTRACT

Psoriasis is a distressing, recurrent disease that significantly impairs the quality of life and has no permanent cure. Traditional Chinese medicine is an alternative method of therapy that can be used in the psoriasis treatment. It has been reported in Chinese literature that indigo naturalis exhibited potential anti-psoriatic effects in systemic therapy. However, its pharmacology was unclear and its gastrointestinal side effects limited its use in clinical practice. This article reviews and summarizes our experience of psoriasis therapy using Chinese herb prescription and the investigation of the anti-psoriatic mechanism of indigo naturalis. Indigo naturalis formulated in ointment has efficacy in treating psoriasis and can avoid adverse systemic effects. The possible mechanism of indigo naturalis action in treating psoriasis includes inhibiting hyperproliferation of epidermal keratinocytes and lymphocytes infiltration, as well as restoring epidermal differentiation and tight junctions in psoriatic skin. We believe that indigo naturalis will become a promising natural product that exerts great therapeutic potential as an alternative therapy in psoriasis.

Key words : Traditional Chinese medicine, psoriasis, indigo naturalis, indirubin, keratinocyte, differentiation

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INTRODUCTION

Psoriasis is a chronic inflammatory disease of the skin characterized by generalized erythematous papules or well-demarcated erythematous plaques topped by silvery scales which vary in severity and clinical manifestation. The most common form, plaque psoriasis, affects 2–3% of the Caucasian population (Lebwohl, 2003) but only 0.3% of the Mongoloid population (Yip, 1984). Many factors such as genetic features, immunological disorders, biochemical changes, environmental effects, trauma, stress, endocrine features, infection, drugs and alcohol play important roles in the pathogenesis of psoriasis (Ortonne, 1996). Conventional treatment for psoriasis has several disadvantages, including disease rebound or worsening after treatment is stopped (*e.g.* steroid) and risk for organ toxicities, such as nephrotoxicity and hepatotoxicity (*e.g.* cyclosporine, methotrexate). The fear of adverse events in long-term use may lead to poor compliance and results in following flares. Therefore, it is important to explore an alternatively new intervention which is relatively safe and can prolong the remission duration of psoriasis or prevent progression.

The earliest description of psoriasis in ancient Chinese medical literature may be found in the 5th century AD. Traditionally, Chinese herbal preparation has been extensively used to treat psoriasis both topically and orally for centuries, and produced promising clinical results (Bedi & Shenefelt, 2002). Most of the key actions of Chinese herbs commonly used in treating psoriasis reflect anti-inflammatory properties, modulation of cytokine production or inhibition of angiogenesis (Tse, 2003). All these actions are potentially relevant in reducing the severity of psoriasis. Nowadays, traditional Chinese medicine (TCM) is still one of the most frequently chosen alternative therapies that can be used in the treatment of psoriasis in China and Taiwan. However, its lack of randomized blinded control trials and its underlying mechanisms of action have not been systemically investigated (Koo & Desai, 2003). The theoretical system and the terminology of TCM are quite different from those of Western medicine. The doctors examine their patients with four traditional diagnostic methods (inspection, auscultation, interrogation, and pulse-taking), determine the principle of treatment according to the differentiation of symptoms and signs, and prescribe a formula according to the principle of treatment. In general, standard TCM practice emphasizes the importance of using many herbs that are combined in different formulations for each individual patient, sometimes mixing more than 10 different herbs. The mixture of herbs prescribed varies individually depending on the subtype of psoriasis ("blood-heat" type, "blood deficiency dryness" type, and "blood stasis" type), which is determined in traditional Chinese by many findings, including the lesions of psoriasis, the pulse, and the condition of the tongue (Tse, 2003). This is vastly different from Western medicine, which uses therapeutic approaches that are standardized and mono-therapy, as well as emphasizing

"average" efficacy in large, double-blinded, placebo-controlled studies. Due to the overwhelming variability in how patients are treated, both between TCM practitioners and within the practice of a single TCM practitioner, it is difficult to conduct controlled trials to study traditional approach of individual polypharmacy.

Indigo naturalis is one of the Chinese herbal remedies that have been reported to exhibit potential anti-psoriatic efficacy (Yuan *et al.*, 1982). However, the poor solubility and low absorption of indigo naturalis, and the adverse effects associated with irritation of the gastrointestinal tract and liver function in long-term systemic use have been occasionally reported (Verucchi *et al.*, 2002). In order to avoid the adverse systemic effects, but retain the demonstrated efficacy of indigo naturalis as an anti-psoriatic medicine, we initiated an alternative approach in 2003 by applying the drug topically on skin lesions. Within the last five years, we have been using indigo naturalis formulated in ointment topically to treat thousands of patients with psoriasis. We also used indigo naturalis as a mono-therapy in treating psoriasis and conducted randomized, observer-blinded, vehicle controlled, intra-patient comparison trials to evaluate the clinical efficacy and safety in patients with psoriasis treated with indigo naturalis. This unique mono-therapy, rather than individual polypharmacy that TCM used to emphasize, hence provided a compelling evidence of indigo naturalis for treating psoriasis. This review aims to provide our clinical experience for the efficacy, safety of indigo naturalis in topical treatment of psoriasis, and our studies on the pharmacology of indigo naturalis and its active components.

INDIGO NATURALIS'S PROFILE

Indigo naturalis (Qing Dai) is a dark blue powder originating from the branches and leaves of various indigo-producing plants, such as *Strobilanthes flaccidifolius*, *Polygonum tinctorium*, *Baphicacanthus cusia* Bremek, and *Isatis indigotica* Fort. The extraction process is based on the natural fermentation method in Taiwan. In brief, the freshly cut plants are soaked in water until they decompose and the contents of indigo naturalis are ready to be extracted. An adequate amount of limestone powder is then added and oxygen is forced into the liquid usually by stirring the soaking solution thoroughly until it turns from blackish green to deep red, extracted products contained in the foaming surface of the mixture are collected and air-dried for later use.

Indigo naturalis was used as textile dyes, paint pigment and medicine in ancient China. Indigo naturalis and indigo concoctions are recorded as being applied externally or taken internally before the first century AD in China and India. Until the mid-twentieth century, when antibiotics and steroids came into the world, indigo naturalis was one of the most important

and efficacious treatments to heal ailments of various infections and inflammatory diseases in China and Taiwan. There are many people today who still have faith in the powers of indigo naturalis to cure various difficult illnesses, such as cancer.

In TCM, indigo naturalis has been described as possessing anti-inflammatory, anti-microbial, analgesic and sedative properties, *i.e.* "clearing away heat, detoxicating, removing blood-heat and ecchymoses, purging liver-fire and arresting convulsion". Indigo naturalis has been used as a remedy for high fever in infectious sickness, infantile convulsions, epilepsy, aphtha, mumps, pharyngitis, tonsillitis, gingivitis, periodontitis, and various dermatoses, including eczema, eruption, erysipelas, carbuncles, furuncles and insect or snake bites. Modern research, particularly in China, is revealing that indigo naturalis, with effects of antivirus, and anti-bacteria, has also been used as an important source of anti-cancer drugs in China (Han, 1994). A well-studied example is Danggui Longhui Wan, a mixture of 11 herbal medicines traditionally utilized against certain types of leukemia. Only one of these ingredients, indigo naturalis, was found to carry the antileukemic activity (Xiao *et al.*, 2002). Although indigo naturalis is mostly constituted of indigo blue, a minor constituent, indirubin, was identified as the active component (Hoessel *et al.*, 1999).

TOPICALLY INDIGO NATURALIS IN PATIENTS WITH PSORIASIS

Clinical Efficacy

Since 2003, we began to use Chinese herbs topically to treat patients with psoriasis. Initially, we used a combination of three herbs, indigo naturalis, *Scutellaria baicalensis-Georgi*, and *Cortes phellodendri*, which was recommended for psoriatic treatment in TCM. The topical ointment combined mixed herbs and vaseline, yellow wax as well as olive oil. We found that many patients with psoriasis, after multiple treatment failures with conventional anti-psoriasis medications, showed clinical improvement after topical treatment with indigo naturalis composite ointment. In addition, remission time was found to last longer than their previous therapies. This novel finding was reported in the journal *Pediatric Dermatology* (Lin *et al.*, 2006). An 8-year-old boy with widespread psoriasis showed remarkable improvement with 8 weeks of topical treatment with this ointment. The psoriatic lesions on his body disappeared completely and the total body surface area involvement (BSA) decreased from almost 80% to 0%. Remission has lasted for over 2 years. In past 6 years observation, only mild disease recurred with no more than 1% of BSA was noticed in this patient which was immediately improved after re-treatment with indigo naturalis composite ointment. Because this formula consists of three herbs, it is difficult to conduct controlled trials and investigations of pharmacological mechanism. We reviewed the published literature, and

found indigo naturalis and its active ingredients, indirubin, have been orally used to treat psoriasis with considerable effectiveness (Koo & Arain, 1998). Therefore, we initiated the indigo naturalis mono-therapy for psoriasis and we reported that two adult patients with severe, recalcitrant psoriasis were successfully treated with topical indigo naturalis in the journal *Clinical and Experimental Dermatology* (Lin *et al.*, 2006). Two patients were male aged 45 and 36. One had an 8-year history of psoriasis and was presented with 40% BSA and a Psoriasis Area and Severity Index (PASI) score of 28.8. The other patient had suffered psoriasis for 22 years. He was presented with more than 80% BSA and a PASI score in excess of 42. The treatment by indigo naturalis ointment resulted in an approximately 95% reduction in PASI scores and BSA in these two patients within 3 months (Fig 1).

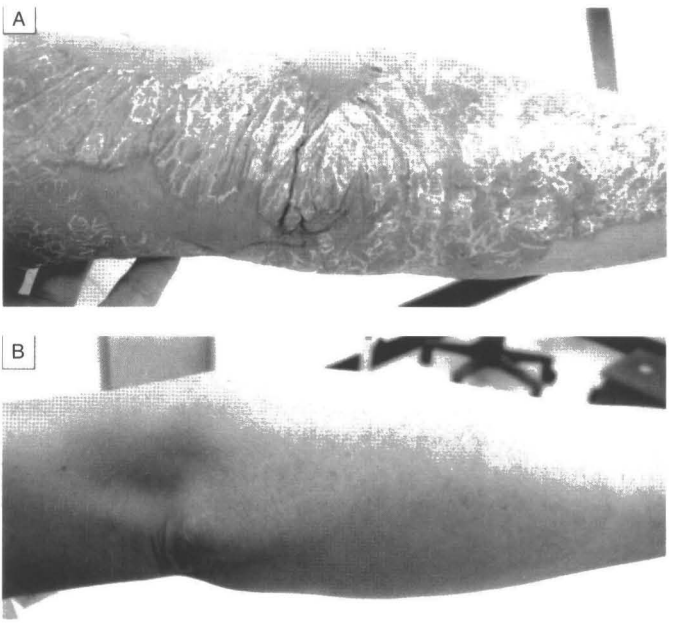

Fig 1. Clinical improvement after the treatment with indigo naturalis. A 40-year-old man with a 22-year history of plaques-type psoriasis. (A) Severe plaques are salmon pink in color, covered by silvery scales on right arm at the initial visit; (B) the same arm after 12 weeks of treatment with indigo naturalis ointment

In order to provide evidence-based data for this alternative therapy in psoriasis, we designed a randomized, vehicle-controlled clinical trial to evaluate the efficacy and safety of topically applied indigo naturalis in treating plaque-type psoriasis. An 8-week pilot clinical trial was performed during April-June 2004 in our hospital. Fourteen patients (10 men and 4 women) with chronic plaque psoriasis were enrolled. The mean patient age was 35.8 years (range, 21-54 years) and the median disease duration

was 10.8 years (range, 2-30 years). The median pretreatment PASI was 11.7, with a median BSA of 13.6%. The patients were topically treated with either indigo naturalis ointment or vehicle ointment on contralateral skin lesions daily for 8 weeks. Efficacy was evaluated on the basis of the clinical scores, including induration, scaling, erythema and clearing percentage. Ten patients completed the trial and the results demonstrated a significant reduction in clinical scores achieved by topically applied indigo naturalis ointment. This interesting result has been published in the journal *Dermatology* (Lin *et al.*, 2007). To further confirm the efficacy of indigo naturalis for psoriasis treatment, another randomized, observer-blinded, vehicle controlled, intra-patient comparative trial was conducted. Forty-two (32 men, 10 women) outpatients with chronic plaque psoriasis were enrolled. The mean patient age was 34.6 years (range, 18-58 years) and the median disease duration was 10 years (range, 2-41 years). The median pretreatment PASI was 14.7, with a median BSA of 18%. Target lesions were located on the upper extremities (17 participants), lower extremities (21 participants), and trunk (4 participants). The patients applied either indigo naturalis ointment or vehicle ointment topically to each of two bilaterally symmetrical psoriatic plaque lesions for 12 weeks. There was no significant difference in score of area, redness, thickness, and scaling between lesions which were assigned for treatment by indigo naturalis and vehicle ointments in baseline. The outcomes were assessed with the sum of erythema, scaling, and in duration scores and the clearing percentage of the target plaque lesion by two blinded professional observers. A significant reduction in the sum of scaling, erythema and in duration scores ($p<0.001$) (mean score 6.3 following indigo naturalis vs. 12.8 in control) and plaque area percentage ($p<0.001$) (mean percentage 38.5% following indigo naturalis vs. 90% in control) were achieved with topical application of indigo naturalis ointment. About 74% patients experienced clearance or near clearance of their psoriasis in the indigo ointment treated-lesion (Lin *et al.*, 2008).

Follow-up and Recurrences

In the follow-up study, withdrawal of the indigo naturalis treatment has been associated with disease recurrence occasionally in most patients, same as current western medicine therapies. However, according to statements of patients who achieve clearing or near clearing of their psoriasis, the median time to get re-treatment observed with indigo naturalis is markedly longer than their prior experience using conventional western medicine. That is to say, indigo naturalis seems to provide a remission time longer than that of traditional western medicine, and with less disease rebound. However, the current clinic data are not enough to assess this additional beneficial effect of indigo naturalis. It is therefore valuable in the future to conduct a clinical trial for direct comparison between indigo naturalis and

other currently used topical medicine (*e.g.* corticosteroid) to further evaluate the response duration and the disease recurrence.

Histological Response to Indigo Naturalis Treatment in Psoriatic Lesions

Based on our histological examination of skin biopsies, we found a marked decrease in epidermal thickness and elongated rete ridges for the lesions treated with indigo naturalis compared with the untreated lesions (Fig 2). We suggested that the anti-psoriatic effect of indigo naturalis might be mediated by controlling hyperproliferation and/or abnormal differentiation of epidermal keratinocytes, and/or exhibiting immunomodulatory effects in psoriatic lesion.

We analyzed and compared the expression of different marker proteins with respect to proliferation (Ki-67, PCNA), differentiation (involucrin, filaggrin), and inflammation (CD3) in psoriatic lesions treated with indigo naturalis ointment and vehicle ointment. Results from immunohistochemical analysis showed a marked decrease in the number of cells with positively Ki-67-stained nuclei in the indigo naturalis ointment-treated lesions compared to that in vehicle ointment-treated lesions (Lin *et al.*, 2007). Similar findings were also demontrated for PCNA marker (Lin *et al.*, 2007). The increased and normalized expression of filaggrin was observed in the stratum coreum and granular layer of the skin lesions treated with the indigo naturalis ointment (Lin *et al.*, 2007). Involucrin also expressed normally in the granular and upper spinous layers after indigo naturalis therapy (Lin *et al.*, 2009). In contrast to the original psoriatic lesions, the number of positively CD-3-stained T lymphocytes was demonstrated to be significantly reduced in the epidermis and papillary dermis of indigo naturalis ointment-treated lesions (Lin *et al.*, 2007).

In addition, based on our histological examination of skin biopsies, we found the presence of wide intercellular clefts only in the epidermis of lesions before treatment and control lesions treated with the vehicle ointment, but not in lesions treated with indigo naturalis ointment. In the treated psoriatic lesions that showed good clinical improvement, the keratinocytes in the granular layers were found to adhere to each other tightly. This finding implied that the impaired epidermal tight junction of psoriatic lesions may recover as a result of indigo naturalis treatment. We analyzed the expression of different marker proteins with respect to tight junction function (such as claudin-1) in psoriatic lesions, and the results showed a significant increase and recovery after treatment with topical indigo naturalis ointment compared with vehicle ointment (manuscript submitted).

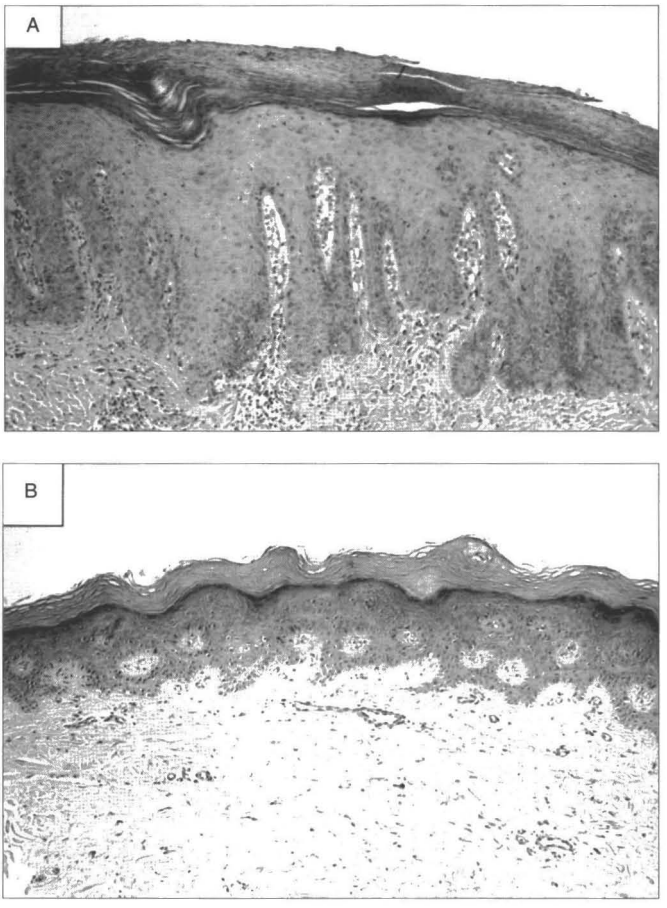

Fig 2. Effect of topically applied indigo naturalis ointment on the histological change of psoriatic lesion. Skin biopsies taken at baseline and after 8 weeks of treatment were processed for histological examination by HE stain method. (A) Untreated psoriatic lesion. (B) Indigo naturalis-treated lesion. Original magnification: ×100

In short, our pathological findings suggested that indigo naturalis has great potential values in the treatment of psoriasis by inhibiting hyperproliferation of epidermal keratinocytes and lymphocytes infiltration, as well as restoring epidermal differentiation and tight junctions in psoriatic skin. Whether these findings are simply a result of skin recovered from psoriasis after indigo naturalis treatment or indigo naturalis might directly modulate these changes in skin is waited to be clarified by investigating the pharmacology of indigo naturalis in much more details.

Adverse Events

Indigo naturalis, as a crude extract, appears to be dark blue in color and has a slightly unpleasant odor. The indigo naturalis ointment might slightly

stain the skin and clothing which can be cleaned thoroughly by common detergents. Repeated application has no significant effect on the skin color and does not change the skin appearance. However, either the color or smell of indigo naturalis may affect the compliance of few patients, therefore benefit comes along with longer treatment can not possibly attain for them.

Among 10 patients who completed an 8-week pilot clinical trial, 2 patients reported a short period (3-5 days) of itching at the beginning of treatment. In the following treatment period, no further adverse events such as scaling, erythema, tenderness, vesicles or other serious problems were found (Lin *et al.*, 2007). Another 12 weeks clinical trial, 4 of 34 patients who completed the treatment reported itching after applying the indigo naturalis ointment to their psoriatic plaque, but the itching was only present for a couple of days at the start of treatment, and no tenderness, vesicles or other serious problems were found in the subsequent treatment period (Lin *et al.*, 2008). In these two study groups, there were no abnormalities seen in the liver function tests, renal function tests, and complete blood counts after trial. Similar results were obtained in our long-term follow-up of these patients for the past 5 years. In our past 5-years experience in clinic, there were no serious events such as hepatic toxicity, renal damage and gastrointestinal problems found in these studies. Hypersensitivity, however, may be the major adverse effect as we have noticed in clinic (less than 15%). Some patients developed erythrodema and itching after using topical indigo naturalis treatment for psoriasis and most of them could recover by discontinuing the indigo naturalis application. Combining the treatment with topical steroids intermittently the development of allergy contact dermatitis may also be avoided or diminished.

PHARMACOLOGICAL PROPERTIES OF INDIGO NATURALIS

Although the effectiveness of indigo naturalis in the treatment of psoriasis is well established, the mechanism of its action is poorly understood. We have utilized cultured normal human epidermal keratinocytes (NHEKs) as a psoriasis-relevant experimental model to investigate the potential effects of indigo naturalis on the gene expression of keratinocytes. In our laboratory data, we found that indigo naturalis could suppress proliferation and promote differentiation of epidermal keratinocytes which were correlated with the down-rgulation of PCNA gene expression and the up-regulation of involucrin gene expression of NHEKs (Lin *et al.*, 2009). We have also made another novel finding that indigo naturalis can improve skin barrier protection, at least in part, by up-regulating claudin-1 gene expression and therefore the tight junction function (manuscript submitted).

The main components of indigo naturalis used in our clinic are indigo blue, indirubin and tryptanthrin as identified in chromatographic fingerprints. Among them, indigo blue in the most abundant one, however,

the activity in psoriasis treatment has not been reported yet (Lin, 1993). Our preliminary data suggest that indigo blue may exert an anti-angiogenic effect which may also partially explain the efficacy of indigo naturalis. Indigo blue extracted from some of the indigo-producing plants was reported to possess a hepatoprotective effect (Sreepriya *et al.*, 2001). Indirubin and its analogues have been used to treat psoriasis orally in China (Lin, 1993). In addition, clinical trials showed that indirubin has a definite efficiency against chronic myelogenous leukemia (Han *et al.*, 1988; Xiao *et al.*, 2002). Indirubin and its analogues selectively inhibit CDKs and other kinases and block cell proliferation in the late G1 and G2/M phase of the cell cycle (Marko *et al.*, 2001). The promotion of neutrophilic differentiation of human myelocytic leukemia HL-60 cells by indirubin has also been reported (Suzuki *et al.*, 2005). Furthermore, recent studies have revealed that indirubin simultaneously targets several signaling/molecules involved in the pathological process of cancer. The targets include those involved with cell cycle regulation (Hoessel *et al.*, 1999), growth factor receptor signaling (Stat3) (Nam *et al.*, 2005) and AhR signaling (Knockaert *et al.*, 2004). However, mechanism underlying the efficacy of indirubin in psoriasis is still unclear, and the pharmacology of indirubin in keratinocytes also is unknown. Tryptanthrin, another bioactive ingredient from indigo-producing plants, has been reported to exhibit anti-tumor, anti-inflammatory and anti-allergic activities (Lin, 1993). Whether tryptanthrin play any role in the anti-psoriatic effect of indigo naturalis is not elucidated and we will further investigate its pharmacology.

SUMMARY

Although the western medicines currently used for psoriasis treatments can produce clinical improvement at controlling the disease, none are universally safe and effective, and each carries a considerable risk profile. The clinical responses are sometimes typically short duration, and may be accompanied by disease rebound after therapy is withdrawn. All these therapies for psoriasis are only remissive, but not curative. Recently, alternative therapy (such as herbal remedies, nutritional supplements, and dietary manipulation) for the treatment of psoriasis has increasingly received much attention (Traub & Marshall, 2007). Most common reason cited for patients with psoriasis who are interested in alternative therapy was the dissatisfaction with their conventional treatment. Chinese herbal medicines have demonstrated their efficacy in treating psoriasis for hundreds of years. Unfortunately, the use of traditional Chinese herbal medicine, a mixture of various herbs individually formulated for the patient makes scientific analysis extremely difficult. The mechanisms of these herbs were unknown and any possible adverse effect associated these herbs has fallen on dead ears. In this article, we attempt to provide evidence-based information on the clinical benefit of using topical indigo naturalis as a

mono-therapy for psoriasis and explore the potential mechanisms of indigo naturalis in treating psoriasis.

In our experience, the use of topical application can avoid first-pass effect and chemical degradation of the drug in the gastrointestinal tract, as well as minimizing the risk of toxic side effects including gastric irritation, and liver damage. As psoriasis is a persistent and recurring disease, patients often require continuous treatment preferably with a low cumulative toxicity potential. The use of topical application not only presents direct and better therapeutic efficacy but also avoids the long-term adverse reactions. The effects for the use of indigo naturalis have been described in Chinese medical terms as "clearing the heat and cooling the blood" (Lin, 1993). However, the mechanisms underlying the effects of indigo naturalis specially for treating psoriasis seem to be very limited in the published literature.

Psoriasis has three principal histological features: epidermal hyperplasia; dilated, prominent blood vessels in the dermis; and an inflammatory infiltrate of leucocytes, predominantly into the dermis (Griffiths & Barker, 2007). Since psoriatic plaques are likely to arise from the failure of normal differentiation process, reagents that suppress hyperproliferation and promote differentiation of epidermal keratinocytes, as well as inhibit inflammation may have a significant impact on the treatment of psoriasis. Our pathological findings verified the above hypothesis that indigo naturalis is indeed effective to improve psoriatic lesion by controlling hyperproliferation and abnormal differentiation of epidermal keratinocytes, as well as lymphocyte infiltration. In addition, as shown in our histological findings, indigo naturalis may have the effect of recovering the epidermal barrier function. In our *in vitro* study, we were able to demonstrate that the proliferation of epidermal keratinocytes was down-regulated and differentiation was up-regulated after indigo naturalis treatment. Further investigation of pharmacological mechanism of indigo naturalis and its active components in treating psoriasis are currently performed in our laboratory.

In general, topical use of indigo naturalis is relatively safe compared to other currently used therapies. However, one should be aware that most of the herb indigo naturalis sold in the Taiwan area (this can happen in any area) is not prepared from natural plant. The chemically synthesized indigo naturalis barely contains any effective ingredient, stains the skin and clothing more easily, and tends to induce hypersensitivity.

In conclusion, indigo naturalis topically used for treating psoriasis is both safe and effective. We are very pleased to share our experience in the clinic and basic studies of psoriasis treatment which may contribute to the development of promising therapeutic agents for psoriasis.

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3

Development of Anti-Angiogenesis Therapies for Treating Cancer from Chinese Medicinal Herbs

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ABSTRACT

A new pharmacological approach for managing cancer should simultaneously target the multiple biochemical and physiologic pathways that promote cancer progression. Complex mixtures of phytochemicals used at relatively low doses can reduce tumor adaptation and resistance to conventional cytotoxic chemotherapy, as well as the prevention of progression of microscopic disease following definitive therapy. Angiogenesis is a key process in the promotion of cancer. Many plant-derived chemical mixtures manifest both antiangiogenic as well as other anticancer activities. The present article focuses on products that have a high degree of antiangiogenic activity, but it also briefly describes some of the many other actions of these agents that can inhibit tumor progression and reduce the risk of metastases. Natural health products target molecular pathways other than angiogenesis, including epidermal growth factor receptor, the HER2/neu gene, the cyclooxygenase- 2 enzyme, the nuclear factor kappa-B transcription factor, the protein kinases, the Bcl-2 protein, and coagulation pathways. Antiangiogenic Chinese medicinal herbs include Artemisia annua (Chinese wormwood), Curcuma longa (curcumin), Scutellaria baicalensis (Chinese skullcap), Magnolia officinalis (Chinese magnolia tree), Camellia sinensis (green tea), Ginkgo biloba, Poria cocos, Zingiber officinalis (ginger), Panax ginseng, and Rabdosia rubescens hora (Rabdosia). Quality assurance of appropriate extracts is essential prior to embarking upon clinical trials. More data are required on dose–response, appropriate combinations, and

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potential toxicities. Given the multiple effects of these agents, their future use for cancer therapy probably lies in synergistic combinations. During active cancer therapy, they should generally be evaluated in combination with chemotherapy and radiation. In this role, they act as modifiers of biologic response or as adaptogens, potentially enhancing the efficacy of the conventional therapies.

Key words : Angiogenesis, antiangiogenic, natural health products, herbal medicine, anticancer, molecular biology

INTRODUCTION

The Importance of Antiangiogenesis Therapies for Preventing Cancer Progression

The induction and promotion of cancer is a multistep process that involves biochemical interactions from the level of the genes, through cell-signaling pathways, intercellular communication mechanisms, supply of nutrients, channels for metastases, and a host immune response. Part of this process is the induction of new blood vessels to supply nutrients and immunocytes. Many natural health products that inhibit angiogenesis also manifest other anticancer activities. This multi-targeted approach may have major advantages for the treatment of cancer by overcoming the therapeutic resistance to the single drug approach caused by multiple redundant physiological pathways in the cancer cell.

Cancer progression requires a source of nutrition and oxygen. Tumors that outgrow their oxygen supply cannot form masses more than 1 to 2 mm in size or they then develop central necrosis. Neoplasms are genetically plastic and often adapt by switching on genes that result in an increased ability to invade and to metastasize. A critical part of this process is the induction of local small blood vessels, termed angiogenesis (Fidler, 2000a; Fidler, 2000b). Tumors do not grow progressively unless they induce a blood supply from the surrounding stroma. Cancers that lack angiogenesis remain dormant. Rapid logarithmic growth follows the acquisition of a blood supply.

The tumor angiogenic switch is activated (Fig 1) when the balance of angiogenic inhibitors to stimulators is shifted (Wallace, 2002). The process of neovascularization is subtly controlled in normal tissues by a series of endogenous polypeptides that are secreted during growth, healing, and tissue renewal (Table 1). Cancers synthesize or induce some of these polypeptides, especially vascular endothelial growth factor (VEGF) and angiopoietin (APN). These are peptides that are stimulated by hypoxia and result in sprouting of endothelial cords. The cancer induces a profuse but immature network of thin endothelial-lined channels, essential for tumor oxygenation. Although these new vessels allow progressive tumor growth, they are less efficient than the vascular supply of normal tissues.

APN normally recruits pericytes and initiates modeling of the vessel wall to more mature forms. However, tumors secrete a relative excess of VEGF, and this results in disorganized and leaky vessels that cause local bleeding and edema. Antiangiogenic therapy is less susceptible to the development of treatment resistance because it is directed to stromal tissue rather than the genomically unstable tumor cells.

Table 1. Endogenous angiogenic polypeptides

• Angiogenin (AG) and Angiotropin (AT)
• Basic Fibroblast Growth Factor (bFGF)
• Granulocyte-Colony-Stimulating Factor (G-CSF)
• Hepatocyte Growth Factor (HGF)
• Interleukin-8 (Il-8)
• Placental Growth Factor (PGF)
• Platelet-Derived Endothelial Cell Growth Factor (PD-ECGF)
• Pleiotrophin (PTN)
• Proliferin
• Transforming Growth Factor-α (TGF-α)
• Transforming Growth Factor-β (TGF-β)
• Tumour Necrosis Factor-α (TNF-α)
• Vascular Endothelial Growth Factor (VEGF)
• Vascular Permeability Factor (VPF)
• Insulin-Like Growth Factor I and II (IGF-1 and II)
• Cyclooxygenase (COX) and Lipoxygenase (LOX)
• Nuclear Factor-Kappa β (NF-κB)
• Activator Protein (AP-1)
• Angiopoietin (APN)

- Tumour angiogenic switch is activated when balance of angiogenic inhibitors to stimulators is shifted
- Reduced production of thrombospondin (TSP-1) from fibroblasts
- Vascular endothelial factor (VEGF) stimulates new vessel formation but exceeds Angiopoietin (APN) that normally induces differentiation (pericytes)
- Matrix metalloproteinase induction
- Increased VEGF relative to APN results in disorganized leaky vessels
- Rapid logarithmic growth follows the acquisition of a blood supply

Fig 1. The angiogenic switch

Single antiangiogenic agents, such as monoclonal antibodies or specific tyrosine kinase inhibitors, seem to have limited efficacy. Natural health products contain a range of complex organic chemicals that have synergistic activity. They inhibit angiogenesis by interacting with multiple pathways, as well as having other activities that can interact with cell signaling, the apoptotic pathway, and the interaction of cancer cells with the immune system. Some antiangiogenic agents also have anticoagulation activity that may also be associated with a reduction of metastases. Heparin is a well known example of a therapy with both anticoagulation and antiangiogenic activities. Instead of developing multiple monoclonal antibodies to target

the various peptides and their receptors, an alternative approach would be to evaluate phytochemicals that influence multiple pathways. The science of pharmacognosy evaluates drugs derived from herbal remedies or phytomedicines. There has been minimal clinical research that evaluates their use as adjuvant therapy to conventional treatment with cytotoxic drugs and radiotherapy. Antiangiogenic natural health products may be most effective in impeding cancer recurrence after cytotoxic therapy, encouraging tumors to remain dormant by changing the balance from cell proliferation to cell death by apoptosis.

The Angiogenic-Metastatic Pathway as a Target for Anticancer Therapies

The process of cancer metastasis consists of a series of sequential interrelated steps. Each step is rate limited and may be a target for therapy. The outcome of the process is dependent on both the intrinsic properties of the tumor cells and the responses of the host. The balance of these interactions varies between tumors and patients. The major steps in the formation of a metastasis are as follows (Sugarbaker, 1979; Hart *et al.,* 1989; Liotta & Stetler-Stevenson, 1991):

1. Transformation of normal cells into tumor cells followed by growth. Initially depends on nutrients supplied by simple diffusion.
2. Extensive vascularization (angiogenesis). This must occur if the tumor mass is to exceed 1 mm in diameter. The production and secretion of proangiogenic factors by tumor cells and host cells play a major role in establishing a capillary network from the surrounding host tissue.
3. Local invasion. Tumor cells invade the host stroma through several mechanisms. Thin-walled venules, fragmented arterioles, and lymphatic channels offer little resistance to penetration and provide the most common pathways for tumor cell entry into the circulation.
4. Detachment and embolization. Single cells or clumps break away. Most circulating tumor cells are rapidly destroyed. Those that survive must arrest in the capillary beds of distant organs by adhering either to capillary endothelial cells or to the exposed subendothelial basement membrane.
5. Extravasation into new host organ or tissue.
6. Proliferation within the new host organ or tissue. To continue growing beyond the size of 1 mm in diameter, the micrometastasis must develop a vascular network and evade destruction by host defenses. The cells can then continue to invade blood vessels, enter the circulation, and produce additional metastases.

The growth of many cancers is associated with the absence of the endogenous inhibitors of angiogenesis, such as interferon-beta (INF-β). INF-

β is a potent inhibitor of angiogenesis through blocking interleukin-8 (IL-8), basic fibroblast growth factor (bFGF), and collagenase type V, which are all potent angiogenic factors that aid tumor development and invasiveness. VEGF stimulates the proliferation and migration of endothelial cells and induces the expression of metalloproteinases and plasminogen activity. Over expression of VEGF in tumor cells enhances tumor growth and metastasis in several animal models by stimulating vascularization (Folkman & Kotran, 1976; Senger *et al.,* 1983; Folkman *et al.*, 1986; Folkman & Klagsbrun, 1987; Nagy *et al.*, 1989; Folkman *et al.,* 1995; Ferrera *et al.*, 1996; Risau, 1997; Kumar *et al.*, 1998). Some cytotoxic chemotherapy agents are being used at lower than normal doses with the intent of inhibiting angiogenesis and minimizing toxicity (Oliff *et al.*, 1996; Miller *et al.*, 2001). This strategy may permit advanced cancer patients to maintain a better quality of life. The low-dose therapy is termed *metronomic* dosing (Hanahan *et al.*, 2000; Maraveyas *et al.,* 2005; Hudis, 2005). The metronomic model of conventional cytotoxic chemotherapy suggests that there may also be advantages for administering combinations of phytochemicals that interact with the multistep process of angiogenesis (Singh *et al.*, 2003). In other words, targeting the vascular endothelium with continuous low-dose non-cytotoxic therapies may maintain tumor control without excessive toxicity. Their potential role for increasing overall survival (but not necessarily disease-free survival) and maintaining quality of life requires evaluation in future clinical trials.

Screening Herbs for Antiangiogenic Activity

One of the first isolated antiangiogenic agents was a phytochemical. Ingber *et al.* (1990) reported the antiangiogenic properties of fumagillin, a secreted antibiotic of the fungus *Aspergillus fumigatus* (Trichocomaceae). Refined fumagillin produces excess toxicity, so analogues of fumagillin were subsequently synthesized. Fumagillin and an analogue labeled TNP-470 are proposed to inhibit angiogenesis by selective inhibition of methionine aminopeptidase type 2 (MetAP-2). However, TNP-470 also demonstrated poor pharmacokinetic behavior and dose-limiting toxicity in clinical trials, and these factors remain obstacles to its use as an anticancer agent. Further modifications of fumagillin have been conducted to develop MetAP-2 inhibitors with desirable pharmacological properties. They have been tested only by *in vitro* assays, and to date, no clinical trials of these analogues have yet been conducted (Ingber *et al.,* 1990; Pyun *et al.,* 2004; Furness *et al.*, 2005). Since the angiogenic cascade is a multistep process, numerous assays have been developed to study potential angiogenic activity. Some analyze a single step in the pathway, whereas others test the angiogenic cascade as a whole. The relationship of each assay to clinical activity is poorly defined. Some agents have profound antiangiogenic effects at low doses; others exhibit antiangiogenic activity only at near cytotoxic concentrations. Some agents have activity in one model but none in others.

Criteria for antiangiogenic activity are listed in Fig 4 and should include (Miller *et al.,* 2001):

- differential cytotoxicity,
- alteration of endothelial cell function,
- critical mechanistic effects, and
- inhibition of angiogenesis *in vivo.*

Traditional Chinese Medicine	Western Biomedicine
• Stagnation of blood and Qi	• Increased fluid content and abnormal vascular supply; blood stasis; ***poor oxygenation***
• Destagnation herbs	• Anticoagulants as adjunct to chemotherapy increase survival in clinical studies; prevent blood-borne metastases in animal models

Fig 2. The traditional chinese medicine versus the biomedical theory of cancer

Various assays (Table 2) are used to screen natural health products for antiangiogenic activity (Miller *et al.*, 2001; Wang *et al.,* 2004; Kruger *et al.,* 2001). Assays used for screening will be briefly discussed.

Table 2. Screening for anti-angiogenesis

- *In vitro* Assays
  - Human umbilical vein endothelial cells (HUVEC)
  - Bovine aortic endothelial cells (BAEC)
  - Tumour endothelial cells (TEC)
- *In vivo* Assays
  - Chick embryo chorioallantoic membrane (CAM) model
  - Cornea implantation
  - Matrigel
  - Tumour xenografts

In vitro Assays

In vitro assays are designed to recapitulate each of the multiple events that constitute the angiogenic process. Some of them are very specific in analyzing a single event (proliferation, apoptosis, migration, production of proteases), whereas others provide a more complex picture of the process involving cell functions and interactions with the environment. *In vitro* assays for the activity of antiangiogenic compounds are usually based on the use of endothelial cells. A critical issue in setting up an *in vitro* assay is the choice of endothelial cells. Immortalized endothelial cells are sometimes used, as they provide an "unlimited" source of cells. Although these cell lines have the obvious advantages of being easy to grow and relatively stable

throughout *in vitro* passages and among batches, they have usually lost some of the characteristics of endothelial cells, including molecular markers, and exhibit changes in function. The most commonly used endothelial cells are from the human umbilical vein, since these are easily available and cell isolation is relatively simple. For the same reasons, bovine or murine aortic endothelial cells are often used too, but these come from large vessels, and they have different phenotypic and behavioral characteristics from those of the microvessels that are more likely involved in angiogenesis. Other common sources of microvascular endothelial cells are the skin, brain, adipose tissue, and adrenal gland. Endothelial cells derived from the microvasculature of different tissues/organs are often heterogeneous, imposing a further constraint on the choice of cell model. Ideally, when developing inhibitors of tumor angiogenesis, tumor-derived endothelial cells should be used. However, practical difficulties in their isolation from tumor tissue and maintenance in culture have limited their use in preclinical studies (Taraboletti *et al.,* 2004). The ability to maintain endothelial cells in culture has allowed the study of endothelial cell proliferation, migration, and cellular function. Angiogenic activity may be represented as endothelial cell migration across a Boyden chamber. Compounds with antiangiogenic potential will inhibit the migration. The bovine aortic endothelial cell (BAEC) and the human umbilical vein endothelial cell (HUVEC) assays are established systems. *In vitro* assays are relatively inexpensive and give more rapid results. However, the ability to inhibit endothelial cell proliferation, migration, and tubule formation *in vitro* may not necessarily predict *in vivo* response. *In vitro* assays are a rapid method for initial screening of large numbers of agents. Definitive conclusions cannot be based on *in vitro* assays alone.

In vivo Assays

These biological assays are more specific for detecting antiangiogenic activity. The chick embryo chorioallantoic membrane (CAM) model is an extraembryonic membrane that is commonly used to study agents that influence angiogenesis. An angiogenic response occurs 72 to 96 h after stimulation in the form of increased vessel density around the implant. On the other hand, an angiostatic compound induces the vessels to become less dense around the implant and even disappear. Other systems include animal cornea implantation, disc angiogenesis, Matrigel systems, and tumor xenograft models. The *in vivo* assays provide a more complete physiologic assessment of angiogenesis but are more time-consuming and expensive.

Criteria for Antiangiogenic Activity

The degree of antiangiogenic activity is dose dependent. Most chemotherapy drugs have antiangiogenic activity when administered at high doses. We

are especially interested in compounds that specifically interact and antagonize the steps involved in angiogenesis when administered at low doses. These agents may have relatively low toxicity at low dose and may exhibit a higher therapeutic gain. Most conventional chemotherapy drugs have some degree of antiangiogenic activity as a consequence of their cytotoxic activity. Ideal botanical derivatives would specifically antagonize new vessel formation in tumors, without significant toxicity to normal tissues and without major adverse reactions. The ideal agent would also inhibit tumor cell proliferation through other physiologic pathways, such as influencing intracellular signaling pathways. Multiple levels of antiangiogenic activity may be required to overcome the development of resistance by tumor-associated endothelial cells (TEC). Survival factors, such as the increased secretion of VEGF and bFGF by the tumor cells, activate intracellular pathways that prevent TEC apoptosis. Maximal antiangiogenic activity usually requires prolonged exposure to low concentrations of the active agent. This approach contrasts with the concept of administering maximum tolerated doses of cytotoxic drugs to maximize tumor cell kill. Some reports have confirmed the utility of combining low, frequent-dose chemotherapy plus an agent that specifically targets the endothelial cell compartment (Hanahan *et al.*, 2000; Maraveyas *et al.*, 2005). The evidence suggests that an antiangiogenic schedule can be more effective than using high-dose cytotoxic drugs alone. We hypothesize that concomitant scheduling of antiangiogenic botanicals with low, frequent-dose cytotoxic therapies may have biological advantages that can increase therapeutic gain.

Natural Health Products that Inhibit Angiogenesis

Further research is necessary to screen herbs that may be useful antiangiogenic therapies. Table 3 lists Chinese herbs with antiangiogenic activity (Wang *et al.*, 2004), and Table 4 lists herbs and their derivatives that inhibit VEGF (Singh *et al.*, 2003). An expert herbalist can advise on potential herbal treatments derived from advanced traditional medical systems, such as traditional Chinese medicine. It will be imperative to develop a new model of modern pharmacology based on traditional pharmacognosy. Our developing knowledge of cancer biology suggests that administering cytotoxic drug therapy at very high doses is not always appropriate. A new approach is to administer lower doses of synergistic organic chemicals. These complexes already exist in myriad botanicals. New laboratory techniques allow more specific assays of activity and enable quality assurance and consistency between batches of botanical preparations to be maintained. This will enable credible clinical trials of antiangiogenic natural health products to be initiated.

Table 3. Antiangiogenesis activity of chinese medicinal herbal extracts (Exhibiting more than 20% inhibition at 0.2 g/herb/mL) (Wang *et al.*, 2004)

Assays: chick embryo chorioallantoic membrane (CAM) and bovine aortic endothelial cells culture models (BAEC)

Name	Used Part	% Inhibition (CAM)	% Inhibition (BAEC)
Berberis paraspecta	Root	25	38
Catharanthus roseus	Leaf	27	30
Coptis chinensis	Rhizome	25	37
Scrophularia ningpoensis	Root	20	34
Scutellaria baicalensis	Root	27	41
Polygonum cuspidatum	Whole plant	-	28
Taxus chinensis	Bark	-	26

Traditional Chinese Medical (TCM) theory describes tumor formation as stagnation of qi and blood (Fig 2). In Western theory, this corresponds to the abnormal vasculature induced by the cancer when it induces the angiogenic switch. The tumor has a stagnant blood supply with abnormal leaky vessels that raises the interstitial fluid pressure, resulting in centrifugal diffusion and poor oxygenation. TCM theory suggests "destagnation herbs" as a potential therapy. A randomized placebo-controlled trial from China showed that the addition of "destagnation" herbs (including *Salvia miltiorrhiza* Bunge [Lamiaceae] and *Angelica sinensis* Diels [Apiaceae]) to radiotherapy doubled both the local control and survival rates of patients with nasopharyngeal cancer (Fig 3) (Xu *et al.*, 1989). In some clinical trials, anticoagulation drugs are associated with a reduction in metastases (Hejna *et al.*, 1999; Smorenburg *et al.*, 2001; Blom *et al.*, 2005). Laboratory evidence now suggests that many of these Chinese herbs have antiangiogenic and anticoagulation properties (Huang *et al.*, 2003; Wang *et al.*, 2004; Samuels, 2005).

Destagnation herbs (Huo Xue Hua Yu) activate blood flow and improve micro-circulation *(huang qi, chi shao, chuan xiong, dang gui, tao ren, hong hua, ji xue teng, ge gen, chen pi, dan shen)*

	N	Local recurrence	5 Year survival
XRT alone	90	29%	37%
XRT + herbs	98	14%	53%
		($p<0.05$)	($p<0.05$)

Fig 3. A randomized controlled trial of chinese herb destagnation formula with radiation in the treatment of nasopharyngeal carcinoma (Xu *et al.*, 1989)

Some herbs that are traditionally used in China as anticancer agents have been screened for their antiangiogenic activity (Fig 4) in the laboratory (Wang *et al.*, 2004). Table 3 lists the most active herbs (exhibiting more than 20% inhibition at 0.2 g/herb/mL), using the CAM and BAEC assays. Other herbs are included in Table 4 and are discussed in more detail.

Table 4. Chinese herbs and their derivatives that specifically inhibit vascular endothelial growth factor and have direct activity against angiogenesis

- *Artemisia annua* (Chinese wormwood; contains 95% artemisinin and other related terpenes and flavonoids)
- *Scutellaria baicalensis* (Chinese Baical skullcap; contains 95% baicalin and flavonoids)
- *Curcuma longa* (turmeric; contains 95% curcumin)
- *Camellia sinensis* (green tea; contains 95% phenols; 50% epigallocatechin)
- *Angelica sinensis* (Dong quai; contains 4-hydroxyderricin)
- *Taxus brevifolia* (Pacific yew; contains taxol)
- *Polygonum cuspidatum* (Japanese knotweed; contains 20% resveratrol)
- *Silybum marianum* (Milk thistle; contains 80% silymarin [silibin])
- *Ginkgo biloba*
- *Magnolia obovata* (contains 90% honokiol)
- *Zingiber officinale* (contains 6-gingerol)
- *Poria cocus*
- *Panax ginseng*
- *Rabdosia rubescens* (Rabdosia)
- *Berberis paraspecta* Ahrendt (Berberidaceae), root
- *Catharanthus roseus* G Don (Apocynaceae), leaf
- *Coptis chinensis* Franch (Ranunculaceae), rhizome
- *Scrophularia ningpoensis* Hemsl (Scrophulariaceae), root
- *Polygonum cuspidatum,* Whole plant
- *Taxus chinensis* Rehder (Taxaceae)

- Degree of antiangiogenic activity is dose dependent
- Specifically interacts and antagonizes angiogenesis at low doses (high therapeutic gain)
- Inhibits tumor cell proliferation through multiple physiologic routes, such as intracellular signaling pathways
- Minimal effect on normal angiogenesis
- Low toxicity with prolonged administration

Fig 4. Criteria for an anti-angiogenic botanical

Botanicals usually act on multiple anticancer targets since they contain a variety of organic chemical complexes. The biochemical signaling pathways of angiogenesis form a complex, interconnected web. Inhibition of one part of this web may result in compensation through another pathway. A potential advantage of phytochemicals is that they may act through multiple pathways and reduce the development of resistance by cancer cells. This model of pharmacognosy recognizes the advantage of administering the whole plant product to maximize activity (Fig 5). Over extraction of a specific chemical constituent may remove this therapeutic gain. The

challenge for modern pharmacognosy is to ensure that the optimum mixture of chemical constituents is maintained when purifying the product. Usually, this will require a combination of both chemical and biological assays. Further anticancer properties of some antiangiogenic botanicals will briefly be discussed. Their effects may also interact with various biochemical pathways that indirectly influence angiogenesis. Traditional practice has been to combine multiple natural health products, and this may scientifically provide a therapeutic advantage.

- Range of complex organic chemicals that may have synergistic activity, alone or in combinations.
- Inhibit angiogenesis by interacting with multiple pathways.
- Bonus activities: interact with cell signaling, apoptosis and interaction of cancer cells with the immune system.
- Anticoagulation activity that may be associated with a reduction of metastases
- Less acquired resistance to therapy.
- Low toxicity: suitable for combination therapy and long term administration.
- Possible economic advantages.

Fig 5. Advantages of anti-angiogenic NHPs

Chinese Medicinal Botanicals for Potential Development into Anti-Angiogenic Therapeutic Agents for the Treatment of Cancer

Artemisia annua (Chinese Wormwood)

Artemisinin is the active constituent extracted from the plant *A. annua* L. (Asteraceae). It has been used clinically as an antimalarial drug (Mueller *et al.,* 2004). More recently, it was shown to be cytotoxic to cancer cells through induction of apoptosis (Singh & Lai, 2004). Artesunate (ART) is a semisynthetic derivative of artemisinin. The *in vitro* effect of ART was tested on the HUVEC model of angiogenesis. It significantly inhibited angiogenesis in a dose-dependent manner. The inhibition of HUVEC proliferation was greater than the effect on cancer cells, fibroblast cells, and human endometrial cells. This indicates that its antiangiogenic activity is greater than its cytotoxicity. The antiangiogenic effect *in vivo* was evaluated in nude mice using transplanted human ovarian cancer (HO-891) cells and immunohistochemical staining for microvessel CD31 antigen, VEGF, and the VEGF receptor (KDR/flk-1). Tumor growth was decreased and microvessel density was reduced without any toxicity to the host animals. Artemisinin also lowered the VEGF expression by tumor cells and the KDR/flk-1 expression by endothelial cells (Chen & Chang, 2004). Artemisinin also has therapeutic effects through other pathways. It inhibits the activation of nuclear factor-κB (NF-κB), an important activator protein in cancer development and progression (Aldieri *et al.,* 2003).

Scutellaria baicalensis (Chinese Skullcap)

Baicalin and baicalein are the main derivatives from the Chinese Skullcap herb, *S. baicalensis* Georgi (Lamiaceae). They are potent antiangiogenic

compounds that reduce VEGF, bFGF, 12-lipoxygenase activity, and MMP (Liu *et al.,* 2003; Miocinovic *et al.*, 2005). It is a component of the traditional Bupleurum formula for stagnation (Xiao Chai Hu Tang):

- Bupleurum (Chai Hu) 12-15 g *Bupleurum chinense*
- Scutellaria (Huang Qin) 9-12 g *Scutellaria baicalensis*
- Pinellia (Ban Xia) 9-12 g *Pinellia ternata*
- Fresh Ginger (Sheng Jiang) 3-6 g *Zingiberis officinalis*
- Ginseng (Ren Shen) 6-9 g *Panax ginseng*
- Licorice (Gan Cao) 3-6 g *Glycyrrhiza uralensis*
- Jujube Dates (Da Zao) 3-5 pcs *Zizyphus jujuba*

S. baicalensis is also one of the herbs found in PC-SPES, a complex of Chinese herbs that may have clinical activity against advanced prostate cancer (Small *et al.*, 2000; Oh *et al.,* 2001). However, although a phase 2 trial demonstrated clinical activity in patients with androgen independent prostate cancer, diethylstilbestrol (DES) and ethinyl estradiol (both known to have potent antiprostate cancer activity) were detected in various lots of PC-SPES (Hsieh *et al.,* 2002). It is still intriguing that the decline in PSA was greater for PC-SPES that was potentially contaminated with DES than for the comparator group that received DES alone, suggesting some independent activity. Although baicalin and baicalein have multiple anticancer activities *in vitro*, their clinical activity is not established, and their contribution to any potential therapeutic effect of PC-SPES is unknown (Cordell, 2002; Oh *et al.,* 2004).

Curcuma longa (Turmeric)

Curcumin is the most active curcuminoid present in turmeric, *C. longa* L. (Zingiberaceae). It interacts with cancer cells at a number of levels and can enhance the tumoricidal efficacy of cytotoxic chemotherapy and radiotherapy (Narayan, 2004; Khafif *et al.,* 2005; Sen *et al.,* 2005). Its anti-invasive effects are partly mediated through the down regulation of MMP-2 and the upregulation of tissue inhibitor of metalloproteinase (TIMP-1) (Shao *et al.*, 2002). These enzymes are involved in the regulation of tumor cell invasion. Curcumin inhibits the transcription of 2 major angiogenesis factors, VEGF and bFGF (Arbiser *et al.,* 1998). It interacts with VEGF and nitric oxide– mediated angiogenesis in tumors (Sreejayan, 1997; Garcia-Cardena *et al.,* 1998). Elevated levels of nitric oxide correlate with tumor growth. Curcumin reduces nitric oxide generation in endothelial cells. CD13/aminopeptidase-N (APN) is a membrane bound enzyme found in blood vessels undergoing active angiogenesis. Curcumin binds to APN and blocks its activity, thereby inhibiting angiogenesis and tumor cell invasion (Gururaj *et al.,* 2002; Shim *et al.,* 2003). Derivatives of curcumin may be

developed to target APN, providing a novel approach to reduce neoplastic angiogenesis (John *et al.,* 2002; Hahm *et al.*, 2004). Curcumin also downregulates the expression of the VEGF and MMP-9 genes that are associated with angiogenesis. Demethoxycurcumin is a structural analogue of curcumin isolated from *Curcuma aromatica* Salisb. (Zingiberaceae). It specifically inhibits the expression of MMP-9 (Kim *et al.,* 2002). Curcumin can interfere with the activity of both MMP-2 and MMP-9, the basis of the angiogenic switch, thereby reducing the degradation of the extracellular matrix (Chen *et al.,* 2004). It also interferes with the release of angiogenic factors that are stored in the extracellular matrix. It inhibits growth factor receptors such as epidermal growth factor receptor (EGFR) and VEGFR and the intracellular signaling tyrosine kinases. This cell-signaling system can promote further angiogenesis through gene activation that increases levels of cyclooxygenase-2 (COX-2), VEGF, IL-8, and the MMPs (Reddy & Aggarwal, 1994; Dorai *et al.,* 2001; Leu *et al.,* 2003). A phase 1 study of curcumin found no treatment-related toxicity up to 8000 mg/d. Beyond 8000 mg/d, the bulky volume of the drug was unacceptable to the patients. The serum concentration of curcumin usually peaked at 1 to 2 h after oral intake of curcumin and gradually declined within 12 h (Cheng *et al.,* 2001). This study suggested that it may prevent cancer progression. Derivatives of curcumin, such as copper chelates of curcuminoids, may have increased antitumor activity (John *et al.,* 2002).

Camellia sinensis (Green Tea)

C. sinensis (L.) Kuntze (Theaceae), contains polyphenols and catechins (mainly epigallocatechin-3 gallate [EGCG]) (Lee *et al.,* 2002). These constituents inhibit MDAMB breast cancer cell and HUVEC proliferation (Sartippour *et al.*, 2002). In addition, they suppress breast cancer xenograft growth and reduce the density of tumor vessels in rodent studies (Cao & Cao, 1999). This is associated with a decrease in VEGF, regulated at the level of transcription. EGCG also suppresses protein kinase C (PKC), another VEGF transcription modulator. Inhibition of VEGF transcription is one of the molecular mechanisms involved in the antiangiogenic effects of green tea that may contribute to its potential use for cancer treatment (Kojima-Yuasa *et al.*, 2003; Tang *et al.*, 2003; Fassina *et al.,* 2004). EGCG may be administered as a powdered extract of green tea. An appropriate dose has been extrapolated from antiangiogenic activity in rodent experiments as well as a phase 1 study in humans (Cao *et al.,* 1999; Pisters *et al.,* 2001). A dose of 1.0 g/m^3 times daily (equivalent to 7-8 Japanese cups [120 mL] 3 times daily) has been recommended. In practice, lower total daily doses of 2 to 4 g of standardized green tea extract (95% polyphenols/60% catechins) are usually prescribed. Each gram of this extract provides 400 to 500 mg of EGCG. The dose-limiting adverse effects are gastrointestinal and

neurological effects of caffeine. However, the caffeine may potentiate the antiangiogenic effect of EGCG (Pisters *et al.,* 2001).

Polygonum cuspidatum (Japanese Knotweed)

This contains 20% resveratrol. It has antiangiogenic activity demonstrated by its ability to inhibit HUVEC division and to decrease the lytic activity of MMP-2 (Cao *et al.,* 2005). Resveratrol inhibits VEGF induced angiogenesis by disruption of reactive oxygen species-dependent Src kinase activation and subsequent VE-cadherin tyrosine phosphorylation (Igura *et al.,* 2001; Lin *et al.,* 2003). Resveratrol inhibits the growth of gliomas in rats by suppressing angiogenesis (Tseng *et al.,* 2004).

Silybum marianum (Milk Thistle)

Silibinin and silymarin are polyphenolic flavonoids isolated from the fruits or seeds of *S. marianum* (L.) Gaertn. (Asteraceae). In the laboratory, silymarin demonstrates strong activity against a variety of tumors through down regulating VEGF and EGFR (Jiang *et al.*, 2002; Singh & Aggarwal, 2003). Silymarin suppresses VEGF when used as a single agent against human ovarian cancer endothelial cells *in vitro* (Gallo *et al.,* 2003). A more detailed discussion can be found elsewhere (Sagar, 2007).

Ginkgo biloba

G. biloba L. (Ginkgoaceae) extract has anticancer effects that are related to its gene-regulatory and antiangiogenic properties. The *G. biloba* extract used in most of the research is EGb 761, which contains about 25% flavonoids (ginkgo-flavone glycosides) and about 5% terpenoids (ginkgolides and bilobalides). The most potent flavonoid is ginkgolide B. This extract inhibits angiogenesis by down regulating VEGF (Zhang *et al.*, 2002; De Feudis *et al.,* 2003).

Magnolia officinalis (Chinese Magnolia Tree)

The seed cones of *M. officinalis* Rehder & E.H. Wilson (Magnoliaceae) contain substances that inhibit the growth of new blood vessels. Honokiol is the active constituent. It may partly reduce angiogenesis through the regulation of platelet-derived endothelial cell growth factor and TGF-β expression. It also inhibits nitric oxide synthesis and TNF-β expression (Son *et al.*, 2000; Lee *et al.,* 2004). In animal experiments, it suppresses the proliferation of blood vessel endothelial cells more than other types of cells and thereby reduces tumor growth (Bai *et al.*, 2000; Chen *et al.,* 2004).

Zingiber officinale (Ginger)

6-Gingerol, from *Z. officinale* Roscoe (Zingiberaceae), inhibits both the VEGF- and bFGF-induced proliferation of human endothelial cells and causes cell cycle arrest. It also blocks capillary-like tube formation by endothelial cells in response to VEGF and strongly inhibits sprouting of endothelial cells in

the rat aorta and mouse cornea *in vitro* models. In mice receiving injections of B16F10 melanoma cells, intraperitoneal administration of 6-gingerol, at doses less than cytotoxic levels, reduces the number of lung metastases (Kim *et al.,* 2005).

Poria cocos

P. cocos F.A. Wolff (Coriolaceae) is a mushroom extract that has been traditionally held to have anticancer activity. It inhibits platelet aggregation and appears to be antiangiogenic by downregulating NF-kappa-B (Jin *et al.,* 2003; Chen *et al.,* 2004; Lee *et al.,* 2004).

Panax ginseng

The lipophilic constituents of *P. ginseng* C.A. Meyer (Araliaceae) are called saponins (or ginsenosides). These extracts possess anticancer activities in tumors that include antiangiogenesis and induction of tumor cell apoptosis (Sato *et al.,* 1994).

Rabdosia rubescens (Rabdosia)

The herb *R. rubescens* H. Hara (Lamiaceae) is used traditionally to treat cancer and is a constituent of the PC-SPES formula. It contains ponicidin and oridonin, two diterpenoids that possess significant antiangiogenic activity (Meade-Tollin *et al.*, 2004).

CONCLUSIONS

Angiogenesis involves multiple interdependent processes operating at the molecular level. These include gene expression, signal processing, and enzyme activities.

Most antiangiogenic natural health products block new vessel formation at multiple levels. Lack of standardization of screening assays may be an obstacle to defining the most effective products for clinical use. Over extraction of constituents may negate some of the advantages of potential synergy. Mainly preclinical data exist for most of the naturally derived antiangiogenic agents. Most of the studies of antiangiogenic activity are based on *in vitro* or animal work, which cannot be readily extrapolated to humans. Phase 1 and 2 studies are required to determine their potential to improve cytotoxic therapies. Quality assurance of appropriate extracts is essential prior to embarking on clinical trials (Fig 6).

Since antiangiogenic agents are mainly cytostatic in nature, the usual paradigm for anticancer drug development, in which tumor response in phase 2 trials prompts further development, is not always appropriate. More data are required on dose response, appropriate combinations, and potential toxicities. Given the multiple effects of these agents, their future use for cancer therapy probably lies in synergistic combinations. They may

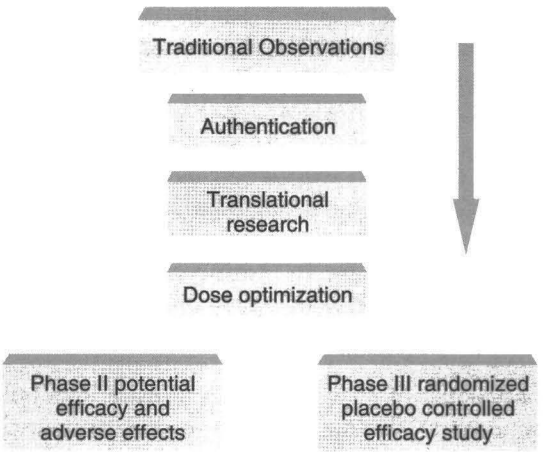

Fig 6. Systematic research of natural health products

be evaluated alone for the prevention of cancer recurrence following definitive treatment. To be suitable for long-term chronic use, these agents should possess minimal toxicity and should be orally administered. However, angiogenesis is also essential for healing of injuries. Most compounds that inhibit tumor angiogenesis are likely to inhibit physiologic angiogenesis, leading to potential side effects, such as ulceration and bleeding. Studies are required to determine distinguishing features of tumor vessels from normal vessels to enable a therapeutic gain to be achieved. During active cancer therapy, they should generally be evaluated in combination with chemotherapy and radiation. In this role, they act as biological response modifiers and adaptogens, potentially enhancing the efficacy of the so-called conventional therapies. The diversity of angiogenic factor expression in different tumors receiving various therapies, combined with the fact that endothelial cells in different tumors are phenotypically distinct, is a major challenge for the development of effective antiangiogenic regimens (Jung *et al.,* 2000; Sweeney *et al.,* 2003). Their effectiveness may be increased when multiple agents are used in optimal combinations. Surrogate markers, such as angiogenic cytokines, are necessary to predict antiangiogenic response. Circulating levels of FGF-2, VEGF, vascular adhesion molecule (V-CAM-1), endothelial intercellular adhesion molecule (ICAM-1), IGF-1, and cytokines such as IL-8 may correlate with tumor angiogenesis (Yoshida *et al.,* 1997; Tang *et al.,* 2001; Brower, 2003; Rüegg, *et al.,* 2003; Ria *et al.,* 2004). In addition, circulating endothelial cells and their progenitors may be a more reliable marker of response to antiangiogenic therapies (Salcedo *et al.*, 2005; Schneider *et al.,* 2005; Shaked *et al.,* 2005). Noninvasive functional imaging, such as positron emission tomography and functional magnetic resonance imaging, may play a role (Neeman, 2000).

Current laboratory evidence suggests a useful role for natural health products in the treatment of cancer. The input of an ethnobotanist, oncologist, laboratory scientist, and a clinical trials methodologist to the research effort is essential to distill the wealth of traditional knowledge into a modern framework that can be evaluated scientifically. The determination of the biologically active dose that may possess least toxicity may be relevant. Combinations of whole herbs or constituent phytochemicals at lower doses are important. In addition, a longer period of exposure to the natural health product may be more effective than a short exposure to the highest possible dose level. New designs for trials to demonstrate activity in human subjects are required. Although controlled trials might be preferred, smaller studies with appropriate end points and surrogate markers for antiangiogenic response could help prioritize agents for the larger resource-intensive phase 3 trials. Because most of the agents are expected to be cytostatic, it is inappropriate to require the standard criteria of measured tumor response. On the other hand, simply confirming stable disease may be misleading. More research on surrogate markers of antiangiogenic response is obviously necessary prior to directing resources to large-scale clinical trials. A multidisciplinary approach by the herbalist and the oncologist is important for implementing and studying natural health products used for the treatment of patients with cancer. We now have a better understanding of their effects at the molecular level. Introducing these interventions into the clinic through appropriate studies will provide more definitive evidence of efficacy and hopefully improve outcome for many cancer patients.

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4

An Anthology of the Studies on the Anti-Inflammatory, Antinociceptive and Febrifuge Effects of Medicinal Plants in Turkey

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ABSTRACT

Inflammatory diseases are among the most common health problems treated with traditional remedies. Therefore it is crucial to evaluate the potential of herbal remedies for the discovery of novel bioactive compounds that might serve as leads for the development of potent drugs. Although several agents are known to treat these types of disorders, prolonged use should be avoided due to severe side effects. Consequently, there is a need to develop safe and new anti-inflammatory agents with minimum side effects. In this context, traditional medicines provide a vast source for the discovery of original drug leads. The present investigation represents a preliminary screening in an ongoing program on plants used in Turkish traditional medicine for the treatment of rheumatism and related inflammatory diseases. In this study, selected plants as the subject of the present study were recorded in the alphabetical order of plant families.

Key words : Antinociceptive, anti-inflammatory, bioassay-guided processing, ethnopharmacology, febrifuge, folk remedies, plants, Turkey

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INTRODUCTION

Acute and chronic inflammations are known to be complicated processes induced by several different classes of chemical mediators, *e.g.* prostaglandins, leukotrienes and platelet-activating factor, etc. Anti-inflammatory agents exert their effect through a spectrum of different modes of action (Samuelsson *et al.*, 1978). However, chronic inflammatory diseases are still one of the major health problems worldwide and non-steroid anti-inflammatory drugs (NSAIDs) are the most prescribed drugs for treatment of inflammatory diseases. Although the NSAIDs provide the patients with symptomatic relief, they do not modify the pathogenesis of inflammation and do not reduce the disabling bone and cartilage damage (Ford-Hutchinson *et al.*, 1981). Therefore, it has become a must to search for new initiatives in the treatment of chronic inflammation. On the other hand, plants have been used by human being since ages in traditional medicine due to their therapeutic potential and the search on medicinal plants have led the discovery of novel drug candidates used against diverse diseases.

The plant kingdom encompasses a wide range of plants that possess biologically active molecules of medicinal value. Many such plant-derived molecules have been isolated, identified, and successfully introduced into international markets by pharmaceutical industries that are mostly located in the developed world. In contrast, plant-based remedies, prescribed by folk doctors in the developing world for the treatment of many diseases, remain largely restricted in their use to the local communities in which such remedies were developed (Sumner, 2000). Although such plant-based remedies have recently received considerable interest from the medical and pharmaceutical industries, only a small fraction of such plants have been assessed. In the past few years, many studies have investigated the healing potential of higher plants with ethnobotanical histories. This stemmed from several reasons such as consumer interest in such plant-based remedies, the concern about the possible side effects of allopathic medicine, the lower cost of phytotherapy, and the fact that many plant-based or herbal remedies succeeded in replacing allopathic medicines in relieving malady symptoms.

The aim of this manuscript is to review the literature of bioactive anti-inflammatory plant extracts and their isolated active components as an addition to the literature typically used in folk medicine in Turkey basin to treat inflammation related ailments.

ANACARDIACEAE

The nuts of *Pistacia vera* L. (Anacardiaceae), commonly referred to pistachio, is a quite popular flavoring foodstuff and snack in Turkey and in the world. The oleoresin of *P. vera*, a widely-distributed tree throughout

the south-east region of Anatolia, has been used to treat asthma by chewing as folk remedy in this part of Turkey as well as against stomachache (by chewing the gum) and hemorrhoids (externally) (Baytop, 1999). In the light of traditional information, the ethanolic and aqueous extracts prepared from different parts (leaf, branch, fruit, stalk) of *Pistacia vera* as well as its oleoresin were evaluated for their *in vivo* anti-inflammatory and antinociceptive activities (Orhan *et al.*, 2006). Only the oleoresin, out of the all extracts screened herein, exhibited a dose-dependent anti-inflammatory activity on carrageenan-induced hind paw edema model in mice ranging between 27.4-34.7% at 250 mg/kg and 32.6-38.7% at 500 mg/kg doses without inducing any gastric damage. The potency of oleoresin at 500 mg/kg dose was almost equal to that observed for indomethacin, a reference agent. As for antinociceptive activity, the oleoresin also displayed 32.1% inhibition at 500 mg/kg dose on *p*-benzoquinone induced abdominal contractions in mice, while inhibitory rate decreased to 21.7% at 250 mg/kg. Fractionation of the oleoresin indicated that *n*-hexane fraction to be active, which further led to recognition of some monoterpenes, mainly α-pinene (77.5%) by capillary gas chromatography-mass spectrometry (GC-MS) as well as the oleoresin itself. α-Pinene was also assessed for its antinociceptive and anti-inflammatory activities in the same manner and exerted a moderate anti-inflammatory effect at 500 mg/kg dose.

APIACEAE

In Turkish folk medicine various species of the *Eryngium* are used for a wide range of ailments; particularly roots are used against various inflammatory disorders, oedema, sinusitis, urinary infections or inflammations etc. and snake or scorpion bites or goiter, roots and leaves for infertility and herbs for wound healing as well as food while fresh (Sezik *et al.*, 1997; Yesilada & Sezik, 2003). *Eryngium* species, particularly *E. campestre* and *E. maritimum* are called in Turkish folk-medicine as "Boğadikeni" and widely distrubuted in all parts of Turkey. Infusion of aerial parts and roots of these species are used in folk remedies as antitussive, diuretic, appetizer, stimulant and aphrodisiac (Baytop, 1999). Aqueous extract of the roots of *E. campestre* is used orally and poultice is applied externally as a remedy aganst scorpion bite in the Balikesir-Turkey (personal interview). Similar utilizations were found in the old documents from the medieval ages such as against wound and burns, pains, hemorrhoids and sexual diseases (Lev, 2002). Effects of the roots from different *Eryngium* species against various inflammatory conditions are also well-known in the traditional medicines worldwide; for example, *E. campestre* as anti-oedema (Leporatti & Ivancheva, 2003) or *E. creticum* for inflammatory wounds (Ali-Shtayeh *et al.*, 1998). It is also documented in the popular phytotherapy books for such treatments, *i.e. E. campestre* is recommended against kidney and urinary tract inflammations and oedema (Gruenwald *et al.* 2000a). To

confirm the traditional usage ethanolic and aqueous extracts obtained from either aerial parts or roots of *E. campestre, E. creticum, E. davisii, E. falcatum, E. isairicum, E. kotschyi, E. maritimum,* and *E. trisectum* were evaluated for their *in vivo* anti-inflammatory and antinociceptive activities (Küpeli *et al.*, 2006a). According to the results of investigations, except *E. falcatum* extracts, ethanol extracts either from the aerial parts or roots of *Eryngium* species showed apparent anti-inflammatory activity on carrageenan-induced hind paw oedema and TPA-induced ear oedema tests and antinociceptive activity on p-benzoquinone-induced writhing test. In spite of potent activity of the ethanol extract from *E. isairicum* aerial parts was induced gastric damage. Aerial parts and roots of *E. maritimum* and *E. kotschyii* were found to possess most promising activities without including any apparent gastric damage. Moreover, Yesilada *et al.* (1989) reported that the methanol extract (150 mg/kg) from the whole plant of *E. billardieri* showed a 44% inhibition on increased vascular permeability induced by acetic acid in mice on oral administration. Further studies were conducted on the various solvent fractions obtained from the aerial parts and roots of the plants and they were tested for their effects on increased vascular permeability induced by acetic acid model, as well as serotonin, bradykinin, and carrageenan-induced paw oedemas in mice. A significant anti-inflammatory activity was observed only in the precipitated part of the n-butanol extract of the roots, which mainly contains saponins (Yesilada *et al.*, 1989).

Seseli is an old Greek name that was called by Hippocrates for certain members of the Apiaceae family (Hamlyn, 1969). The genus *Seseli* L., is composed of aromatic herbs and economically important species that are used as foods, spices, condiments and ornamentals (Lawrence, 1969; Crowden *et al.*, 1969; Pimenov & Leonov, 1993). Several *Seseli* species are reported in ancient literature for various healing effects. The roots of *S. mairei* Wolff., have been used against inflammations, swelling, rheumatism, pain and common cold in Chinese traditional medicine (Hu *et al.*, 1990). The seeds of an Indian species, *S. indicum*, have been reported to possess anthelmintic, carminative, stomachic and stimulant properties (Tandan *et al.*, 1990). In Turkish folk medicine, the fruit of *S. tortuosum* is used as emmenagogue and anti-flatulence (Baytop, 1999), while the leaves of *S. libanotis* (Kelemkesir or Kelemenkesir in Turkish) are consumed as a vegetable in the eastern Turkey (Baytop, 1999). Traditional usage of the genus in folk medicine led us to search other species for their biological activies. The ethyl acetate and methanol (80%) extracts obtained from ten *Seseli* L. species (Apiaceae) growing in Turkey, *Seseli andronakii* Woron., *S. campestre* Besser, *S. gummiferum* Pall. ex Sm. subsp. *corymbosum* (Boiss & Heldr) P. H. Davis, *S. gummiferum* Pall. ex Sm. subsp. *gummiferum*, *S. hartvigii* Parolly & Nordt, *S. libanotis* (L.) W. Koch, *S. petraeum* M. Bieb., *S. peucedanoides* (Bieb.) Koso-Pol., *S. resinosum* Freyn & Sint., *S. tortuosum*

L. were evaluated for their *in vivo* anti-inflammatory and antinociceptive activities (Küpeli *et al.*, 2006c). Among the plant extracts, the ethyl acetate extracts from *S. andronakii, S. campestre, S. gummiferum* subsp. *corymbosum, S. petraeum, S. resinosum* and *S. tortuosum* showed 30.1%, 32.3%, 36.9%, 39.8%, 35.1%, 37.6% inhibition in *p*-benzoquinone-induced abdominal constriction test, respectively. The ethyl acetate extracts of *S. gummiferum* subsp. *corymbosum, S. petraeum, S. resinosum* and *S. tortuosum* also exhibited notable inhibition, ranging between 24.5-29.7%, 28.1-33.3%, 17.4-27.5%, 27.9-31.3%, respectively, in carrageenan-induced hind paw edema model at 100 mg/kg dose without inducing any gastric damage. During the acute toxicity evaluation, neither death nor gastric bleeding was observed for any of the plant extracts. Results have supported the traditional use of some *Seseli* species against inflammatory disorders.

APOCYNACEAE

Nerium oleander L., oleander, is widely distributed in the Mediterranean region. Due to the toxic nature, limited number of utilization have been recorded in Turkish folk medicine. Among them a speculative assertion on cancer treatment by a Turkish medical doctor became very popular and eventuated recently the leaf extract undergoing Phase I clinical evaluation as a potential treatment against cancer in USA (Smith *et al.*, 2001). During our expeditions, an informant described that fresh oleander flowers was put in alcohol in summer and kept as a home-remedy to alleviate her severe pain and paralysis in her legs. In a reference survey in the database program of Turkish folk medicine (TUHIB), a similar utilization was also encountered by another informant, *i.e.* flowers kept 40 days in olive oil was applied on joint against rheumatic pain (Yesilada, 2002). Moreover, leaves or flowers are used to stop pain or eczema, sap obtained from fresh leaves for abscess or rheumatism were recorded. Besides, another part of oleander, the fruits, is reported as antirheumatic and as a remedy for skin diseases in Saudi folk medicine (Adam *et al.*, 2001). In this context, Zia *et al.* (1995) reported that two purified fractions (B_1 and B_3) obtained from the methanol extract of fresh oleander leaves possess a CNS depressant activity, *i.e.* produced reduction in locomotor activity, rotarod performance and potentiate hexobarbital induced sleeping time. Moreover, they showed significant analgesic activity as indicated by inhibitory effects on acetic acid-induced writhings and increased reaction time to thermal test. Our results also supported that both aqueous and ethanol extracts of oleander leaves possess significant antinociceptive activity as decided by using another writhing test model, but the effect of EtOH extract was more pronounced (Erdemoglu *et al.*, 2003). However, both extracts were shown gastric ulcerogenicity in mice. On the other hand, EtOH extracts from either dried or fresh flowers exhibited potent antinociceptive activity without inducing any gastric damage and potency was more pronounced than that of reference

drug aspirin. Experimental results have clearly confirmed that liposoluble fractions of flowers, as decided by ethanol or olive oil extracts were used in folk medicine, possess remarkable antinociceptive activity. Anti-inflammatory activity assessment experiment has also verified the conclusion that liposoluble components of flowers may have the activity since only the EtOH extracts were found active even than that of reference drug indomethacin.

ASTERACEAE

The genus *Achillea*, named after the mythological Greek warrior Achilles, comprises of approximately 85 species, most of which are endemic to Europe and the Middle East. In the Turkish flora 40 *Achillea* species have been recorded and 20 of them are endemic (Davis, 1982). On the otherhand, seveal *Achillea* species have been known to be used in folk remedies for various purposes such as haemorrhoid and wound healing (Baytop, 1999). Especially, *A. millefolium* is frequently used against diarrhoea, abdominal pain and stomachache in Turkish traditional medicine (Yesilada *et al.*, 1993; Fujita *et al.*, 1995; Honda *et al.*, 1996). In order to evaluate their folkloric utilization, both antinociceptive and anti-inflammatory activities of five *Achillea* species including *Achillea wilhelmsii* C. Koch, *A. setacea* Waldst & Kit, *A. vermicularis* Trin., *A. phrygia* Boiss. & Bal. and *A. sipikorensis* Hausskn. et Bornm. were investigated (Küpeli *et al.*, 2007a). The ethanol extracts of *A. wilhelmsii, A. setacea* and *A. vermicularis* showed significant antinociceptive (p-benzoquinone-induced writhing test) and anti-inflammatory (carrageenan-induced hind paw edema model) activity at 500 mg/kg dose, per os, without inducing any apparent acute toxicity as well as gastric damage. *A. phrygia* was shown to possess only significant antinociceptive activity, while *A. sipikorensis* did not show any remarkable anti-inflammatory and antinociceptive activity.

Furthermore, the essential oils obtained by water distillation from aerial parts of *Achillea schischkinii* Sosn. and *Achillea aleppica* DC. subsp. *aleppica* were analysed by GC and GC/MS. 1,8-Cineole (32.5% and 26.1%, respectively) was the main component in both oils. Their antimicrobial, anti-inflammatory and antinociceptive activities were also studied and the oil of *A. aleppica* subsp. *aleppica* showed significant anti-inflammatory, antinociceptive and moderate antimicrobial activities. The oil of *Achillea aleppica* subsp. *aleppica* contains 6.6% of α-bisabolol and its derivatives. These compounds might be at least partially responsible for anti-inflammatory activity of the oil (Iscan *et al.*, 2006).

In folk medicine, *Helichrysum* species have been used for gall bladder disorders as medicinal tea, because of their bile regulatory and diuretic effects (Süzgeç *et al.*, 2005). Some of them are used for anti-inflammatory and anti-allergic properties (Carini *et al.*, 2001; Tepe *et al.*, 2005). Moreover,

this genus is traditionally used in the treatment of wounds, infections and respiratory conditions (Lourens *et al.*, 2004). In Turkey, several *Helichrysum* species are used in folk medicine as diuretic, cholagogue, and against kidney stones (Baytop, 1999). In order to evaluate the anti-inflammatory and antinociceptive activities of *Helichrysum* species (Küpeli *et al.*, 2006b), aqueous extracts from *H. arenarium* (L.) Moench. subsp. *aucheri* (Boiss.) Davis & Kupicha and *H. plicatum* DC. subsp. *plicatum* were investigated and they showed 37.0%, 30.4% inhibition at 100 mg/kg doses and 31.2%, 28.3% at 200 mg/kg doses in p-benzoquinone-induced abdominal constriction test, respectively, without inducing any gastric damage. The aqueous extracts of all the plants did not show any anti-inflammatory activity in carrageenan-induced hind paw edema model at 50, 100 and 200 mg/kg doses. During the acute toxicity evaluation, neither death nor gastric bleeding was observed for any of the plant extracts.

BERBERIDACEAE

Roots and barks of various *Berberis* species are used as folk remedy for the treatment of various inflammatory diseases such as lumbago, rheumatism and to reduce fever. In Semerkand Bazaar (Uzbekistan) a black tough material prescribed for the treatment of lumbago attracted our attention during our scientific expeditions. The herb dealer described that it was the concentrated aqueous extract of *Berberis oblonga* roots. In a reference survey, several other *Berberis* species were also found to be used in the treatment of various inflammatory conditions, including rheumatism, fever and pyrexia. In order to evaluate this information, the effect of a widespread Turkish species, *B. crataegina* DC. was studied and found that the roots of the plant possessed significant anti-inflammatory, analgesic and febrifuge effects. Through bioassay-guided fractionation and isolation procedures berberine was isolated as the main active ingredient responsible from the relevant effects. In that paper, however, it was addressed that the role of other alkaloids in the effect of the roots should also be investigated (Yesilada & Küpeli, 2002). In the study of Kosar *et al.* (1999) three types of isoquinoline alkaloids were detected in the roots, barks and branches of Turkish *Berberis* species: protoberberine (berberine, palmatine, jatrorrhizine, columbamine), bisbenzylisoquinoline (berbamine, oxyacanthine, aromoline) and aporphine (magnoflorine) types. In a following study, the effects of these alkaloids were studied using various *in vivo* models in mice (Küpeli *et al.*, 2002). All alkaloids inhibited inflammations in varying degrees, among them berberine, berbamine and palmatine were shown to possess significant and dose-dependent inhibitory activity against serotonin-induced hind paw oedema both on oral and topical applications and acetic acid-induced increase in vascular permeability on oral administration. Moreover, these three alkaloids were also shown to possess dose-dependent antinociceptive activity,

which assessed by using the model based on the inhibition of p-benzoquinone-induced writhing movements as well as antipyretic activity on FCA-induced increased rectal temperature on subacute administration. However, all alkaloids induced gastric lesions in varying degrees (Küpeli *et al.*, 2002). Since it is well known that anti-inflammatory agents acting through inhibition of cyclooxygenase-1 (COX-1) enzyme induce such gastric ulceration, the effect of isoquinoline alkaloids might also be based on this mechanism. As a matter of fact, berberine was found to inhibit castor oil-induced diarrhoea in rats, which supported this conclusion (Yeşilada & Küpeli, 2002). Fukuda *et al.* (1999) demonstrated that berberine inhibits cyclooxygenase-2 (COX-2) transcriptional activity in colon cancer cells in a dose-dependent manner, however, the effect on constitutive enzyme (COX-1) was not determined. It is noteworthy that powdered roots of *B. lycium* is suggested to be taken with milk for the treatment of rheumatism and muscular pains in Pakistan folk medicine, probably to protect the gastric mucosa from damage (Ikram *et al.*, 1966). The results of the previous study (Küpeli *et al.*, 2002) revealed that berberine, berbamine and palmatine were the main anti-inflammatory, antinociceptive and antipyretic alkaloids in the roots of *Berberis* species. Especially the effect of first two was more pronounced. Since alkaloid composition of the roots showed great variations from species to species, the relevant activity would highly be species-specific. Thus, utilization of *B. integerrima* roots in therapy would not be beneficial due to the higher concentrations of the alkaloids with weak activity, *i.e.* magnoflorine and oxyacanthine. On the other hand, due to the higher concentration of effective alkaloids in *B. cretica* and *B. vulgaris* roots, more beneficial effects would be expected. Berberine and related isoquinoline alkaloids have been reported to exhibit a broad distribution in the plant kingdom (Keys, 1985), such as; *Epimedium* sp. leaves (Berberidaceae); *Phellodendron* sp. barks and *Xanthophyllum* sp. roots (Rutaceae); *Coptis* sp. rhizomes and *Hydrastis* sp. roots (Ranunculaceae); *Sanguinaria* sp. rhizomes (Papaveraceae); etc. In traditional medicine, some of these plants are known to be employed in the treatment of various inflammatory ailments and results of the present study would also be helpful to explain the relevant effects as well as safety evaluation of these plant remedies especially regarding to the ulcerogenic effect of these alkaloids reported in that study (Küpeli *et al.*, 2002). Contrary to the observations of the present study that berberine type alkaloids induce remarkable gastric lesions, berberine was reported to be effective in the treatment of gastritis and gastric ulcers in some documents (PDR, 2000), which needs further studies and explanations.

BRASSICACEAE

In Turkish folk medicine, *Isatis* sp. is known to be used for wound healing and against constipation (Baytop, 1999). In Iran, another species of the

plant, *I. cappadocia*, has also been reported against rheumatism, asthma, eczema, fever, headache and wound healing. Rezaeipoor *et al.* (2000) showed that the plant possess *in vivo* suppressive effect on humoral primary immune response at the dose of 250 mg/kg, while stimulated secondary immune responses at the dose of 500 mg/kg. Additionally, the activity of a Turkish species, *Isatis glauca* Aucherx Boiss. ssp. *glauca*, was studied and ethanol extract demonstrated a significant antinociceptive activity (37.2%) at 500 mg/kg, and a medium anti-inflammatory activity (24-28% inhibition) (Kupeli *et al.*, 2007c).

CISTACEAE

In Turkish folk medicine, the leaves of the *Cistus laurifolius* are also used externally as an effective remedy against several inflammatory ailments such as rheumatic pain, high fever and urinary inflammations. For relieving rheumatic pain a warm decoction of leaves is used as a bath or wilted leaves are externally applied on joints. For alleviation or treating of urinary inflammations, a poultice is prepared by boiling the leaves and then mixing with flour. This poultice is then applied externally as a plaster on the dorsal part of the body in a line with the kidneys (Sezik *et al.*, 1991; Yesilada *et al.*, 1995). Effects of the extracts and fractions from the leaves with non-woody branches of *Cistus laurifolius* L. (Cistaceae) were studied using two *in vivo* models of inflammation in mice. For the preliminary anti-inflammatory activity assessment of *C. laurifolius* leaves two types of extracts (aqueous and ethanolic) were prepared and investigated against carrageenan-induced hind paw oedema and acetic acid-induced increased vascular permeability models in mice. Aqueous extract did not show any remarkable effect against carrageenan-induced hind paw edema model, while EtOH extract was significantly active both in 250 and 500 mg/kg doses. A comparable result was obtained against another inflammatory model based on the inhibition of increased vascular permeability, induced by the intraperitoneally injection of acetic acid. Subsequently, through bioassay-guided fractionation and isolation procedures three flavonoids; 3-*O*-methylquercetin, 3,7-*O*-dimethylquercetin and 3,7-*O*-dimethylkaempferol were isolated as the main active ingredients from the ethanol extract (Fig 1). Moreover, these flavonoids were shown to possess potent antinociceptive activity, which was assessed through inhibition of p-benzoquinone-induced writhing reflex. Results of the present study have clearly supported the utilization of *C. laurifolius* in Turkish traditional medicine and three flavonoids were shown to have strong antinociceptive and anti-inflammatory activities, per os without inducing any apparent acute toxicity as well as gastric damage (Küpeli & Yesilada, 2007).

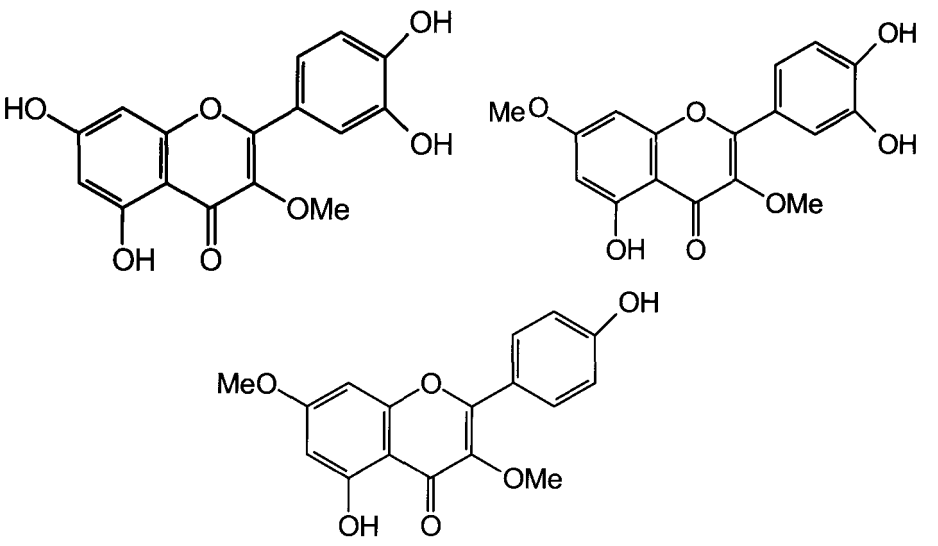


Fig 1. Chemical structures of active compounds

DIOSCOREACEAE

Tamus communis root is used to treat rheumatism after pounded in Turkish folk medicine (Yesilada *et al.*, 1995). In a previous report, the ethanol extract from *Tamus communis* roots was shown to possess significant inhibitory effect on granuloma formation when tested on cotton pellet granuloma model in rats (Capasso *et al.*, 1983; Mascolo *et al.*, 1987). However, in our study neither aqueous nor ethanol extracts prepared from both roots and aerial parts of that plant did show any remarkable anti-inflammatory or antinociceptive activity (Kupeli *et al.*, 2007c).

ERICACEAE

The sap obtained from a fresh branch of *Rhododendron ponticum* L. is dropped into the toothcave against toothache in Turkish folk medicine (Yesilada *et al.*, 1999). The flowers and fruits of another *Rhododendron* species, *R. molle*, have also been recorded in ancient and modern monographs as analgesic and insecticide in Chinese Traditional Medicine (Li *et al.*, 2000). Moreover, the leaves of *R. chrysanthum* in Siberia (Grieve, 1994) and *R. ferrugineum* in Germany (Chosson *et al.*, 1998) have been reported as a remedy against rheumatism. In a reference survey, we did not find any experimental study related to this subject, however, Manjeet and Ghosh (1999) reported that quercetin isolated as a major component of *R. cinnabarium* inhibited LPS-induced nitric oxide and tumor necrosis factor-production in murine macrophages, which might be related with the aforementioned effects. *In vivo* studies also confirmed that leaves of the

plant possess a significant antinociceptive activity. The EtOH extract showed the highest antinociceptive activity among the plants investigated as well as a potent anti-inflammatory activity without inducing any gastric lesion was recorded (Erdemoglu *et al.*, 2003).

The genus *Erica* is represented only four species of which one taxon (*E. bocquetti* (Peşmen) P. F. Stevens) is endemic in Turkey. Among the other species, *E. arborea* L. and *E. manipuliflora* Salisb. are widespread species common in the coastal sides in Turkey, while *E. sicula* Guss. subsp. *libanotica* (C. & W. Barbey) P. F. Stevens has a limited distribution in South West Anatolia (Stevens, 1978). Herbal tea prepared from aerials parts of *E. arborea* or *E. manipuliflora* has been popularly used as diuretic, astringent and treatment of urinary infections in Turkey (Baytop, 1999; Tuzlaci & Eryaşar Aymaz, 2001). In particular, a glass of 5% infusion or decoction of *E. arborea* leaves is suggested after meals to discharge the edema from the body in slimming formulations (Başer *et al.*, 1986; Antonone *et al.*, 1988). In the light of traditional usage anti-inflammatory and antinociceptive activities of different extracts were prepared with methanol, chloroform, ethyl acetate, *n*-butanol and water from the aerial parts of *E. arborea* L., *E. manipuliflora* Salisb., *E. bocquetii* (Pesmen) P. F. Stevens and *E. sicula* Guss. subsp. *libanotica* (C. & W. Barbey) P. F. Stevens (Ericaceae) of Turkish origin were investigated by using *in vivo* methods (Küpeli Akkol *et al.*, 2008c). The ethyl acetate extracts of *E. arborea* (EAE), *E. bocquetii* (EBE) and *E. manipuliflora* (EME) exhibited notable inhibition against carrageenan-induced (24.1-32.3%, 23.8-36.1%, 29.2-35.1%, respectively) and PGE_2-induced (21.2-37.7%, 6.8-29.7%, and 6.2-34.1%, respectively) hind paw edema as well as TPA-induced mouse ear edema models in mice, while the ethyl acetate extract of *E. sicula* subsp. *libanotica* (ESE) (10.7-29.7%) displayed potent anti-inflammatory activity only on the PGE_2-induced hind paw edema model. However, the remaining extracts were found to be inactive against inflammatory models. Same extracts *i.e.*, EAE, EBE and EME were also found to exhibit remarkable antinociceptive activity in *p*-benzoquinone-induced abdominal constriction test at a dose of 100 mg/kg (46.5%, 27.7% and 36.3%, respectively) (Küpeli Akkol *et al.*, 2008c). The anti-inflammatory and antinociceptive activities were mainly determined in the ethyl acetate extracts. Previous studies have shown that flavonoids, anthocyanoids, coumarins, terpenic compounds especially ursolic acid and essential oils were present in *Erica* species (Vieitez & Ballester, 1972; Ballester *et al.*, 1975; Bennini *et al.*, 1993; Chulia *et al.*, 1995; Dufor *et al.*, 2007). Flavonoids exert significant scavenging properties on oxygen radicals and can interfere with the production of arachidonic acid metabolites trough lipoxygenase enzyme inhibition and reduce the concentration of leukotrienes (Reyes Ruiz *et al.*, 1996; Halliwell & Chirica 1993).

Calluna vulgaris L. (heather), which is also known as "the true heather of Europe", is the sole member of the genus *Calluna* (Ericaceae) in Turkey (Baytop, 1999). Heather has a long history of medicinal use in folk medicine. In Anatolia, infusion of the plant has been used as urinary disinfectant and diuretic (Baytop, 1999). Different ethnobotanical uses of the plant have been documented as antiseptic, astringent, cholagogue, depurative, diaphoretic, diuretic, expectorant, mildly sedative, vasoconstrictor and antirheumatic as well as for the treatment of gout symptoms in European societies (Mabey, 1979; Chiej, 1984; Kumarasamy *et al.*, 2002). A homeopathic remedy is prepared from the fresh branches is prescribed in the treatment of rheumatism, arthritis and insomnia (Launert, 1981). The plant is also suggested among the Bach flower remedies and the keywords for prescribing it are "self-centeredness" and "self-concern" (Chancellor, 1985). In order to evaluate this ethnobotanical information, its anti-inflammatory and antinociceptive activities were studied using *in vivo* experimental models in mice (Orhan *et al.*, 2007b). The ethanolic extract of the plant was first fractionated into five extracts; namely, *n*-hexane, chloroform, ethyl acetate (EtOAc), *n*-butanol, and water fractions. Among them, the EtOAc fraction was found to be the most effective and was further subjected to bioassay-guided fractionation and isolation procedures. After successive column chromatography applications, on Sephadex LH-20 and silica gel, a component, which is responsible for the above-mentioned activities of this species of Turkish origin, was isolated and its structure was elucidated as kaempferol-3-*O*-β-*D*-galactoside, a common flavonol derivative by means of spectral techniques (Fig 2) (Orhan *et al.*, 2007b). A reference survey has revealed that *in vitro* anti-inflammatory activity of *C. vulgaris* was previously

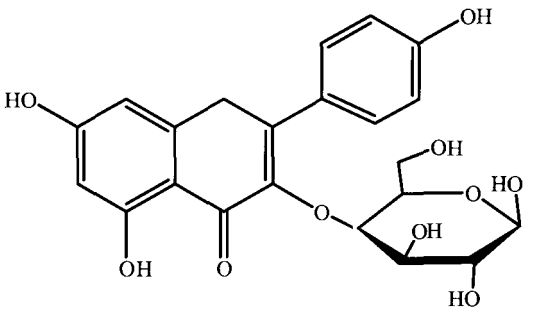


Fig 2. Chemical structure of kaempferol-3-O-β-D-galactoside

reported by two separate studies. In the first study, the water extracts of 52 plants from 28 different families, where selections were based upon the records for their use in inflammatory diseases and treatment of wounds in Swedish traditional medicine, were investigated for their inhibitory activities on prostaglandin biosynthesis and platelet activating factor (PAF)-induced exocytosis *in vitro* and was found highly efficient on both assays (Tunon *et*

al., 1995). In particular, *C. vulgaris* was one of the three plants, which exhibited the most potent cyclooxygenase inhibition in that study. In connection to these data, the flowers of *C. vulgaris*, was tested *in vitro* for its effect on arachidonic acid metabolism using mouse peritoneal macrophages, human platelets, and differentiated HL60 leukemic cells (Najid *et al.*, 1992). Among the several organic and aqueous extracts of heather flowers, the acetone extract was found to inhibit lipoxygenase (LOX) enzyme and further analysis of this extract led to the isolation of ursolic acid as an active anti-lipoxygenase component. Ursolic acid was shown a higher efficiency on 5-LOX inhibition than 12- and 15-LOX, whereas it had a 38% inhibition on cyclooxygenase.

Many flavonoids, isolated from medicinal herbs used in traditional medicines, are endowed with desired biological activities such as antimicrobial, anti-hepatotoxic, anti-inflammatory, cardioprotective, anti-osteoporotic, and anti-cancer (Middleton, 1998). Amongst them, quercetin and kaempferol are known to be the most common flavonols present in many plants in different glycosidic forms. In one study, two coumaryl glycosides of kaempferol (platanoside and tiliroside) were reported to exhibit strong antiproliferative effect suppressing DNA synthesis in a dose- and time-dependant manner (Dimas *et al.*, 2000). In a very good accordance with the above data (Orhan *et al.*, 2007b), Gil *et al.* (1994) also figured out that kaempferol-3-*O*-β-*D*-galactoside possessed a remarkable anti-inflammatory activity on both *in vivo* carrageenan-induced hind paw edema and 12-*O*-tetradecanoylphorbol-3-acetate-induced ear edema as well as *in vitro* inhibitory effect on phospholipase-A_2 enzyme. In some similar studies, various kaempferol glycosides have also shown to possess significant inhibitory activities on TNF-α production and nitric oxide (NOS) release (Liang *et al.*, 1999; Authore *et al.*, 2001; Matsuda *et al.*, 2001).

GENTIANACEAE

Isoorientin is a common C-glycosyl flavone, luteolin-6-C glucoside, and has been reported in many different plant species such as *Gentiana*, *Asphodelus*, *Rumex, Swertia* and *Vitex* species (Harborne, 1994). During the studies on a Turkish folk medicine, *Gentiana olivieri* Griseb. (Gentianaceae), isoorientin was isolated as the active anti-hepatotoxic component from the flowering herbs through *in vivo* bioassay-guided fractionation procedures against carbon tetrachloride-induced hepatic damage (Deliorman-Orhan *et al.*, 2003). In a following bioassay-guided activity assessment study on the same plant material same compound was again isolated as the active hypoglycaemic component (Sezik *et al.*, 2004). In a reference survey, several other biological activities were attributed to isoorientin including potent antioxidant (Ko *et al.*, 1998), antimicrobial (Afifi *et al.*, 1997), myolitic activity on smooth muscle-containing preparations from the rat and guinea pig uterus (Afifi *et al.*, 1999), etc. Isoorientin (Fig 3) was also shown to possess significant

antinociceptive and anti-inflammatory activities without inducing any apparent acute toxicity as well as gastric damage. Isoorientin induced inhibition between 28.4-37.1% and 36.4-43.4% against carrageenan-induced inflammation at 15 and 30 mg/kg doses, respectively. The most remarkable point was the anti-inflammatory activity of isoorientin was found almost equal to that of indomethacin (10 mg/kg) which exerted 30.4-40.3% activity, in a very close dose level. Maybe more important point was isoorientin did not induce any visible gastric damage. Due to the high gastric lesion risk of nonsteroidal anti-inflammatory agents, *i.e.* indomethacin and acetyl salicylic acid, this is a very important peculiarity. On the other hand, isoorientin was also found to possess significant antinociceptive activity, though not potent as acetyl salicylic acid (Küpeli *et al.*, 2004). Literature survey demonstrated that isoorientin possesses a wide range of biological activities. Due to the adjacent hydroxyl groups at ring B, *i.e.* catechol functions, the most prominent biological activity reported for isoorientin is a potent antioxidant activity (Diena *et al.*, 2003). Isoorientin showed a strong anti-peroxidative activity towards linoleic acid (Mun'im *et al.*, 2003). The antioxidant activity of isoorientin was also reported by Budzianowski *et al.* (1991) through evaluating the inhibitory effect on MDA concentration stimulated by Fe^{+3} ions and ascorbic acid in rat liver microsomes. Ko *et al.* (1998) studied the antioxidant activity of isoorientin-6"-O-glucoside, a water-soluble form, from *Gentiana arisanensis* (Gentianaceae) using various models and reported a potent antioxidant and radical scavenging activity and suggested as an antioxidant therapy against a wide range of free radical-induced disorders particularly to protect human LDL from oxidative attack. Isoorientin was isolated as one of the active anxiolytic ingredients from a Peruvian folk remedy, *Jatropha cilliata* M. Arg. (Euphorbiaceae) at 40 and 100 mg/kg doses and the activity at the later dose was identical to that of diazepam. Under the same conditions, a similar effect was observed for orientin obtained from another fraction, however luteoline, aglycone of these two flavonoids, was ineffective. In the same work, analgesic effect was also investigated and isoorientin was found to have more potent effect than orientin (Okuyama *et al.*, 1996).

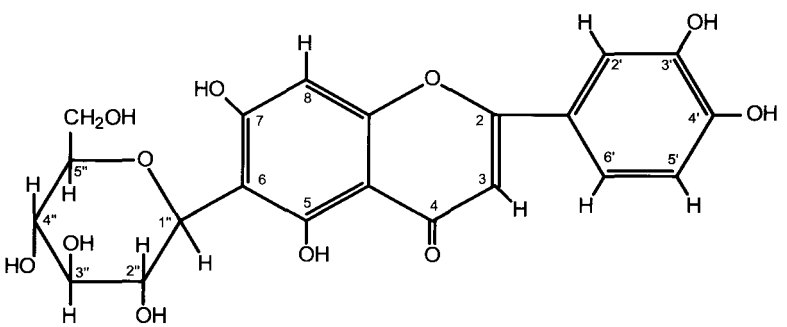
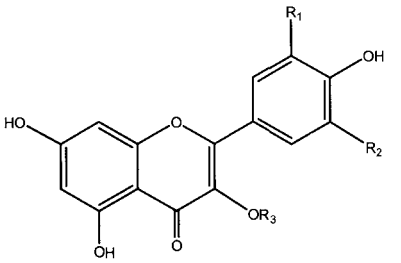


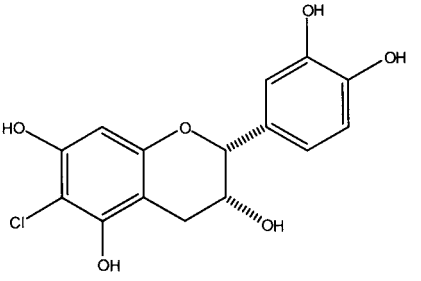
Fig 3. Structure of isoorientin

GERANIACEAE

Geranium species are used as antiasthmatic, antiallergic, antioxidant, antidiarrhoeic, antihepatotoxic, diuretic, tonic, haemostatic, stomachic and antidiabetic in folk medicine (Baytop, 1999). *G. pratense* ssp. *finitimum* is particularly used to treat stomachache in in Turkey (Özaydin *et al.*, 2006). As a relevant activity, *G. maculatum* has been reported to treat duodenal ulcers as well as in diarrhoea and hemorrhoids in Canada (British Herbal Pharmacopeia, 1987). The leaves of *G. thunbergii* have been used as an antidiarrhoeic in Japan (Fujiki *et al.*, 2003) and *G. sanguineum* was reported to possess a potent effect against influenza virus (Sokmen *et al.*, 2005). To obtain an experimental evidence on the therapeutic efficacy of *Geranium* species and its phenolic compounds for inflammatory diseases, the aqueous extract of aerial parts of *G. pratense* subsp. *finitimum* (Woronow) Knuth, its fractions and isolated compounds, *i.e.* the mixture of quercetin 3-*O*-α-



	R_1	R_2	R_3
1	OH	H	α-arabinopyranose
	H	H	β-galactopyranose
2	OH	H	β-glucopyranose
	OH	H	β-galactopyranose
3	H	H	β-glucopyranose
5	OH	H	(2″-*O*-galloyl)-β-glucopyranose
	OH	H	(2″-*O*-galloyl)-β-galactopyranose
6	OH	OH	(2″-*O*-galloyl)-β-glucopyranose



4 (-)-6-chloroepicatechin

Fig 4. The chemical structures of the isolated compounds from *Geranium pratense* subsp. *finitimum*

arabinopyranoside, kaempferol 3-*O*-β-galactopyranoside (**1**), the mixture of quercetin 3-*O*-β-glucopyranoside, quercetin 3-*O*-β-galactopyranoside (**2**), kaempferol 3-*O*-β-glucopyranoside (**3**), (-)-6-chloroepicatechin (**4**), the mixture of quercetin 3-*O*-(2-*O*-galloyl)-β-glucopyranoside, quercetin 3-*O*-(2-*O*-galloyl)-β-galactopyranoside (**5**) and myricetin 3-*O*-(2-*O*-galloyl)-β-glucopyranoside (**6**) (Fig 4) were examined on carrageenan-, PGE_2- and TPA-induced inflammation in mice and *p*-benzoquinone induced writhing reflex to assess anti-inflammatory and antinociceptive activities. The effective dose of materials for the inhibition of carragenan-induced hind paw edema assay was determined to be 100 mg/kg, which was also used in the assays with the extract, its fractions and isolated compounds in all other experiments. The aqueous extract as well as compounds **1** and **2** (100 mg/kg) significantly inhibited the formation of the carrageenan-induced hind paw edema. Significant reductions were also exerted by the aqueous extract and the compounds **1**, **2** and **5** against PGE_2-induced hind paw edema and TPA-induced ear edema models. The same compounds were shown to possess antinociceptive activity (Küpeli *et al.*, 2007d).

JUGLANDACEAE

Juglans regia L. leaves have been used mostly in worldwide traditional medicines as antifungal, antihelmintic, astringent, keratolytic, antidiarrhoeal, hypoglycaemic, depurative, tonic and for the treatment of sinusitis, cold and stomachache (Girzu *et al.*, 1998; Mouhajir *et al.*, 2001). In Turkish folk medicine, however, leaves are frequently used to reduce fever in sunstroke or to alleviate rheumatic pain, externally. Fresh leaves are spread to form a bed for unclothed patient, who is covered with fresh leaves or pounded with/without raw salt and applied on the naked body or forehead to reduce fever or on swelled joint to alleviate the rheumatic pain, or boiled in water and used for bathing in both conditions (Fujita *et al.*, 1995; Yeşilada, 2002). Both aqueous and EtOH extracts showed significant antinociceptive activity without inducing any gastric damage, but the effect of later extract was more pronounced than the former as well as that of aspirin (Erdemoglu *et al.*, 2003). EtOH extract also showed a potent anti-inflammatory activity as potent as indomethacin. Since direct application of fresh leaf is suggested for the treatment, less polar components of the leaves might possibly be more active (Erdemoglu *et al.*, 2003).

LAMIACEAE

Prunella vulgaris L. is used against rheumatism, colds and cardiac disorders in Turkish folk medicine (Yesilada *et al.*, 1993). In a previous study, a herbal combination (SKI 306X) consisting of *Clematis mandshurica*, *Trichosanthes kirilowii* and *Prunella vulgaris*, which was used in Chinese traditional medicine for the treatment of various inflammatory disorders, *i.e.* lymphadenitis and arthritis, was studied for their *in vitro* effects as anti-inflammatory, analgesic,

antiarthritic, blood microcirculation-enhancer as well as inhibition of cartilage degeneration enzyme activities on proteoglycan degradation in cartilage explant culture and collagenase-induced rabbit ostheoarthritis model (Choi *et al.*, 2002). The results revealed that SKI 306X inhibited proteoglycan degradation in a concentration-dependent manner and it also showed protective effect on the knee joint of rabbit from ostheoarthritis-like changes on prophylactic administration. In another study, the aqueous extract of *P. vulgaris* was shown to possess strong antioxidant potency in inhibiting rat erythrocyte hemolysis and lipid peroxidation in rat kidney and brain homogenates (Liu & Ng, 2000). On the other hand, in a study was performed by our group, *in vivo* anti-inflammatory or antinociceptive activity of the plant was found to be weak (Kupeli *et al.*, 2007).

The genus *Lamium* L. (Lamiaceae) is characterized by almost 40 annual or perennial herbaceous plants spread throughout Europe, Asia, and Africa (Willis, 1973). *Lamium album*, *L. maculatum,* and *L. purpureum* exhibit antispasmodic, anti-inflammatory, antioxidant and antiproliferative properties and have been extensively used in folk medicines as a remedy for the treatment of several disorders such as trauma, fractures, putrescence, paralysis, leucorrhoea, hypertension, as well as women afflictions, such as menorrhagia, uterine hemorrhage, vaginal or cervical inflammations (Bremness, 1995; Matkowski & Piotrowska, 2006; Paduch *et al.*, 2006; Shuya *et al.*, 2003; Weiss, 1988). In Turkish flora, thirty *Lamium* species have been recorded (Duman, 2000a; Mill, 1982). Among them, *L. album*, *L. maculatum*, and *L. purpureum* have been reported to be used as tonics and for the treatment of constipation in Anatolia (Baytop, 1999). In western Anatolia (in particular in Manisa) whole plant of *L. album* and several other *Lamium* species are used to relieve pain in rheumatism and other arthritic ailments (Ozaydin *et al.*, 2006). In order to assess the traditional usage various extracts were prepared from several *Lamium* species. The *n*-butanol extracts of *L. garganicum* subsp. *laevigatum* (LGL-BuOH), *L. garganicum* subsp. *pulchrum* (LGP-BuOH), and *L. purpureum* var. *purpureum* (LPP-BuOH) exhibited notable inhibition (16.5-28.9%, 14.5-26.9%, 12.3-21.5%, resp.) in carrageenan-induced hind paw edema model at doses of 200 mg/kg without inducing any gastric damage. The LGL-BuOH (7.1-30.4%) and LGP-BuOH (5.9-24.1%) extracts also displayed potent anti-inflammatory activity against PGE_2-induced hind paw edema model. However, the remaining extracts were found to be inactive against carrageenan-induced hind paw edema and PGE_2-induced hind paw edema models. On the other hand, all the extracts of the title plants failed to display any anti-inflammatory activity on TPA-induced mouse ear edema model. LGL-BuOH and LGP-BuOH were also found to exhibit remarkable antinociceptive activity in *p*-benzoquinone-induced abdominal constriction test at a dose of 200 mg/kg (25.0% and 24.3%, respectively). The experimental data demonstrated that *L. garganicum* subsp. *laevigatum* and *L. garganicum* subsp. *pulchrum* displayed remarkable anti-inflammatory and antinociceptive activities (Küpeli Akkol *et al.*, 2008b).

Salvia species, Sage, features prominently in the national pharmacopoeias of many countries throughout the world and several species have been used as a remedy against perspiration and fevers; as a carminative; a spasmolytic; an antiseptic/bactericidal; an astringent; as a gargle or mouthwash against the oral inflammations such as tongue or throat; a wound-healing agent; in skin and hair care; and against rheumatism (Kintizios, 2000). *S. virgata* is known as "yilancik" in Turkey and used to alleviate dermatological problems and wounds. The decoction of *S. virgata* is used against blood cancers in Western Turkey (Baytop, 1999). *S. halophila* is an endemic plant of Turkey and traditionally being used as herbal tea. To provide a scientific evidence for traditional uses, the aerial parts of *S. halophila* and *S. virgata* were subjected to Soxhlet extraction with different solvents *i.e. n*-hexane, ethyl acetate, methanol and aqueous methanol (50%). Plants were also extracted with water under reflux. The effects of the extracts were studied in *p*-benzoquinone-induced abdominal constriction test for the assessment of antinociceptive activity and carrageenan-induced hind paw oedema and 12-*O*-tetradecanoyl-13-acetate (TPA)-induced ear oedema models in mice for assessing the anti-inflammatory activity. Results showed that methanol extract significantly inhibited carrageenan-induced paw oedema and *p*-benzoquinone-induced abdominal constrictions at 100 mg/kg dose, while it was ineffective against the TPA-induced ear oedema. However, the other extracts did not show any inhibitory antinociceptive and anti-inflammatory activities on these *in vivo* models. The extracts were then analysed using a HPLC-PDA method. Rosmarinic acid was found as the main constituent in the extracts, while caffeic acid and luteolin derivatives were also detected in small ratio (Küpeli Akkol *et al.*, 2008a). In a literature survey, Hosseinzadeh *et al.* (2003) reported that the aqueous extract of *Salvia leriifolia* seeds showed significant and dose-dependent (1.25–10 g/kg) antinociceptive activity over 7 h, and the activity was inhibited by naloxone pretreatment in hot-plate and tail flick tests. They also observed significant and dose-dependent (2.5–10 g/kg) activity against acute inflammation induced by acetic acid and in the xylene ear oedema test. In the chronic inflammation test (cotton pellet test), however, the extract (2.5–5 g/kg) demonstrated considerably significant and dose-dependent anti-inflammatory activity. Consequently, a supraspinal antinociceptive effect mediated by opioid receptors has been suggested as the activity mechanism of extract (Hosseinzadeh *et al.*, 2003). On the other hand, in a previous study, the methanol extract of *Salvia miltiorrhiza* was found to inhibit production of PGD_2 and the ethyl acetate subfraction exerted the strongest inhibition. From the ethyl acetate subfraction, tanshinone I was isolated as an active principle through activity-guided isolation procedures (Kim *et al.*, 2002).

The genus *Sideritis* L., widely distributed in Mediterranean-Macronesian region, are traditionally used in Spanish folk medicine for their anti-inflammatory and gastroprotective properties to treat certain

disorders that are accompained by inflammation. Several anti-inflammatory compounds have been obtained from the members of this genus, mainly flavonoids and terpenoids (Godoy *et al.*, 2000). In Turkey, this genus is represented by 46 species (Duman, 2000b; Aytaç & Aksoy, 2000) and some of which are used in the treatment of gastrointestinal ailments, common colds (including bronchitis, sore throat, flu) and as a diuretic as well as a herbal tea for pleasure in Turkish folk medicine (Baytop, 1999; Yesilada *et al.*, 1995). In the light of traditonal usage, acetone extract from the aerial parts of *Sideritis ozturkii* Aytac & Aksoy and its fractions were investigated for its *in vivo* anti-inflammatory and antinociceptive activities. Acetone extract of the plant and its phenolic fraction were found to possess significant inhibitory activity on carrageenan-induced hind paw oedema and p-benzoquinone induced writhing reflex models in mice. Ozturkoside A (chrysoeriol 7-*O*-[2‴-*O*-caffeoyl-6‴-*O*-acetyl-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside]) **(1)**, ozturkoside B (chrysoeriol 7-*O*-[2‴-*O*-caffeoyl-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside]) **(2)** and ozturkoside C (chrysoeriol 7-*O*-[2‴-*O*-*p*-coumaroyl-6‴-*O*-acetyl-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside]) **(3)** were isolated from the active phenolic fraction (Fig 5). Ozturkoside C showed notable antinociceptive and anti-inflammatory activities without inducing any apparent acute toxicity or gastric damage.

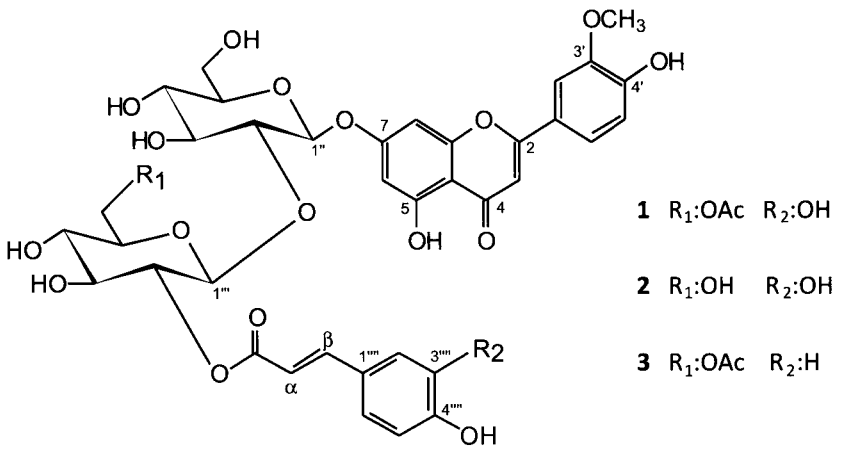


Fig 5. Chemical structures of compounds **1-3**

Although the activity of ozturkosides A and B were not significant in statistical analysis, some inhibitory effect was observed. Accordingly, it is suggested that these components in phenolic fraction might possibly share the antinociceptive and anti-inflammatory activities together (Küpeli *et al.*, 2007b). In spite of a high number of studies reporting the anti-inflammatory and antinociceptive activities of several *Sideritis* species only two flavonoids have been isolated and defined as the active constituents; hypolaetin-8-

glucoside (Villar *et al.*, 1984) and 5-*O*-demethylnobiletin (Bas *et al.*, 2006). As shown in Fig 6, hypolaetin-8-glucoside shows very close chemical structure to ozturkoside C, both having luteoline type flavone glycoside structure.

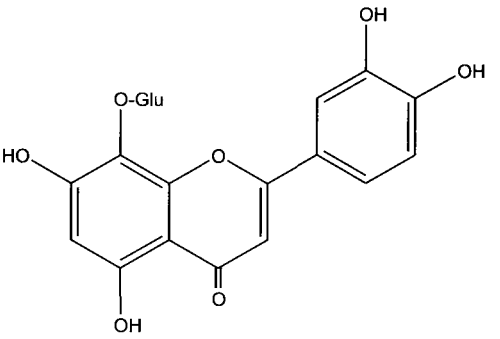


Fig 6. Chemical structure of hypolaetin-8-glucoside

Additionally, acetone extract of *S. stricta* Boiss. & Heldr. apud Bentham and its phenolic fraction exhibited potent inhibitory activity against both bioassay models in mice. From the active phenolic fraction a well-known phenylethanoid glycoside, verbascoside (= acteoside) (**1**) and two flavonoid glycosides, isoscutellarein 7-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl-(1→2)]-β-D-glucopyranoside (**2**), and isoscutellarein 7-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl-(1→2)]-6″-*O*-acetyl-β-D-glucopyranoside (**3**) were isolated. During phytochemical studies we also isolated a methoxyflavone, xanthomicrol (**4**) from the non-polar fraction (Fig 7). Although antinociceptive and anti-inflammatory activities of phenolic components were found not significant in statistical analysis, compounds **1** to **3** showed a notable activity without inducing any apparent acute toxicity as well as gastric damage. Furthermore, a mixture of flavonoid glycosides (**2** + **3**) exhibited a significant inhibitory effect in both models at a higher dose (Küpeli *et al.*, 2007c).

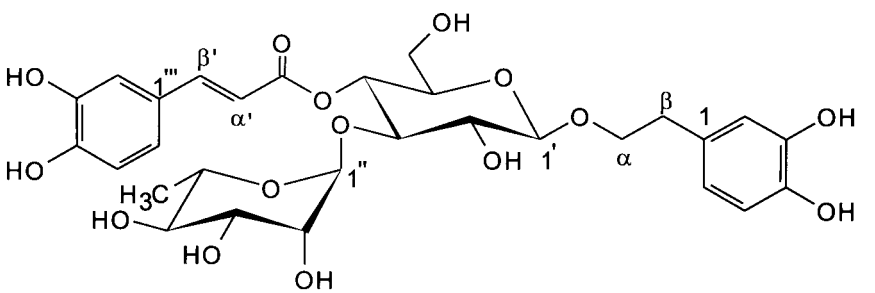


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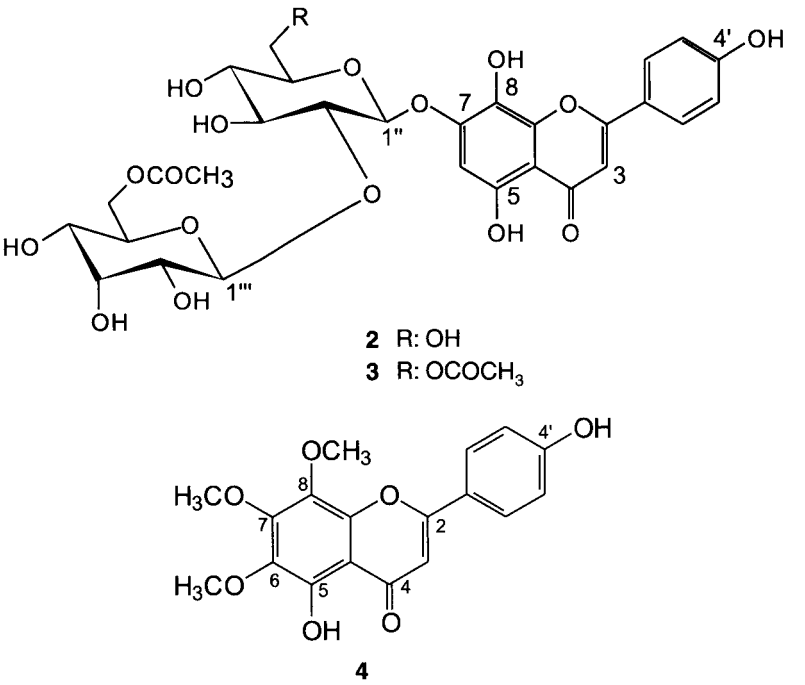
Fig 7. *Contd.*



4

Fig 7. Chemical structures of compounds **1** – **4**

LAURACEAE

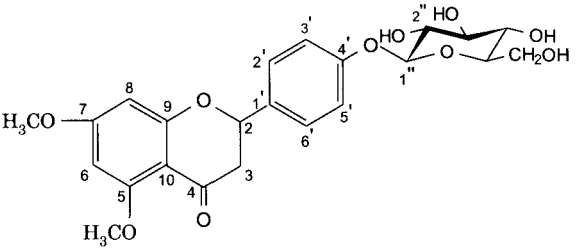
Laurus nobilis L., laurel, is a spice plant widely distributed in the Mediterranean area and the seed oil is used against rheumatic pain in Turkish folk medicine as well as in Europe. Previously, the oil obtained from laurel fruit was also reported to be used for treatment of furuncles, sprains, bruises and rheumatism (Simic *et al.*, 2003). The experimental results indicated that the ethanol extract of the seeds possesses a remarkable anti-inflammatory (29.1-37.3%) and antinociceptive (34%) activity at a dose of 500 mg/kg (Kupeli *et al.*, 2007c). On the other hand, extract prepared from the leaves were also studied for comparison and was found completely devoid of any activity (Kupeli *et al.*, 2007c).

LORANTHACEAE

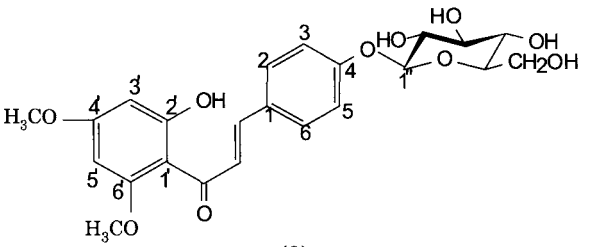
The genus *Viscum* L. (Loranthaceae) comprises semi-parasitic plants which grow on various host tree and shrubs (Miller, 1982). *Viscum album* L. is the most widespread species worldwide and has been reputed against cardiovascular diseases, *i.e.* hypertension and atherosclerosis; various bone and joint disorders including periarthritis, spondylitis, and arthritis; to

alleviate headache; for immune system stimulation; in nervous disorders as sedative or to combat epilepsy (Bartram, 1995; Murray, 1995; Wichtl & Bisset, 1994). In order to evaluate this information, antinociceptive and anti-inflammatory activity of the five flavonoids [5,7-dimethoxy naringenin (**1**, **3**, and **5**) or 4′, 6′-dimethoxy chalcononaringenin (**2**, **4**)] derivatives; (5,7-dimethoxy-flavanone-4′-*O*-β-D-glucopyranoside (**1**), 2′-hydroxy-4′,6′-dimethoxy-chalcone-4-*O*-β-D-glucopyranoside (**2**), 5,7-dimethoxy-flavanone-4′-*O*-[2″-*O*-(5‴-*O*-*trans*-cinnamoyl)-β-D-apiofuranosyl]-β-D-glucopyranoside (**3**), 2′-hydroxy-4′,6′-dimethoxy-chalcone-4-*O*-[2″-*O*-(5‴-*O*-*trans*-cinnamoyl)-β-D-apiofuranosyl]-β-D-glucopyranoside (**4**), 5,7-dimethoxy-flavanone-4′-*O*-[β-D-apiofuranosyl(1→2)]-β-D-glucopyranoside (**5**) isolated from ethyl acetate fraction of *V. album* ssp. *album* were investigated (Fig 8). The ethyl acetate fraction showed a remarkable and dose-dependent anti-inflammatory activity on carrageenan-induced hind paw oedema model (between 24.7 to 37.2%). Same pharmacological profile was also observed for the compound **2** (30.7-33.6%) from the ethyl acetate fraction. The anti-inflammatory activity of **2** was almost as potent as that of indomethacin which exerts 33.3-42.7% anti-inflammatory activity. Other flavonoids, **1**, **3**, and **5**, isolated from the ethyl acetate fraction, also showed significant but weaker anti-inflammatory activity against the same model. Among the isolated compounds from the ethyl acetate fraction were administered in 30 mg/kg dose, compounds **2** and **5** were found to possess highest and significant antinociceptive activity on p-benzoquinone induced writhing reflex test, but not as potent as ASA. The ethyl acetate fraction and isolated compounds did not induce any apparent acute toxicity during the 24 h observation period. It is noteworthy that, in spite of a weak gastric lesion incidence in the higher dose of ethyl acetate fraction (gastric lesions were observed in the stomach of 1 out of 6 rats), all the isolated flavonoids were found completely safe from the view point of gastric damage (Deliorman *et al.*, 2006). Herencia *et al.* (1999), investigated the effects of a series of chalcone derivatives on various *in vivo* and *in vitro* inflammatory models (*i.e.* human neutrophil functions, eicosanoid release, TNF-α production, air-pouch, etc.) and reported inhibitory effects on iNOS and COX-2. Correa *et al.* (2001) reported potent antinociceptive activity for 3,4-dichlorochalcones using the writhing test in mice. In the study of Pelzer *et al.* (1998) it was concluded that the anti-inflammatory activity of flavonoids increase depending upon the catechol or guaiacol-like substitution to B ring (3′, 4′-dihydroxy or 3″-hydroxy-4′-methoxy or 3′-methoxy-4′-hydroxy). Accordingly flavanones such as eriodictyol, 7-*O*-methyleridictyol and hesperidin showed the highest activity against carrageenan-induced edema. However, flavanone and chalcone derivatives demonstrated to possess anti-inflammatory activity in the present study were shown to possess one glucosylated hydroxyl group at B ring [5, 7-dimethoxy naringenin (**1**, **3**, and **5**) or 4′, 6′-dimethoxy chalcononaringenin (**2**, **4**) derivatives]. Therefore, results showed herein present additional structural data for the anti-inflammatory

and antinociceptive activity evaluation of chalcone and flavanone derivatives.



(1)



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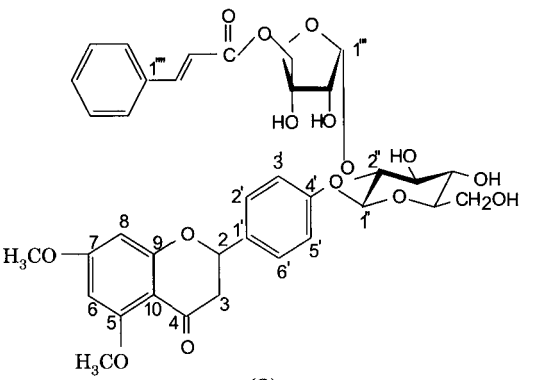

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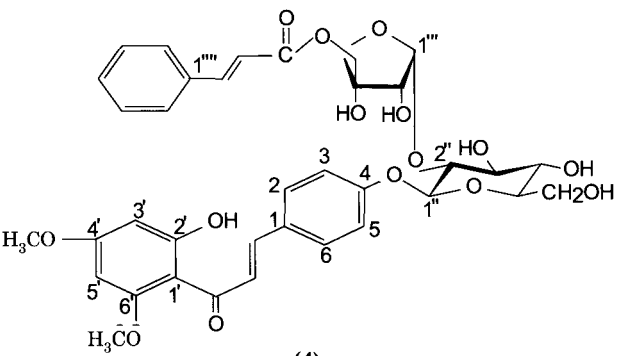

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Fig 8. *Contd.*

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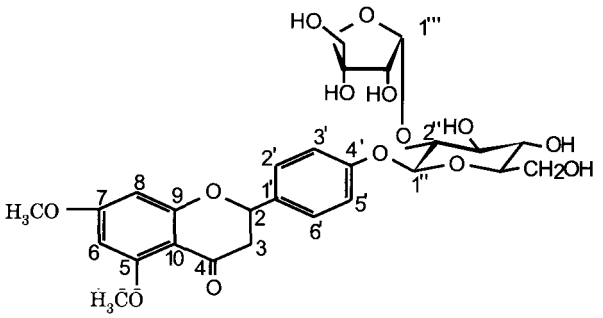


(5)

Fig 8. Structures of investigated compounds

Arceuthobium oxycedri (DC.) Bieb., is a semi-parasitic plant grows on *Juniperus oxycedri* and the shape and color of the plant looks very similar to host plant. In Anatolia this plant is believed to be effective against a wide range of diseases; from inflammatory to infectious (Yesilada *et al.*, 1995; Fujita *et al.*, 1995; Honda *et al.*, 1996). To confirm the conventional usage, prepared ethanol and aqueous extracts of the plant were studied and both extracts exhibited significant anti-inflammatory effect at the ranges of 28.9-34.2% and 21.1-30.5%, respectively. The plant also showed a potent antinociceptive activity; for the aqueous extract 34.2% at 500 mg/kg dose, while for the ethanol extract 28.6% at 250 mg/kg and 39.9% at 500 mg/kg doses. Results supported the folkloric utilization of the plant as no such study has been carried out on this plant previously (Kupeli *et al.*, 2007c).

LYCOPODIACEAE

Lycopodium genus (syn. *Huperzia*), commonly known as "club moss, ground pine, devil's claw, devil ash" in English, is a pteridophyte abundantly found in subtropical and tropical forests in the world, which is under the risk of extinction (Lawrence, 1989). In Turkey, the genus is represented by five species, namely *L. alpinum* L., *L. annotinum* L., *L. clavatum* L., *L. complanatum* ssp. *chamaecyparissus* (A. Br.) Döll, and *L. selago* L. (Davis & Cullen, 1984). Among them, *L. clavatum* (LC), and has been reported to be used for wound-healing effect as well as against nappies occurring in babies and, therefore, also called "belly powder" (Baytop, 1999). The spores of the plant were stated to have a protective effect as dusting powder for tender skin (Vasudeva, 1999). In Papua New Guinea, LC is used against stomach pain (Holdsworth & Giheno, 1975). Some other *Lycopodium* species have also been reported for similar or related ethnobotanical utilizations

worldwide. According to an ethnobotanical survey conducted in Sabah region of Malaysia, *Lycopodium* sp. was recorded to be used to treat skin burns (Kulip, 1997). In Wisconsin (USA), the leaf decoction of *L. obscurum* was reported to be used against muscle pain and rheumatism (Coffey, 1993). Studies on four extracts prepared with petroleum ether, chloroform, ethyl acetate and methanol as well as the alkaloid fraction from the aerial parts of *L. clavatum* L. using acetic acid-induced increase in capillary permeability assessment in mice revealed that only the chloroform extract and the alkaloid fraction displayed marked anti-inflammatory effect at a dose of 500 mg/kg having percentage of inhibition 24.3 and 32.1, respectively, as compared to indomethacin which exhibited 44.6% of inhibition at 10 mg/kg dose. The alkaloid fraction of *L. clavatum* was submitted to bioassay-guided fractionation procedures and revealed that the alkaloidal-type of compounds might possibly be responsible for the anti-inflammatory activity, which supports the folk medicinal utilization of the plant. Gas chromatographic-mass spectrophotometric analysis of the active alkaloid fraction have demonstrated that lycopodine, an alkaloid isolated from several *Lycopodium* species, was the major alkaloid component (84.5%) (Fig 9). Accordingly, these experimental results point out that the anti-inflammatory activity of the alkaloid fraction of the aerial parts of *Lycopodium clavatum* are primarily due to the alkaloidal components, which might most probably be lycopodine as the major responsible compound (Orhan *et al.*, 2007a).

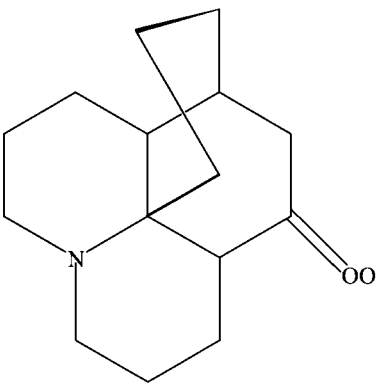


Fig 9. Chemical structure of lycopodine

LYTHRACEAE

Lythrum salicaria (purple loosestrife) was known as medicinal plant already in ancient Greek and Roman times and it has been an important drug for centuries. The whole flowering plant and the flowering branch tips of this

plant are used not only in folk medicine but also in pharmaceuticals. Decoction or some other fluid extracts from *Lythrum salicaria* is used internally for diarrhoea, chronic intestinal catarrh, hemorrhoid and eczema. It is also used externally to treat varicose veins, bleeding of the gums, hemorrhoids and eczema (Baytop, 1999; Mantle *et al.*, 2000; Rauha *et al.*, 2000). Dried aerial parts of *L. salicaria* L. were sequentially extracted with different solvents such as petroleum ether, ethyl acetate, methanol and 50 % aqueous methanol. Water extract of *L. salicaria* was also prepared under reflux. The extracts were then submitted to bioassays, *i.e.* free radical scavenging activity [1,1-diphenyl-2-picrylhydrazyl, DPPH% assay, iron(III) reductive activity, capacity of the inhibition of linoleic acid peroxidation and MDA formation], antinociceptive activity [*p*-benzoquinone-induced abdominal constriction test] and anti-inflammatory activity [carrageenan-induced hind paw edema model]. In addition, the content of total phenolics, flavonoids and flavonols in the extracts were determined by spectrophotometric methods. Although the methanol extract showed anti-inflammatory and antinociceptive activity other extracts remained inactive. None of the extracts caused any gastric damage. The qualitative and quantitative compositions of all the extracts were analysed using a HPLC-PDA system, polar fractions were found to be rich in flavonoids such as isovitexin and isoorientin (Tunalier *et al.*, 2007). In a literature review, *L. salicaria* tannins, flavone-C-glycosides and anthocyanins have been identified in *L. salicaria* extracts. Isoorientin, isovitexin and their derivatives have been reported as its main flavonoids (Rauha *et al.*, 2000; Rauha, 2001). Rauha (2001) and Tunalier *et al.* (2007) reported the concentration of flavonoids in *L. salicaria* to be high and isoorientin was reported to possess anti-inflammatory while isovitexin antioxidant activities (Ko *et al.*, 1998; Arimoto *et al.*, 2000; Küpeli *et al.*, 2004).

MORACEAE

Maclura pomifera (Rafin.) Schneider (*syn. M. aurantiaca* Nutt.), *Ioxylon pomiferum* Raf., *Toxylon pomiferum* Raf. ex Sargis a widely cultivated hardwood tree in Turkey for ornamental purposes. Various parts of the *Maclura* species are used in folkloric medicine worldwide. Decoction prepared from the roots of *M. pomifera* is used for the treatment of sore eyes by Comanche Indians in the North America (Carlson & Volney, 1940). The sap of the plant is used for the treatment of toothache and the barks and leaves for uterine haemorrhage in Bolivia (Bourdy *et al.*, 2004). While the bark of *M. tinctoria* has been reported to be used against toothache by Kaiowa and Guarani indigenous people living in the Caarapo Reserve in Brazil as well as the in the other parts of Amazon region, it was also recorded to be used

in southern Ghana for dental health (Elwin-Lewis *et al.*, 1980; Elvin-Lewis & Lewis, 1983; Bueno *et al.*, 2005). Aqueous, ethanolic and chloroform extracts and two prenylated isoflavones; scandenone (I) and auriculasin (II), isolated from the fruits of *Maclura pomifera*, were investigated for their *in vivo* anti-inflammatory and antinociceptive activities. The ethanol and chloroform extracts and scandenone had 37.2%, 42.0% and 29.7% inhibition, respectively, while acetylsalisilic acid (ASA), the reference compound, showed 53.5% inhibition at 100 mg/kg dose. Extracts and scandenone also exhibited significant inhibition, ranging between 23.3-41.7% for the ethanol and 16.9-40.3% for the chloroform extracts as well as 23.9-35.4% for scandenone in carrageenan-induced hind paw edema model at 100 mg/kg dose. All the extracts and compounds were found completely safe from the viewpoint of gastric damage and no acute toxicity was observed within 48 h observational period. A similar activity pattern was also observed in TPA-induced ear edema model, the EtOH extract (49.2% and 54.9%), chloroform extract (45.1% and 49.6%) and scandenone (45.7% and 49.3%) displayed potent anti-inflammatory activity as measurement of edema weight as well as swelling thickness, compared to indomethacin (62.7% and 70.5%). However, the H_2O extract and auriculasine were found to be completely inactive against this model. Results of the present study have revealed that isoflavonoids are the major anti-inflammatory and antinociceptive principles of the fruits. Scandenone showed a potent activity profile against all the models employed, while that of other isoflavonoid, auriculasin, was not significant, but within reasonable limits, *i.e.* 20.6% on *p*-benzoquinone-induced writhing test (Küpeli *et al.*, 2006). Among the various biological activity profiles reported for flavonoids so far, potent antioxidant activity was particularly attributed to the existence of a catechol group in the B ring (Brandi, 1997; Hom-Ross *et al.*, 2003; Tsao *et al.*, 2003; Cioffi *et al.*, 2003). Since inflammation has been found to elevate the levels of free radicals, antioxidant agents might be also considered possibly to display anti-inflammatory effect (Guo *et al.*, 2002). As shown in Fig 10, auriculasin possesses a catechol group in the molecule; however the anti-inflammatory activity was less potent, not significant, than that of scandenone which does not possess this function. Tsao *et al.* (2003) studied the antioxidant activity of isoflavones from *M. pomifera* fruits. Accordingly, they isolated two linear isoflavones; osajin and pomiferin, as the main constituents from the ethyl acetate extract. These compounds were chemically constitutional isomers of scandenone and auriculasin. Pomiferin, possessing catechol group on ring B, was found to display a strong antioxidant activity, as expected, comparable to the antioxidant vitamins C and E, while osajin (bearing single hydroxyl group in ring B), genistein and daidzein showed no antioxidant activity.

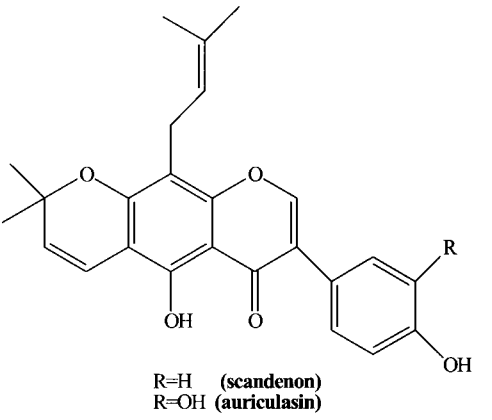


Fig 10. Chemical structures of scandenone and auriculasin

POLYGONACEAE

Rumex crispus L. is reported to treat headache, wound healing and to promote maturation of abscess in folkloric medicine in Turkey (Yesilada *et al.*, 1995). Besides, a literature survey revealed that several other *Rumex* species have also been traditionally used in the treatment of eczema, wound healing, swelling, tonsillitis, leprosy and hemorrhoids in Ethiopia or as analgesic and antipyretic in India and to treat burns and to remove pain from the back and lumber region in Bangladesh (Gebrie *et al.*, 2004; Ghosh *et al.*, 2003; Rouf *et al.*, 2003). In a previous study on the anti-inflammatory activity of two *Rumex* species based on the cell viability and measurement of PGE_2 concentration, the results verified that the root extract of *R. abyssinicus* to inhibit PGE_2 synthesis, whereas the leaf extract of *R. nervosus* showed no inhibition at all (Getie *et al.*, 2003). In another study, anti-inflammatory activity of *R. patientia*, a plant used for wound healing and anti-inflammatory properties in Turkish folk medicine, was studied against carrageenan, histamine, dextrane, serotonin, formaldehyde-induced oedema, cotton pellet granuloma and Kabak test models in rats and the extract was found to possess significant anti-inflammatory activity which was concluded to be dependant on its rich anthraquinone and tannin content (Suleyman *et al.*, 1999). Conversely, the experiments performed by our group, demonstrated that both aqueous and ethanol extracts of *R. crispus* displayed no remarkable anti-inflammatory or antinociceptive activity (Kupeli *et al.*, 2007).

RANUNCULACEAE

The *Clematis* species have been extensively used in the traditional medicines worldwide due to its widespread distribution at the northern hemisphere. The aerial parts of various *Clematis* species are particularly used in Europe

and Eastern Asia as a remedy to reduce pain and fever, as diuretic, used in the treatment of rheumatic pain, eye infections, gonorrhoeal symptoms, bone illnesses, chronic skin disorders, gout and varicosity (Keys, 1985; PDR, 2000). Several species of *Clematis* have been used as remedy in Anatolia as well. The most salient application related to folkloric usage is the one that is applied in the treatment of rheumatic ailments. In Northern Anatolia, *C. cirrhosa* vs *C. flammula* leaves or the aerial part are used to provide temporary relief of joint pains. Ground fresh aerial parts or leaves are applied on inflammatory joints for about 15-30 minutes. The ensuing irritation on the skin opens a gap and drains the edema. In some particular cases, the wound is plugged by inserting a grape dregs in order to provide continuous drainage of the edema for 20-25 days. The plug is removed occasionally to drain the accumulated inflammation out. Then, to cure this open wound a fresh leaf of *Plantago major* ssp. *major* L. is applied (Yesilada *et al.*, 1991). Moreover, *C. vitalba* branches are also used to stop tooth pain by smoking like a cigarette in northwestern Anatolia (Yesilada *et al.*, 1999). In order to test the effectiveness of extracts, fractions and subfractions from dried *Clematis vitalba* L. aerial parts were studied in mice. Ethanolic extract exerted a potent effect on carrageenan-induced hind paw edema and acetic acid-induced increased vascular permeability models. Through bioassay-guided fractionation procedures a new C-glycosyl flavone, 4′-O-coumaroyl-isovitexine (vitalboside) was isolated as the main active ingredient of the aerial parts (Fig 11). Vitalboside showed a potent and dose-dependent (in 75 and 150 mg/kg does, per os) *in vivo* anti-inflammatory activity against acute (carrageenan-, serotonin- and PGE_2-induced hind paw edema model, castor oil-induced diarrhoea), subacute (subcutaneous air-pouch) and chronic (Freund's complete adjuvant-induced arthritis) models of inflammation. The same compound was also isolated as the main antinociceptive principle which was assessed by using the models based on the inhibition of p-benzoquinone-induced writhings, as well as antipyretic activity against Freund's complete adjuvant-induced increased body temperature. Acute and subchronic toxicity studies were also performed (Yesilada & Küpeli, 2007). Previously, Li *et al.* (2002, 2003) studied the *in vitro* activity of several plants on cyclooxygenase-1 (COX-1) enzyme, one of the key enzymes in inflammatory pathway, including *Clematis* species which are used against various inflammatory disorders such as asthma, arthritis, rheumatism, fever or snake bite in Australia and China. They reported that *C. chinensis* and *C. glycinoides* stems did not show any apparent activity against COX-1 enzyme, while *C. pickeringii* leaves were found to possess a potent inhibition. In spite of the worldwide practice of using dried aerial part of *Clematis* species against inflammatory disorders in traditional medicines, fresh aerial part of *Clematis* species is known to possess severe irritating in nature. Such irritation caused by the enzymatic transformation of the ranunculin glycoside into protoanemonin after crushing the fresh plant (PDR, 2000). Ranunculin and eventually protoanemonin which are also known as the most characteristic

components of some other Ranunculaceae plants such as *Ranunculus, Anemone* or *Helleborus* species are ultimately irritating and volatile substances (Didry *et al.*, 1993; Southwell & Tucker, 1993). In fact, some *Ranunculus* species are also practiced in the same way as of *Clematis* species for the same purpose among locals in Anatolia (Yesilada *et al.*, 1995). Protoanemonin is an utterly weak substance and transforms into anemonin after being rapidly dimerized. On the other hand, no protoanemonin has been found in the dried plant (PDR, 2000). At the monograph of the plant (Traveller's Joy) in PDR Gruenwald *et al.*, (2000) for Herbal Medicines it is reported that no side effect was observed on either internal or external application of the dehydrated plant, while even contact with the crushed or cut fresh aerial part induces irritation on the skin. Accidental consumption of the fresh aerial part leads to strong irritation at the gastrointestinal and urinary tract and provokes colic, diarrhoea and related symptoms. Higher doses are reported to induce death on experimental animals depending upon the protoanemonin content in the plant. On the other hand, Pieroni *et al.* (2002) reported that *C. vitalba* sprouts are boiled in large amounts of water in order to cleave protoanemonin prior to eaten in central Italy.

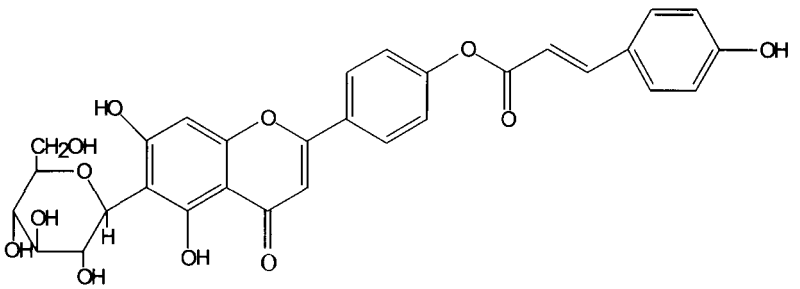


Fig 11. Chemical structure of 4′-O-coumaroyl-isovitexine (=vitalboside)

In Turkish folk medicine, *Helleborus orientalis* Lam. roots are used to treat edema in cattle. A small piece of root is sharpened like a nail is inserted into a hole punched in the ear/or tail of cattle and kept 24 h to reduce inflammation in extremities or applied externally on swelled joints or dried and pounded root is orally administered (Fujita *et al.*, 1995; Yesilada, 2002). Alternatively, the extract of roots mixed with *Salvia tomentosa* herbs is used as a bath for both man and animals. Although roots of the plant are preferentially used to alleviate the inflammatory conditions in cattle, it is not restricted to animals. A small piece of root is inserted inside a cavity against toothache in man (Fujita *et al.*, 1995). The utilization of another *Helleborus* species, *H. purpurascens*, for the treatment of inflammatory stages of rheumatism and autoimmune diseases has also been reported in Romania (Olinescu *et al.*, 1986). This plant was also shown to have *in vivo* anti-inflammatory activity in rheumatism as well as immunosuppressant

activity (Linke *et al.*, 1998). Another species, *H. niger*, was also reported to possess immunomodulating properties (Büssing & Schweizer, 1998). In order to assess the anti-inflammatory and antinociceptive activities aqueous and ethanolic extracts were prepared. Our experiments proved that both roots and herbs of *H. orientalis* to possess significant antinociceptive activity, but that of EtOH extract was more pronounced and did not induce any gastric damage. However, only the EtOH extract of roots showed potent anti-inflammatory activity (Erdemoglu *et al.*, 2003).

Ranunculus species are used in Turkish folk medicine against rheumatic pain, in particular to drain the pus from the inflammed joint through applying the fresh flowering herb after pounding on joint for about 20 min (Baytop, 1999; Yesilada *et al.*, 1995). In an *in vivo* study, ethanol extract from *R. tricophyllus* Chaix herb showed a weak anti-inflammatory and antinociceptive activity (Kupeli *et al.*, 2007).

ROSACEAE

The leaves of *Laurocerasus offficinalis* Roemer are used for asthma, coughs, indigestion and dyspepsia (Grieve, 1994). The fresh leaves of this plant is used in Turkish folk medicine as analgesic for headache and as antipyretic externally. Fresh leaves are applied on the forehead, after being wilted over a fire (Yesilada *et al.*, 1999). Conventional usage of this plant in folk medicine led us to search the biological activity of the plant extracts in a scientific platform. Even though the activity of aqueous extract was weak, EtOH extract showed potent antinociceptive and anti-inflammatory activity without inducing any gastric lesion which supports the claimed activity (Erdemoglu *et al.*, 2003).

Different parts of *Rubus* species are used in folk medicine for the treatment of numerous diseases such as diabetes mellitus, inflammatory disorders, diarrhoea, hemorrhoids and ulcers (Jouad *et al.*, 2002; Marquina *et al.*, 2002; Panizzi *et al.*, 2002). Recently, the novel anti-inflammatory triterpenoids, tormentic acid and euscaphic acid, were isolated from the fresh leaves of *R. sieboldii* (Murakami *et al.*, 2002). In a previous study, the aerial parts of *R. sanctus*, *R. hirtus* and their hybrid were evaluated for anti-inflammatory activity using carrageenan-induced hind paw edema in mice and polar fractions, n-butanol and remaining aqueous fractions obtained by solvent extraction, were shown to possess significant activity (Akcos *et al.*, 1998). Decoction of the roots *Rubus sanctus* is used to alleviate pain and against rheumatism as tea (Honda *et al.*, 1996). In an experimental study, EtOH extracts of *R. sanctus* Schreber (root and aerial part) and *R. hirtus* Walds. et Kit (aerial parts) showed potent antinociceptive activity, while that of aqueous extracts were weak. However, it should be noticed that both plants extracts may induce gastric damage. Meanwhile, neither roots nor aerial parts of both species showed a significant anti-inflammatory

activity against carragenan-induced hind paw edema in 500 mg/kg dose (Erdemoglu *et al.*, 2003).

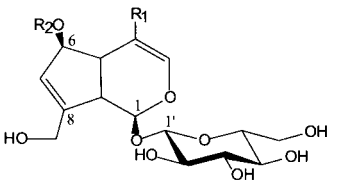
In the German Commission E Monographs, fruits (rose-hips, with seeds) of *Rosa canina* L. (Rosaceae), the dog rose, are stated to possess prophylactic and therapeutic activities against a wide range of ailments, including the inflammatory disorders arthritis, rheumatism, gout, and sciatica, for diseases with fever; for colds and infectious diseases including influenza, against gastro-intestinal disorders, to aid digestion, prevention of inflammation of the gastric mucosa and gastric ulcer, for gallstones, biliary complaints, as a laxative, for disorders of the kidney and the lower urinary tract, as a diuretic, for dropsy and as an astringent (Blumenthal *et al.*, 1998). The plant has a widespread distribution in Turkey and was reported as the most commonly utilized remedy following *Plantago* species in Turkish folk medicine (Yesilada, 2002). In addition to the effects of fruits against the disorders described above, the fruit is known as the most effective remedy against hemorrhoids and diabetes mellitus in Turkish folk medicine. Besides, the roots and leaves of the plant have also been used against bronchitis (Tabata *et al.*, 1994; Fujita *et al.*, 1995; Yesilada *et al.*, 1995, 1999; Honda *et al.*, 1996; Sezik *et al.*, 2001). This knowledge led us to analyze the plant extracts for their biological activity. The aqueous and ethanol extracts of *Rosa canina* L. (Rosaceae) fruits and the fractions prepared from the latter were investigated for their anti-inflammatory and antinociceptive activities in several *in vivo* experimental models. The ethanolic extract was shown to possess significant inhibitory activity against inflammatory models (*i.e.* carrageenan-induced and PGE_1-induced hind paw edema models, as well as on acetic acid-induced increase in a capillary permeability model) and on a pain model based on the inhibition of *p*-benzoquinone-induced writhing in mice. The active EtOH extract was then fractionated through solvent extractions with increasing polarity and five fractions were obtained; hexane, $CHCl_3$, ethylacetate (EtOAc), *n*-BuOH, and the remaining water fraction. Ethylacetate and *n*-butanol fractions displayed potent anti-inflammatory and antinociceptive activities at a dose of 919 mg/kg without inducing acute toxicity. Further attempts to isolate and define the active anti-inflammatory and antinociceptive component(s) from the EtOAc and *n*-BuOH fractions through bioassay-guided isolation procedures have resulted in failure. Both fractions were further submitted to chromatographic separation, but the activities weakened to a large extent, probably due to the synergistic interaction of components in the active fractions (Deliorman Orhan *et al.*, 2007).

SCROPHULARIACEAE

Since the ancient times, *Verbascum* extracts, decoctions and infusions, commonly known as "mullein", have been used as medicinal herbs in traditional medicines worldwide. The leaves and flowers of *Verbascum* are

reported to have expectorant, mucolytic and demulcent properties which are used to treat respiratory problems such as bronchitis, dry coughs, tuberculosis and asthma in traditional Turkish medicine (Baytop, 1999; Turker & Camper, 2002). *Verbascum* flowers are boiled in milk and are applied externally for pruritic conditions affecting the urogenital organs. In addition, as a traditional Anatolian cure for anal fistulae, the flowers are boiled in a cauldron and then the anus is exposed to the vapors. These species are reported to be mildly diuretic and to have a soothing and anti-inflammatory effect on the urinary tract, as well as acting as a mild sedative. Oil made from the flowers is used soothing earache, and can be applied externally for eczema and other types of inflammatory skin conditions. These species are commonly used to treat hemorrhoids, rheumatic pain, superfacial fungal infections, wounds and diarrhoea. They are traditionally consumed as a tea to relieve abdominal pains (Tuzlaci & Erol, 1999; Sezik *et al.*, 2001; Turker & Camper, 2002). In addition to the above mentioned common uses for the *Verbascum* family without referring a particular species, *Verbascum lasianthum* Boiss. ex Bentham flowers are reported to be used against hemorrhoids in southwest Anatolia (Tuzlaci & Erol, 1999). To evaluate the scientific basis for this practice, *in vivo* anti-inflammatory and antinociceptive activities were investigated. A methanolic extract of the flowers was shown to possess significant inhibitory activity in the carrageenan-induced hind paw oedema model and in *p*-benzoquinone-induced writhings in mice. Through bioassay-guided fractionation and isolation procedures eight compounds, 6-*O*-(4‴-*O*-*trans*-*p*-coumaroyl)-α- L-rhamnopyranosylaucubin (1), 6-*O*-(4‴-*O*-*trans*-*p*-methoxycinnamoyl)-α-L-rhamnopyranosylaucubin (2), sinuatol (3), aucubin (4), geniposidic acid (5), catalpol (6), ajugol (7) and ilwensisaponin A (8) were isolated and their structures were elucidated by spectral techniques (Fig 12). An iridoid glucoside, aucubin (4) and a triterpenoid saponin, ilwensisaponin A (8) were found to possess significant antinociceptive and anti-inflammatory activities, per os without inducing any apparent acute toxicity or gastric damage (Kupeli *et al.*, 2007a). Aucubin, one of the major iridoid glycosides of the flowers of *Verbascum lasianthum*, was previously shown to inhibit the TNF-α and IL-6 production in RBL-2H3 mast cells. Aucubin induced an enlarged IkB pool through inhibition of IkB inhibited translocation of TNF-κB/*Rel A* to nucleus, and therefore inhibited expression of inflammatory cytokines TNF-α and IL-6 (Jeong *et al.*, 2002). Moreover, Recio *et al.* (1994) reported that aucubin was effective against carrageenan-induced edema. In the present study, these results supported the previous findings that aucubin is a specific inhibitor of carrageenan induced hind paw edema, which might explain its beneficial effect in the treatment of inflammatory diseases. On the other hand, aucubin derivative and catalpol have no effect against

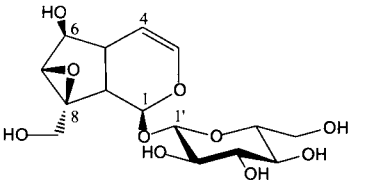
carrageenan-induced edema, while ajugol was found to possess a weak activity in similar experimental protocols. A double bond between C-7 and C-8 is one of the most positive characters for activity, and its oxidation to an epoxy derivative leads to a remarkable decrease in anti-inflammatory activity, as occurs in catalpol (6). If an ester linking appears by acylation with an aromatic acid derivative at the sugar moiety, anti-inflammatory activity is augmented, whereas if acylation occurs directly in the monoterpenoid skeleton, this activity diminishes (Recio *et al.*, 1994). However, in contrast with previous studies, our results showed that aucubin derivative (1) had no activity. Introduction of a hydroxyl function at C-8, as occurs in ajugol (7), decreases topical activity. The absence or presence of the extra–annual carboxyl or carboxymethyl group at C-4 is of no relevance, as is apparent result when the activity of aucubin is compared with those of geniposidic acid (5), but the conversion of a –COOH compound to its –COOMe derivative notably increases the topical activity (Recio *et al.*, 1994). The potency of ilwensisaponin A was similar to that of indomethacin in acute anti-inflammatory experimental models and consistent, *i.e.* the effect was not reduced with time. It has recently been reported that other closely related saponins, verbascosaponin A and verbascosaponin, suppressed the edematous response 1 h after carrageenan injection, but this effect diminished slightly in due time (Giner *et al.*, 2000). The mechanism of action, however, has not yet been established. It may be speculated that, the glucose molecule in the sugar residue connected to the C-3 position of the aglycone is crucial for its acute anti-inflammatory effect (Hostettmann & Marston, 1995; Gepdiremen *et al.*, 2005). Outcome of the study, in connection with the role of aucubin and ilwensisaponin A as the active principles of *V. lasianthum*, it seems that they could be synergistic with other active substances in its fractions and extracts (Kupeli *et al.*, 2007a).



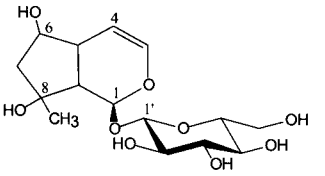
		R_1	R_2
(1)	6-*O*-(4‴-*O*-*trans*-*p*-coumaroyl)-α-L-rhamnopyranosylaucubin	H	(4‴-*O*-*trans*-*p*-coumaroyl)-α-L-rhamnopyranose
(2)	6-*O*-(4‴-*O*-*trans*-*p*-methoxy-cinnamoyl)-α-L-rhamnopyranosylaucubin	H	(4‴-*O*-*trans*-*p*-methoxy-cinnamoyl)-α-L-rhamnopyranose
(3)	Sinuatol	H	α-L-rhamnopyranose
(4)	Aucubin	H	H
(5)	Geniposidic acid	COOH	H

Fig 12. *Contd.*

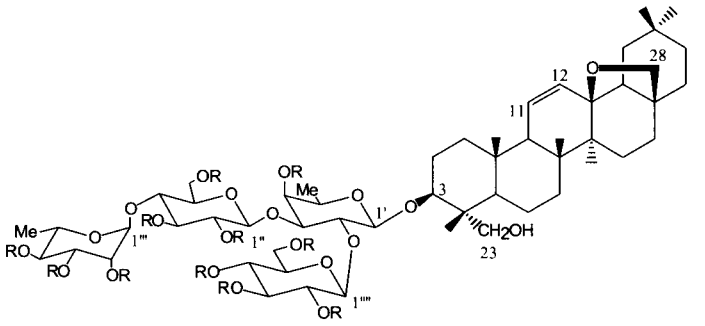
Fig 12. *Contd.*



(**6**) Catalpol



(**7**) Ajugol

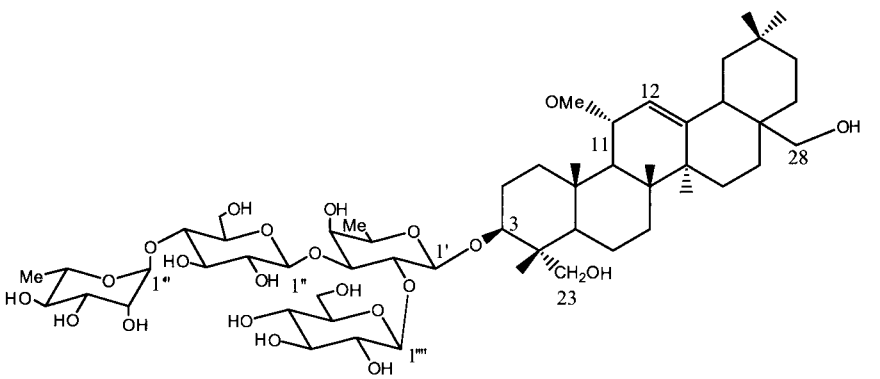


(**8**) Ilwensisaponin A

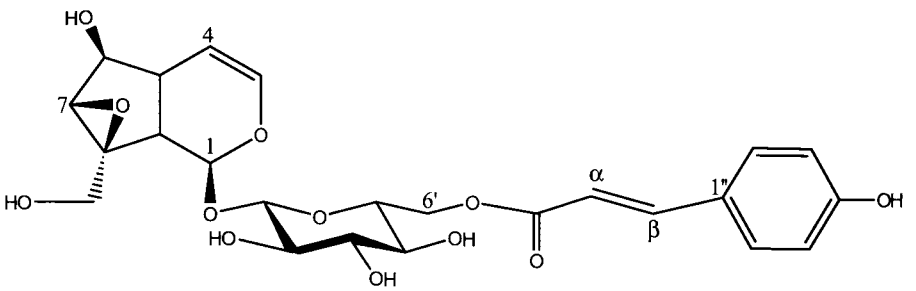
Fig 12. Structures of the compounds (**1-8**)

In a different study, saponin glycosides called ilwensisaponin A and C and iridoid glycosides known as ajugol and picroside IV (Fig 13) were isolated from the *Verbascum pterocalycinum* var. *mutense* Hub.-Mor. methanolic extract. A dose-related anti-inflammatory and antinociceptive response were obtained in the study at doses of 100 and 200 mg/kg. The results of the evaluation of the anti-inflammatory activities induced by carrageenan and PGE_1 showed that this species possesses active constituents that could diminish cyclooxygenase activitiy. No effects were seen in the 12-*O*-tetradecanoyl-13-acetate (TPA)-induced ear edema model. Ilwensisaponin A and C could explain in part the anti-inflammatory and analgesic activities of this species. Although antinociceptive and anti-inflammatory activities of ajugol and picroside IV were not significant in statistical analysis, ilwensisaponin A and C showed notable activity without inducing any apparent acute toxicity as well as gastric damage at 200 mg/kg dose (Küpeli Akkol *et al.*, 2007). In a reference survey, some triterpenes have been reported

to have anti-inflammatory, cytotoxic, antitumor activities and to be chemopreventive. Papillomas in mouse skin were initiated with 390 nmol of 7,12-dimethylbenz(a)anthracene and 1 week later, were promoted 2 times a week with 4.1 nmol of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Five saponin-related compounds used as potential anti-inflammatory agents from *Verbascum songaricum* were applied in the same position with 82 nmol. Saponin-related compounds effectively inhibited tumor formation in the sensitive mouse stock even when these compounds were given 1 h prior to TPA treatment. These results suggested that there was a general correlation between the anti-inflammatory and antitumor-promoting activities of saponins (Tokuda *et al.*, 1991). Songarosaponins and their acylated derivatives, which had been previously isolated from *Verbascum songaricum*, have also been tested for anti-inflammatory activity using the croton oil ear model. Acylated derivatives showed excellent anti-inflammatory activity as compared to that of saponins (Anam, 2001). Giner *et al.* (2000) also demonstrated that verbascosaponin A and verbascosaponin, isolated from *Scrophularia auriculata*, significantly inhibited the mouse paw edema induced by carrageenan and ear edema induced by single and multiple doses of TPA (Giner *et al.*, 2000).



Ilwensisaponin C

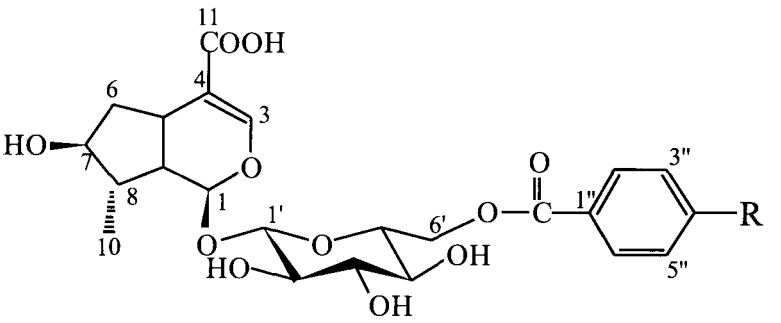


Picroside IV

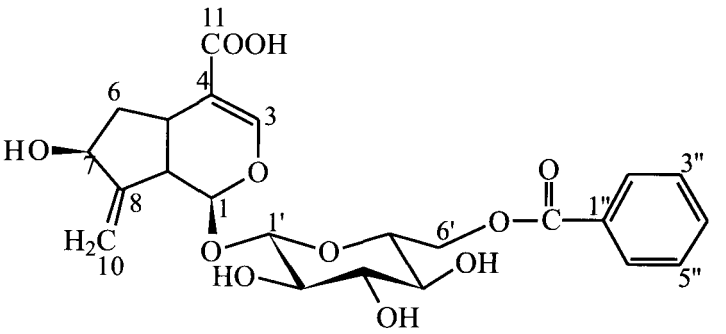
Fig 13. Chemical structures of isolated compounds

Several *Veronica* species are reported to possess application in traditional medicines worldwide for the treatment of a wide range of disorders; as expectorant in respiratory diseases or as antiscorbutic, as diuretics and for wound healing (Baytop, 1999; Harput *et al.*, 2002). In Chinese traditional medicine, *V. anagallis-aquatica* L. is used for the treatment of influenza, hemoptysis, laryngopharyngitis and hernia (Su *et al.*, 1999). During our field expeditions on Turkish folk medicine, we have recorded that aerial parts of *V. anagallis-aquatica* is boiled in milk to obtain poultice and then is applied on abdomen for abdominal pain or warm aqueous extract of leaves without removing the boiled herbs is used as a bath remedy to alleviate rheumatic pain in northwest Anatolia (Fujita *et al.*, 1995). Previously, *in vitro* anti-inflammatory activity of five *Veronica* species (*V. cymbalaria, V. hederifolia, V. pectinata* var. *glandulosa, V. persica* and *V. polita*) were investigated through evaluation of the inhibitory effects on nitric oxide (NO) production in lipopolysaccharide-stimulated mouse peritoneal macrophages as well as cytotoxic activity against KB epidermoid carcinoma and B16 melanoma (Harput *et al.*, 2002). MeOH extracts of five plant materials were found to possess inhibitory effects on NO synthesis in varying degrees. MeOH extracts were further partitioned between water and chloroform, and aqueous fractions showed the activity without inducing any cytotoxicity, while chloroform fractions were cytotoxic dose-dependently. Moreover, water fractions were found to possess remarkable effect on DPPH (2,2-diphenyl-1-picryl-hydrazyl), suggesting that the inhibitory effect on NO production might be due to their radical scavenging activity. In a study performed by our group, methanolic extract of the *Veronica anagallis-aquatica* L. plant was shown to possess significant inhibitory activity against carrageenan-induced hind paw edema model and of p-benzoquinone-induced writhings in mice. Through bioassay-guided fractionation and isolation procedures eight compounds, aquaticoside A (**1**), aquaticoside B (**2**), aquaticoside C (**3**), veronicoside (**4**), catalposide (**5**), verproside (**6**), verminoside (**7**) and martynoside (**8**) were isolated and their structures were elucidated by spectral techniques (Fig 14). Catapol derivative iridoid glucosides, catalposide (**5**) and verproside (**6**) were found to possess potent antinociceptive and anti-inflammatory activities at 500 mg/kg dose, per os without inducing any apparent acute toxicity as well as gastric damage (Küpeli *et al.*, 2005). Previously, anti-inflammatory effect of several iridoid derivatives has been reported by several other authors. Recio *et al.* (1994) studied the anti-inflammatory activity of twelve iridoid glycosides against two inflammation models in mice, carrageenan-induced paw edema and TPA-induced mouse ear edema. Loganic acid and two catalpol derivatives, aucubin and 6′-vanilloylcatalpol, were found to possess potent inhibitory activity on the former, while catalpol derivatives, aucubin, verbenalin and loganin, were found remarkably active on the latter model. According to

their experimental results, systemic anti-inflammatory activity of iridoids was modest, while a higher efficacy could be observed through topic application as evidenced by TPA-edema model. They have further suggested a structure-activity relationship for topic anti-inflammatory activity of iridoids. Accordingly, OH-substitution as C_5, unsaturation as C_7-C_8, methyl substitution of carbonyl C_{11}, and integrity of the cyclopentane ring were essential for higher activity. Some of the results suggested in the study of Recio *et al.* (1994) were later supported by other authors as well; *i.e.* picroside II, a catalpol derivative, was found active only topically and exerted a moderate topic anti-inflammatory activity in mouse ear-swelling assay (Jia *et al.*, 1999). This conclusion also supports the folkloric application of the plant in Turkey, where people applied boiled herbs of *V. anagallis-aquatica* on their extremities, while taking bath in warm aqueous extract to alleviate rheumatic pain (Fujita *et al.*, 1995). Furthermore, the conclusion suggested by Recio *et al.* (1994) reporting that a higher efficacy could be observed through topic application of iridoid glucosides than oral administration provides a further support to folkloric utilization in Turkey,

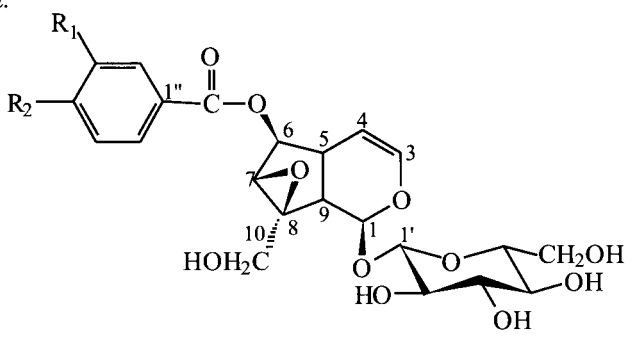


R H (**1**) Aquaticoside A OH (**2**) Aquaticoside B

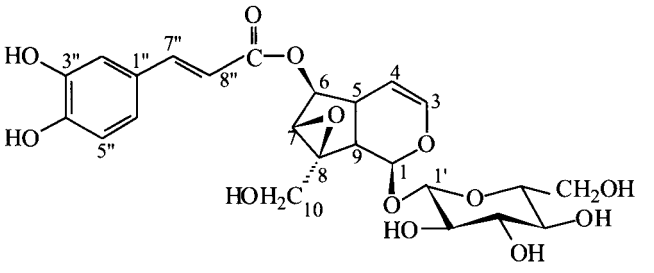


(**3**) Aquaticoside C

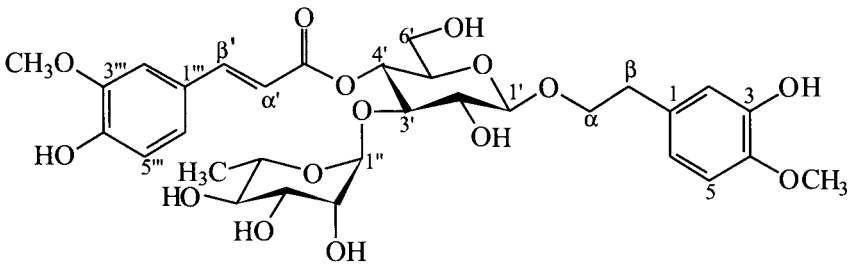
Fig 14. *Contd.*



R_1	R_2	
H	H	(**4**) Veronicoside
H	OH	(**5**) Catalposide
OH	OH	(**6**) Verproside



(**7**) Verminoside



(**8**) Martynoside

Fig 14. Chemical structures of isolated compounds **1–8**

TAXACEAE

There are eight *Taxus* species and two hybrids in the world (Van Rozendall *et al.*, 1999) and *T. baccata* L. (European yew) is the single representative in Turkey (Davis & Cullen, 1965). A large number of taxoids possessing different skeleton systems, as well as lignans, flavonoids, steroids and sugar derivatives were isolated from various *Taxus* species (Baloğlu & Kingston, 1999; Parmar *et al.*, 1999). During the course of studies on the bioactive

components, the chloroform soluble portion of ethanolic extract of the heartwood of *T. baccata* afforded four taxoids; taxusin, baccatin VI, baccatin III and 1b-hydroxybaccatin I, along with five lignans; lariciresinol, taxiresinol (3′-demethyllariciresinol), 3′-demethylisolariciresinol-9'-hydroxyisopropylether, isolariciresinol and 3-demethyl isolariciresinol (Erdemoğlu, 1999). Lignans are known to possess various biological activities, including antibacterial, antifungal, antiviral, antioxidant, anticancer and anti-inflammatory effects (Cho *et al.*, 2001a). As related with the above-presented data, a study was designed to investigate *in vivo* anti-inflammatory and antinociceptive activity of lignans and taxoids isolated from the chloroform-soluble portion of ethanolic extract of the heartwood of *T. baccata*. All the isolated lignan derivatives and taxoid compounds by Erdemoglu *et al.* (1999) were shown to possess significant antinociceptive activity against p-benzoquinone induced abdominal contractions, while only lignan derivatives significantly inhibited carrageenan-induced hind paw edema in mice (Küpeli *et al.*, 2004). These results were in accordance with the previous studies. Two of the studied lignans; lariciresinol and isolariciresinol, were reported to possess potent *in vitro* inhibitory effect on TNF- production, a pro-inflammatory cytokine (Cho *et al.*, 2001b); and thus lignan derivatives in the present study might show their anti-inflammatory effects via the same mode. On the contrary, the weak anti-inflammatory effect was observed for the taxoids studied in the present study, which might be ascribed to their TNF-inducing effect when stimulated with *Salmonella* LPS, as reported by Kirikae *et al.* (2000).

THYMELAEACEAE

In Traditional Chinese Medicine, several *Daphne* species have been frequently added in formulations prescribed against inflammatory disorders; *i.e. D. genkwa* to dispel edema (Zheng *et al.*, 2006), *D. acutiloba* for wounds and bruises (Taniguchi *et al.*, 1998), *D. tangutica* for treatment of rheumatoid arthritis (Chen *et al.*, 2004), seeds and barks, rarely leaves, of *D. mezerium* in chronic rheumatism, skin diseases, gout and inflammations in the lymph tissue (Chen *et al.*, 2004). A piece of root of the latter species was also recommended to stop pain in toothache by chewing (Grieve & Leyel, 1967; Schildknecht *et al.*, 1970). Aerial parts and roots of *Daphne oleoides* ssp. *oleoides* are used for the treatment of rheumatic pain, lumbago, and to reduce fever in Turkish folk medicine. In Inner Anatolia, the fresh stem of the plant was employed to reduce swellings in the legs of cripple animals (Baytop, 1999; Yesilada *et al.*, 2001). In an activity screening study on a number of plant extracts used for treatment of various inflammatory conditions, *D. oleoides* ssp. *oleoides* of Turkish origin was found to be the most effective against interleukin-1α and TNF-α (Yesilada *et al.*, 1997). Then, through *in vitro* activity-guided fractionation procedures assessing the inhibitory effects on inflammatory cytokines two diterpenes and one

coumarin derivative were isolated and chemically defined as the main principles that supported its the folkloric usage (Yesilada *et al.*, 2001). In another study the *in vivo* anti-inflammatory and antinociceptive activities of *D. pontica* were studied. For this purpose, effects of extracts and fractions obtained through successive solvent extractions with *n*-hexane, diethyl ether, ethyl acetate and methanol from either the roots, leaves, stems or flowers with young leaves were investigated against carrageenan-induced hind paw edema, PGE_2-induced hind paw edema and 12-*O*-tetradecanoyl-13-acetate (TPA)-induced mouse ear edema models for the anti-inflammatory activity, and *p*-benzoquinone-induced abdominal constriction test for the antinociceptive activity assessment. Only ethyl acetate extracts of the roots showed significant anti-inflammatory activity on carrageenan-induced (22.7-32.0% inhibition) and PGE_2-induced hind paw edema (3.2-27.3% inhibition) as well as 12-*O*-tetradecanoyl-13-acetate (TPA)-induced mouse ear edema (47.8-43.3% inhibition) models at 50 mg/kg dose without inducing any apparent gastric lesion or acute toxicity, whereas the other extracts were shown to be ineffective. In addition to roots, ethyl acetate extracts of the stems also exhibited 19.5-29.9%; 5.3-23.9%; 36.6-28.1% inhibition on carrageenan-induced and PGE_2-induced hind paw edema as well as 12-*O*-tetradecanoyl-13-acetate (TPA)-induced mouse ear edema models, respectively. On the other hand, none of the extracts showed antinociceptive activity (Kupeli *et al.*, 2007b).

TILIACEAE

Silver linden, *Tilia argentea* Desf. ex DC. (Tiliaceae) [synonym: *T. tomentosa* Moench] inflorescence is a popular remedy worldwide against the symptoms of common cold, bronchitis and cough (Gruenwald *et al.*, 2000b). Moreover, it is also claimed to be effective as a diaphoretic for feverish colds and infectious diseases where a sweating cure is needed as well as occasionally used as a stomachic, antispasmodic, diuretic and sedative, although these effects are notified to need experimental proof (Gruenwald *et al.*, 2000). The use of leaves as medicine is not common as that of flowers, but suggested to be employed as a diaphoretic, however the effect has not been evaluated scientifically so far. It is also reported that the *Tilia* flavonoids to possess some anti-edema effect, however the composition was not described (Gruenwald *et al.*, 2000). In order to assess this information, antinociceptive and anti-inflammatory activity of the main two flavonoid glycosides; kaempferol-3,7-O-α-dirhamnoside and quercetin-3,7-O-α-dirhamnoside isolated from the leaves were investigated. Both compounds were shown to possess potent antinociceptive and anti-inflammatory activity in 50 mg/kg dose, per os, without inducing any apparent acute toxicity as well as gastric damage (Toker *et al.*, 2004). The potent anti-inflammatory activity of different types of flavonoids was reported previously. The anti-

inflammatory activity of quercetin was reported by Simoes *et al.* (1988) against carrageenan-induced paw edema model at a dose of 20 mg/kg *i.p.* and was noted a more pronounced protective effect on the earlier stages of the oedematogenic response. It is suggested that some flavonoids including quercetin blocked both the cyclooxygenase and lipoxygenase pathways of the arachidonate cascade at relatively high concentrations, while at lower concentrations only the lipoxygenase pathway (Di Carlo *et al.*, 1999). Actually these data were supported by the conclusion that quercetin is an effective inhibitor of phospholipase A_2 which catalyses the hydrolysis of phospholipids to release arachidonic acid as the precursor of the inflammatory response (Harborne, 1994). Guardia *et al.* (2001) studied the anti-inflammatory activity of three flavonoids; quercetin (flavonol), hesperidin (flavanone glycoside) and rutin (quercetin rutinoside) in 80 mg/kg doses using an acute/subacute inflammation models. Accordingly, rutin was found very active in suppressing the edema, nodules and ankylosis as evidenced from arthrogram scores in subacute phase, while quercetin and hesperidin were shown to possess low activity. Rotelli *et al.* (2003) conducted an *in vivo* comparative study on the anti-inflammatory activity of various flavonoids (quercetin, rutin, morin, hesperetin and hesperidin) and found that quercetin (in 75 mg/kg dose) showed a remarkable inhibitory activity on carrageeenan-induced paw edema model on acute administration (between 55 to 66% inhibition) as well as on the chronic model against adjuvant-induced arthritis model, while rutin (in 150 mg/kg dose) was only effective against the later. Sanchez de Medina *et al.* (2002) pointed out that quercitrin exerts anti-inflammatory activity in trinitrobenzene sulfonic acid-induced colitis with a narrow dose-response. As described above, quercitrin is also one of the flavonoid components of silver linden leaves. Matsuda *et al.* (2002) reported that the kaempferol glycosides (tiliroside and astragalin) to possess potent inhibitory effect on TNF-production. However, there is no experimental study on the anti-inflammatory activity of kaempferol glycosides. Detailed information on the absorption features of flavonoids have not yet to be available. Although it has been claimed that only the flavonoid aglycones be able to pass through the gut wall, this might be largely depends on the chemical structure of flavonoid (Di Carlo *et al.*, 1999). However, once absorbed, flavonoids influence many biological functions including protein synthesis, cell proliferation differentiation and angiogenesis for the benefit of mankind (Di Carlo *et al.*, 1999). If this hypothesis is correct, α-glycosidic bonds of the flavonoid glycosides **1** and **2** isolated by Toker *et al.* (2004) might be subjected to hydrolysis in the gut through α-glycosidase type enzymes to give aglycone then pass the gut wall to exert its anti-inflammatory and antinociceptive activity.

VITACEAE

The leaves of *Vitis vinifera* (vine leaves) have been traditionally used as a food in both fresh and brined forms in Turkey. The leaves, due to astringent and haemostatic properties, are used in the treatment of diarrhoea, haemorrhage and varicose veins, and the juice of leaves has been used as an eye bath (Felicio *et al.*, 2001). In order to evalute the biological effects of vine leaves, extracts were submitted to bioassay models such as antioxidant activity [free radical scavenging activity (1,1-diphenyl-2-picrylhydrazyl, DPPH% assay), iron (III) reductive activity (reducing power activity assay), capacity of inhibition of linoleic acid peroxidation (ferric thiocyanate and thiobarbituric acid method)], antinociceptive activity (*p*-benzoquinone-induced abdominal constriction test) and anti-inflammatory activity (carrageenan-induced hind paw oedema model). All the vine leaf extracts were able to scavenge DPPH% radicals at physiological pH and did so in a concentration-dependent fashion as well as reduced iron (III) to iron (II). However none of the extracts was as effective as the positive controls ascorbic acid, BHT, and gallic acid. The aqueous extract prepared from fresh leaves showed moderate inhibition at 100 mg/kg dose on carrageenan-induced hind paw oedema and *p*-benzoquinone-induced abdominal constriction tests without inducing any gastric damage. The content of phenolic compounds in the extracts were also determined and hydroxycinnamic acids (*e.g.* caffeic acid), and flavonoids (*e.g.* quercetin) were verified by a reverse phase HPLC-PDA method in the extracts (Koşar *et al.*, 2007).

CONCLUSIONS

Ethnobotanical and ethnopharmacological surveys conducted by our research group have explicitly revealed that Turkish traditional medicines provide a vast source for the discovery of new leads to develop effective and safety agents for the treatment of diverse diseases. Detailed and programmed investigations on this subject along with clinical evaluation, derivatization and formulation studies may yield efficient products for the benefit of human health.

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Comparative Study of the Antigenotoxic Effects of Some Selected Natural Plant Products Against Methylmethane Sulphonate Induced Genotoxic Damage in Cultured Mammalian Cells

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ABSTRACT

In the present study the effect of ascorbic acid (20, 40 and 80 μM), nordihydroguaiaretic acid (NDGA) (0.5, 1.0 and 1.5 μM) and apigenin (5, 10 and 20 μM) was studied against the genotoxic damage induced by 60 μM of methylmethane sulphonate on human lymphocytes culture using chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) as a parameter. The treatments result in a dose dependent decrease in the CAs and SCEs, suggesting a protective role during the chemotherapy of cancer patients, as there are chances of developing secondary tumors. Ascorbic acid was most effective in reducing the genotoxic damage as compared to NDGA and apigenin, and apigenin was more effective as compared to NDGA i.e. Ascorbic acid > Apigenin > NDGA. The results of the present study suggest that ascorbic acid, NDGA and apigenin can modulate the genotoxicity of MMS in human lymphocytes in vitro.

Key words : Ascorbic acid, nordihydroguaiaretic acid, apigenin, chromosomal aberrations, sister chromatid exchanges

INTRODUCTION

The herbal preparations are traditionally used for the treatment of various diseases and for that reason it becomes necessary to assess the mutagenic

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potential or modulating action of natural plant products when associated with other substances. Herbal medicine is the root of various traditional medicine systems around the world. About 25% of the crude drugs are directly obtained from the botanicals and another 25% derived from chemically altered natural products. Various traditional medicine systems around the world, including ancient Chinese medical system, Indian medicinal system and Amazonial ethnomedicine, rely heavily on herbs for health preservation (Jain *et al.*, 2008). Despite the advances in pharmacology and synthetic organic chemistry the dependence on natural plant products remain unchanged (Fostar, 1999).

Apigenin is one of the several active ingredients found naturally in many fruits and vegetables, including apples and celery. It is found in several popular spices, including basil oregano, tarragon, cilantro and parsley. A high amount of apigenin is found in parsley, peppermint, lemon, berries and fruits (Peterson & Dwyer, 1998). It is a member of flavone family of the flavonoid. Apigenin is recognized in traditional or alternative medicine for its pharmacological activity (Hoftman, 2000). In several strains of *Salmonella typhimurium* it was found negative for reverse mutation assay (NLM, 2000). It induced SOS repair system in *E. coli* K-12 stain in PQ37 with and without metabolic activation (Czeczot & Bilbin, 1991). Apigenin has also been reported to have anti-inflammatory effects, free radical scavenger, anticarcinogenic (Kim *et al.*, 1998), grown inhibitor in various cancer lines (Yim *et al.*, 2001; Wang *et al.*, 2000) and antigenotoxic properties (Siddique *et al.*, 2008; Khan *et al.*, 2006).

Nordihydroguaiaretic acid (NDGA) is a phenolic lignan, present in the evergreen shrubs, *Larrea divaricata* and *Guaiacam officinale* (Agarwal *et al.*, 1991). NDGA possesses a number of interesting biological properties, that are of potential use for humans such as enzyme inhibitor (Capdevilla *et al.*, 1988), antimicrobial (Hurtado *et al.*, 1979), a protector from neurotoxicity and bladder toxicity (Frazier & Kehrer, 1993), stimulator of corpus luteum for the secretion of progesterone (Carlson *et al.*, 1995), potential vas and branchiodialator (Nagano *et al.*, 1996), antimutagenic action against alkylating drugs (Wang *et al.*, 1991), synthetic progestins (Siddique *et al.*, 2006; Siddique *et al.*, 2007) and estrogens (Siddique & Afzal, 2004).

Ascorbic acid is a powerful antioxidant, which plays an important role in intra cellular oxidation/reduction systems, and in binding of free radicals produced endogenously (Laurence *et al.*, 1997). Methylmethane sulphonate is a monofunctional alkylating agent with both neoplastic and

mutagenic activities (Mittal & Musarrat, 1990). It alkylates DNA at the N-7 position of guanine and the N-3 position of adenine. All these changes may give rise to abnormal base pairing at DNA replication (Madrigal-Bujaidar *et al.*, 1998). The present study deals with the comparative study of ascorbic acid, NDGA and apigenin against the genotoxic damage induced by methylmethane sulphonate in cultured human lymphocytes.

MATERIALS AND METHODS

Chemicals

Apigenin (Sigma), NDGA (Sigma), Ascorbic acid (SRL); Phytohaemagglutinin-M, antibiotic-antimycotic mixture, RPMI-1640 (Gibco); dimethylsulphoxide, 5-Bromo 2-deoxyuridine, colchicine (SRL, India); Giemsa stain (Merk).

Human Lymphocyte Culture

Duplicate peripheral blood culture were performed according to Carballo *et al.* (1993). Briefly, heparinized blood samples (0.5 mL), were obtained from a healthy female donor and were placed in a sterile culture tubes containing 7 mL of RPMI-1640 medium, supplemented with fetal calf serum (1.5 mL), antibiotic-antimycotic mixture (1.0 mL) and phytohaemagglutinin (0.1 mL). The flasks were kept in an incubator at 37 °C for 24 h.

Chromosomal Aberration Analysis

After 24 h, 60 μM of methylmethane sulphonate was given with 20, 40 and 80 μm of ascorbic acid, 0.5, 1.0 and 1.5 μM of NDGA and 5, 10 and 20 μM of apigenin separately and kept for another 48 h. The treatment of selected doses of MMS, ascorbic acid, NDGA and apigenin were also given separately. After 47 h, 0.2 mL of colchicine (0.2 μg/mL) was added to the culture flask. Cells were centrifuged at 1000 rpm for 10 min. The supernatant was removed and 5 mL of prewarmed (37 °C) KCl hypotonic solution (0.075 M) was added. Cells were resuspended and incubated at 37 °C for 15 min. The supernatant was removed by centrifugation at 1000 rpm for 10 min., and 5 mL of chilled fixative (methanol: glacial acetic acid; 3:1) was added. The fixative was removed by centrifugation and the procedure was repeated twice. The slides were stained in 3% Giemsa solution in phosphate buffer (pH 6.8) for 15 min. 300 metaphases were examined for the occurrence of different type of abnormality. Criteria to classify the different types of aberrations were in accordance with recommendation of EHC 46 for environmental monitoring of human population (IPCS, 1985).

Sister Chromatid Exchanges Analysis

For sister chromatid exchange analysis, bromodeoxy uridine (10 μg/mL) was added at the beginning of the culture. After 24 h, 60 μM of MMS treatment was given with 20, 40 and 80 μM of ascorbic acid, 0.5, 1.0 and 1.5 μM of NDGA and 5, 10 and 20 μM of apigenin separately and kept for another 48 h in an incubator. Mitotic arrest was performed by adding 0.2 mL of colchicine (0.2 μg/mL). Hypotonic treatment and fixation were performed in the same way as for the chromosomal aberration analysis. The slides were processed according to Perry and Wolff (1970). The sister chromatid exchange average was taken from an analysis of metaphase during second cycle of division.

Statistical Analysis

Students t-test was used for the analysis of CAs and SCEs. Regression analysis was also performed for the dose effect of ascorbic acid NDGA and apigenin against the genotoxic dose of MMS using statistical software (Statsoft Inc.).

RESULTS

The results of the present study reveal that the treatment of ascorbic acid, NDGA and apigenin reduced the genotoxic effect of MMS. The used dosage of ascorbic acid, NDGA and apigenin were not genotoxic *per se*. The treatment of 20, 40 and 80 μM of ascorbic acid, 0.5, 1.0 and 1.5 μM of NDGA and 5, 10 and 15 μM of apigenin with 60 μM of MMS results in a dose dependent decrease in number of abnormal metaphases (Table 1). The chromatid exchanges and dicentric chromosomes were completely eliminated even at the lowest tested doses of ascorbic acid, nordihydroguaiaretic acid and apigenin (Table 1). In sister chromatid exchange analysis the treatment of 20, 40 and 80 μM of ascorbic acid, 0.5, 1.0 and 1.5 μM of NDGA and 0.5, 10 and 15 μM of apigenin with 60 μM of MMS results in a dose dependent decrease in SCEs/cells (Table 2). Regression analysis was also performed to determine the dose of effect of ascorbic acid, NDGA and apigenin on 60 μM of MMS for number of abnormal metaphases and sister chromatid exchanges. A decrease in the slope of linear regression was observed as the dose of ascorbic acid, NDGA and apigenin was increased. For abnormal metaphases the treatment of 20, 40 and 80 μM of ascorbic acid ($F = 3.85$; $p<0.10$; Fig 1), 0.5, 1.0 and 1.5 μM of NDGA ($F = 27.0$; $p<0.02$; Fig 2) ad 5, 10 and 20 μM of apigenin ($F = 7.68$; $p<0.08$; Fig 3) with the increase in the dosage a decrease in the slope of linear regression lines was observed. For sister chromatid exchange analysis, the treatment of 20, 40 and 80 μM of ascorbic acid ($F = 6.57$; $p<0.063$; Fig 4), 0.5, 1.0 and 1.5 μM of NDGA ($F = 30.37$; $p<0.02$; Fig 5) and

5, 10 and 20 μM of apigenin (F = 24.14; p<0.024; Fig 6) with the increase in the dosage the decrease in the slope of linear regression lines was observed

Table 1. Effects of ascorbic acid, nordihydroguaiaretic acid and apigenin on chromosomal aberrations induced by methylmethane sulphonate

Treatments	Abnormal metaphases without gaps		Chromosomal aberrations				
	Number	Mean% ± SE	Gaps	CTB	CSB	CTE	DIC
MMS (μM)							
60	38	12.67 ± 1.92[a]	23	22	16	5	4
MMS (μM)+ AA (μM)							
60 + 20	18	6.00 ± 1.37[b]	12	10	8	-	-
60 + 40	12	4.00 ± 1.13[b]	8	8	4	-	-
60 + 80	10	3.33 ± 1.03[b]	6	7	3	-	-
MMS (μM) + NDGA (μM)							
60 + 0.5	28	9.33 ± 1.67[b]	16	16	14	-	-
60 + 1.0	24	8.00 ± 1.56[b]	14	14	12	-	-
60 + 1.5	22	7.33 ± 1.50[b]	10	13	9	-	-
MMS (μM) + Apigenin (μM)							
60 + 5	23	7.67 ± 1.53[b]	9	13	10	-	-
60 + 10	16	5.33 ± 1.29[b]	8	9	7	-	-
60 + 20	12	4.00 ± 1.13[b]	5	7	5	-	-
AA (μM)							
20	2	0.67 ± 0.47	1	2	-	-	-
40	3	1.00 ± 0.57	2	2	1	-	-
80	4	1.33 ± 0.66	2	2	2	-	-
NDGA (μM)							
0.5	4	1.33 ± 0.66	2	2	2	-	-
1.0	5	1.67 ± 0.73	2	3	2	-	-
1.5	6	2.00 ± 0.80	3	3	3	-	-
Apigenin (μM)							
5	2	0.67 ± 0.47	1	2	-	-	-
10	3	1.00 ± 0.57	2	2	1	-	-
20	4	1.33 ± 0.66	2	2	2	-	-
Untreated	2	0.67 ± 0.47	1	2	-	-	-
Negative control (DMSO, 5μl/mL)	2	0.67 ± 0.47	1	2	-	-	-

CTB: Chromatid break; CSB: Chromosome break; CTE: Chromatid exchange; DIC: Dicentric; MMS: Methylmethane sulphonate; AA: Ascorbic acid; NDGA: Nordihydroguaiaretic acid; DMSO: Dimethylsulphoxide; SE: Standard error.
[a]Significant with respect to untreated (p<0.01)
[b]Significant with respect to MMS (p<0.05)

Table 2. Effects of ascorbic acid, nordihydroguaiaretic acid and apigenin on chromosomal aberrations induced by methylmethane sulphonate

Treatments	SCEs/cell (mean ± SE)
MMS (μM)	
60	17.22 ± 0.98[a]
MMS (μM) + AA (μM)	
60 + 20	13.24 ± 0.74[b]
60 + 40	10.34 ± 0.68[b]
60 + 80	8.84 ± 0.59[b]
MMS (μM) + NDGA (μM)	
60 + 0.5	14.12 ± 0.84[b]
60 + 1.0	12.28 ± 0.76[b]
60 + 1.5	11.32 ± 0.74[b]
MMS (μM) + Apigenin (μM)	
60 + 5	13.68 ± 0.78[b]
60 + 10	12.02 ± 0.72[b]
60 + 20	10.42 ± 0.70[b]
AA (μM)	
20	2.14 ± 0.27
40	2.36 ± 0.32
80	2.94 ± 0.36
NDGA (μM)	
0.5	2.56 ± 0.34
1.0	3.22 ± 0.40
1.5	3.98 ± 0.46
Apigenin (μM)	
5	1.72 ± 0.28
10	1.76 ± 0.30
20	1.80 ± 0.31
Untreated	1.52 ± 0.20
Negative control (DMSO, 5 μl/mL)	1.58 ± 0.26

MMS: Methylmethane sulphonate; AA: Ascorbic acid; NDGA: Nordihydroguaiaretic acid; DMSO: Dimethylsulphoxide; SE: Standard error. [a]Significant with respect to untreated ($p<0.01$). [b]Significant with respect to MMS ($p<0.05$).

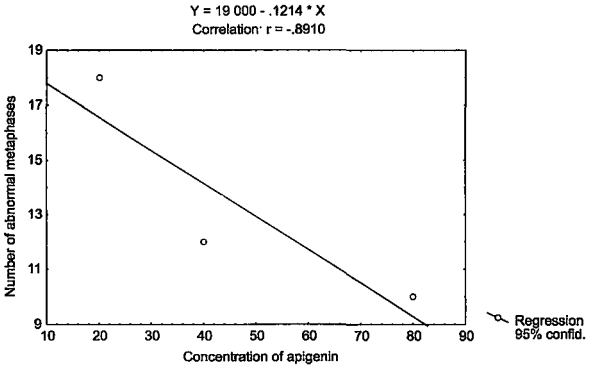

Fig 1. Effect of ascorbic acid on number of abnormal metaphases induced by 60 μM of methylmethane sulphonate

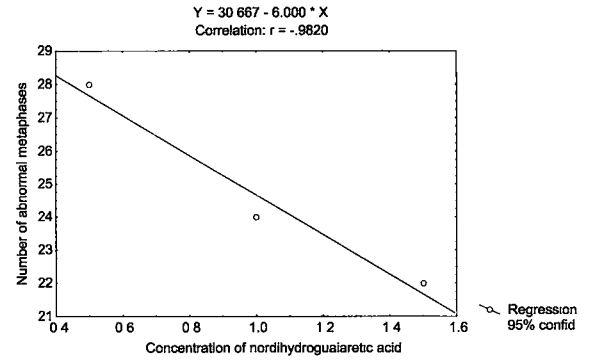

Fig 2. Effect of nordihydroguaiaretic acid on number of abnormal metaphases induced by 60 μM of methylmethane sulphonate

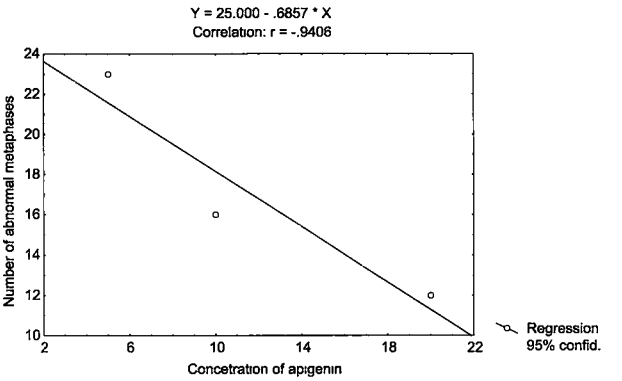

Fig 3. Effect of apigenin on number of abnormal metaphases induced by 60 μM of methylmethane sulphonate

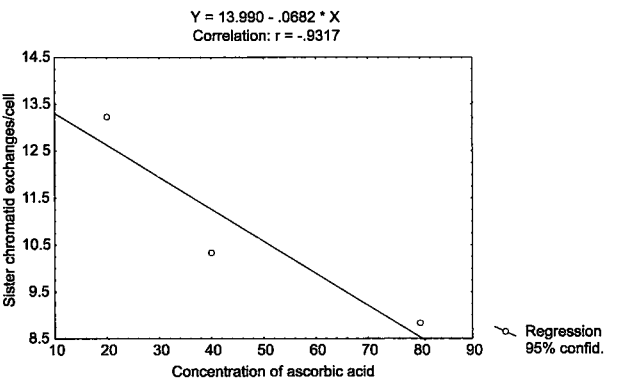

Fig 4. Effect of ascorbic acid on sister chromatid exchanges induced by 60 μM of methylmethane sulphonate

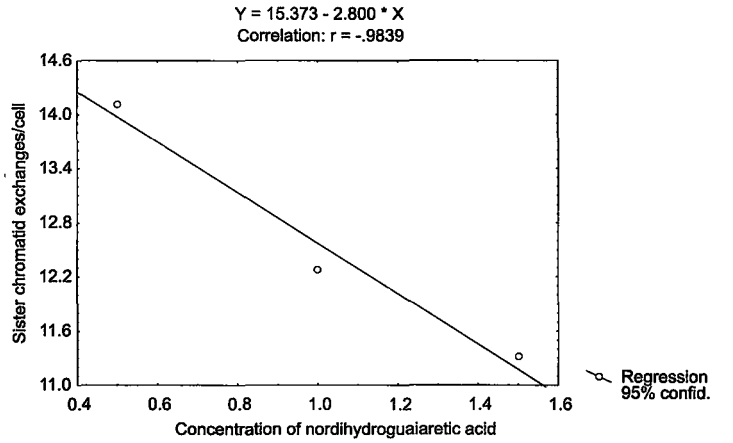

Fig 5. Effect of nordihydroguaiaretic acid on sister chromatid exchanges induced by 60 μM of methylmethane sulphonate

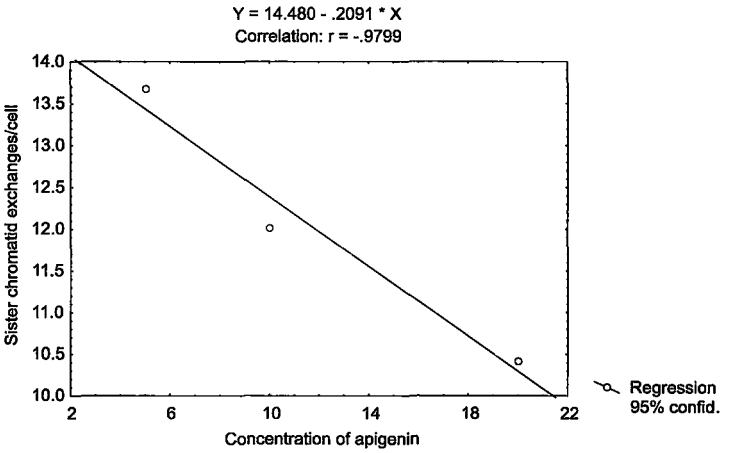

Fig 6. Effect of apigenin on sister chromatid exchanges induced by 60 μM of methylmethane sulphonate

DISCUSSION

The results of the present study reveals that all the natural plant product are effective in reducing the genotoxic damage of methylmethane sulphate. Ascorbic acid is most effective in reducing the genotoxic damage as compared to apigenin and NDGA. In our present study the following trend was found in the potency of reducing the genotoxic damage induced by methylmethane sulphonate–Ascorbic acid > Apigenin > Nordihydroguaiaretic acid. Vitamins act as antioxidants and free radical scavengers, thereby acting as anticarcinogenic, anticlastogenic and antimutagenic agents. Ascorbic acid

possesses a substantial nucleophilic character and it has been suggested that ascorbate might protect against electrophilic attack or cellular DNA by intercepting reactive agents (Edgar, 1974) or that ascorbyl anion radical with a high extent of unpaired electron delocalization is responsible for the scavenging of free radicals (Bielski, 1961; Szeto *et al.*, 2002). As the treatment with ascorbic acid results in a decrease of the genotoxic damage induced by MMS, it may be due to the scavenging property of ascorbic acid. NDGA possesses antioxidant (Olivetto, 1972) and free radical scavenging property (Nakadate, 1989; Marthy *et al.*, 1990), which may be useful in reducing the genotoxic damage induced by MMS. The doses of NDGA were selected on the basis of study performed by Madrigal-Bujaidar *et al.* (1998). Genotoxicity testing provides human a risk assessment. An increase in the frequency of chromosomal aberrations in peripheral blood lymphocytes is associated with an increased overall risk of cancer (Hagmar *et al.*, 1984). Most of the chromosomal aberrations observed in the cells are lethal, but there are many other aberrations that are viable and cause genetic effects, either somatic or inherited. The ready quantifiable nature of sister chromatid exchanges with high sensitivity for revealing toxicant-DNA interaction and the demonstrated ability of genotoxic chemicals to induce significant increase in sister chromatid exchanges in cultured cells has resulted this endpoint being used as indicator of DNA damage in blood lymphocytes of individuals exposed to genotoxic carcinogens (Albertini *et al.*, 2000). The above genotoxic endpoints are well known workers of genotoxicity of any reduction in the frequency of these genotoxic endpoints gives an indication of the antigenotoxicity of a particular compound (Albertini *et al.*, 2000). Genotoxic effect of anti cancer drugs in non-tumor cells are of special significance due to the possibility that they may induces secondary tumors in cancer patients. Therefore, the antigenotoxic effects of these natural plant products are of special significance, that they are potent enough in reducing the genotoxic damage, thereby reducing the possibility of developing secondary tumors in the patients undergoing chemotherapy, but care should be taken with regard to their concentration if consumed in isolation.

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Inflammation and Mediators

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ABSTRACT

Inflammation is the normal protective response to tissue injury caused by any noxious stimulus to destroy the invading organisms, remove irritants and set stage for tissue repair. It is triggered by the release of different cellular and humoral mediators from the injured tissues and migrating cells. The specific mediators vary with the type of inflammatory process. Enormous numbers of them are known today. It has been said that medical knowledge doubles every 10 years. With this assumption, if we start with one mediator in the early twenties we end up with a very high number till today. The concept of inflammatory mediators may sound reasonably limited yet the field has become so specific that no single individual is known who masters it all. As the number of mediators has grown, so has the number of targets. Until 1930's the microcirculation attracted all the attention, in late 30's existence of accumulation of leukocytes in terms of mediators i.e., endogenous substances capable of attracting white blood cells out of the blood vessels. 70's was the era of prostaglandins which created revolution in the history of inflammation and ended with COX-1 and COX-2. Today any cell is a fair target for mediators. The functional response may be extremely subtle but a cell can be induced to express or reveal a new antigen on its surface without any visible change or the target cell simply becomes activated. It is well known that all inflammatory cells can exist in these two conditions - resting and activated - and this may ultimately become true for all cells. Activated cells then produce new mediators of their own and the situation become more complicated. An attempt has been made in this review

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to explain some of the hidden facts of inflammation through explaining the mediators of inflammation.

Key words : Inflammation, mediators, cells, prostaglandins, cytokines, leukotrienes, selectins, integrins

INTRODUCTION

Ever since the dawn of history, man has always tuned to plants for alleviation of his sufferings. Herbal drugs have been used in the treatment of diseases since time immemorial. Written history on the use of medicinal and aromatic plants can be traced back to about 1000 years. Four fifth of the population in the world lives in the developing countries where majority of the people benefit from traditional medicines and only 15% of them benefit from allopathic medicines. Asia is the most important continent for the traditional medicinal plants. India, being an agricultural country, is richly endowed with a wide variety of indigenous floras, some of which have long been employed for their medicinal use. Several indigenous systems of medicines are practiced in India such as Ayurveda, Unani, and Siddha etc. All of them use a variety of herbs, minerals and other biological products as medicaments since long.

These natural products are used in traditional medicines on the basis of subtle principles and concepts. These principles are basically similar in all the indigenous systems of Ayurveda, Unani, and Siddha. These are vividly described in Ayurvedic classics as a series of texts called "nighantus" especially devoted to the usage of herbal drugs and their properties. The recent upsurge of global interest in herbal drugs has created a fresh impetus on the development of plant-based medicines and health products.

As science progresses, the unknown diseases are being discovered and reported. The diseases, which were rarely heard of, are becoming very common. With the advancement in the drug development system, many new drugs are flooding the world market but still they have not succeeded over the drudgery of diseases. Uncounted number of dreadful diseases has been listed in the medical history; inflammation is one among them and a concerned disease particularly rheumatoid arthritis. Although, inflammatory diseases have afflicted a large proportion of world population thus crippling the activity of human beings without killing, yet, no permanent cure.

Every individual, a human or an animal must defend itself against multitude of different pathogens including viruses, bacteria, fungi, protozoan, metazoan parasites as well as tumours and a number of various harmful agents which are capable to derange its homeostasis. Inflammation is a complex stereotypical body's reaction to invasion by any infectious agent, antigen challenge or even just physical or traumatic damage.

To talk of inflammation, war is the perfect metaphor for it. Both are necessary evils, more-or-less displaying stereotyped responses to outside threats. The specialized troops (white cells), suicide-commandos (neutrophils), long term siege armies (granulomas), supply routes (vessels), communications and intelligence (mediators), and a huge array of lethal weapons (inflammatory enzymes) come to patrolling the site of inflammation and surmount an inflammatory response. Despite idealistic rhetoric about "the laws of war", when the fighting starts, there is really only one law: "kill your enemy". Like it or not, if you want peace, you must be prepared to fight under certain conditions. If you want to be healthy, your body must be able to mount an inflammatory response. Force will always rule our world. Our best hope is that this will be the force of good laws. And the best for which we can hope from the inflammatory response is that, for most of our lives, it will do us more good than harm.

"Big Robbins" defines "inflammation as the reaction of vascularized living tissue to local injury". Inflammation is the name given to the more or less stereotyped ways our tissues respond to noxious stimuli, with blood vessels and white blood cells as its twin center pieces and a host of proteins as actors. Inflammation destroys, dilutes, or walls off the injurious agents and sets in motion the limited powers of the body to heal it. The main purpose of inflammation is to bring fluid, proteins and cells from the blood into the damaged tissue for the defense.

In other words inflammation, a localised protective event, elicited by injury, is a complex and multimediated process. It may be considered as an essential protective and normal process to any noxious stimulus that may threaten the well being of the host ranging from a transient, self limiting and localised to a complex sustained response involving a part or the whole organism that may lead to the immunological rejection of foreign tissue. When tissue injury is caused by a single finite event, the inflammation and reparative process progresses smoothly from injury to healing. In these circumstances, the whole inflammatory process is truly beneficial and provides an example of homeostatic mechanism restoring the affected tissue to its former normal healthy state. In contrast, when the injurious agent is self replicating parasite, the inflammatory response becomes much more complex because some form of tissue injury will continue till the agent persists. Living tissue's early local response to an injury is *acute inflammation*, whereas, if acute inflammation persists for longer time it turns to *chronic inflammation* which is often systemic and biochemically different from acute inflammation, meaning thereby that inflammation can be acute or chronic depending upon the injurious agents, although acute and chronic inflammatory events often occur simultaneously.

The physiological features of inflammation have been recognised since Celsus described them in first century A.D. Acute inflammation is

recognised by cardinal signs of *redness, oedema, heat, pain and loss of function*. In fact acute inflammation is not a disease, but a defensive response to injury; however, it is not always beneficial. Acute inflammation comprises the immediate and early response to an injurious agent. The events in the acute inflammatory process are largely mediated by the production and release of a variety of chemical mediators. Despite the diversity, the injurious influence and the tissue involved in inflammation, similar chemical mediators are released, and hence the acute inflammatory process is virtually stereotyped. Further more, the acute inflammation may remain confined to the site of injury and evoke only local signs and symptoms or may be extensive and induce systemic signs as well as evolve secondary line of defense, such as lymphoid tissue. The protein rich fluid and white cells usually polymorphs, migrate from blood that accumulate in the extravascular spaces as a result of an inflammatory reaction, constitute an exudate indicating that acute inflammatory reactions are exudative, while on the other hand chronic inflammation is largely a proliferative one.

Furthermore, inflammatory process has been described in terms of the immediate reaction to injury, which gives rise to acute inflammation. The acute response is characterised principally by the vascular and exudative changes. The white cells that participate in acute inflammation are usually neutrophils and macrophages. In contrast, chronic inflammation results from injurious stimuli that are persistent, often for weeks or months leading to a predominating proliferative rather than an exudative reaction. Chronic inflammatory reactions are also characterized by white cell accumulations, chiefly macrophages and lymphocytes and some times plasma cells. Thus, leucocytic exudate in chronic inflammation is mononuclear, whereas in acute inflammation it is polymorphonuclear.

Chronic inflammation may arise following acute inflammation or the response may be chronic almost from the onset. Acute to chronic transition occurs when the acute inflammatory response cannot be resolved, due to either the persistence of the injurious agent or to some interference in the normal process of healing. Chronic inflammation is associated with a range of inflammatory diseases, such as rheumatoid arthritis, hepatitis, chronic pulmonary disease, various inflammatory diseases of gastrointestinal tract and chronic periodontal diseases. Chronic inflammatory diseases show a pathological immune response probably due to the presence of perpetuating antigenic determination. Under certain conditions immune reactions are set up against the individual's own tissue, leading to autoimmune diseases. In these diseases autoantigens evoke a self perpetuating immunological reaction that results in several chronic inflammatory diseases, such as rheumatoid arthritis and chronic thyroiditis.

Some inflammations are difficult to categorise as acute or chronic because there is no sharp line that divides them. Arbitrarily it is said that when an inflammation lasts more than four to six weeks it is said to be a chronic inflammation. However, much depends on the effectiveness of the host response and nature of the injury. Time limits are without meaning. The differentiation of acute and chronic inflammation can best be made on the basis of morphological pattern of the reaction.

INFLAMMATION

It is presumed that inflammation is a salutary process. It may have the injurious effects on the body. Teleologically, it is a process of self defenses against noxious stimulus to a certain limit, beyond that it requires drug therapy to curtail the limits of the process. Broadly inflammation is a complex cellular and biochemical response to injurious stimuli that involves a multitude cell types and mediators. It is classified into acute and chronic types. The account of acute and chronic inflammation often separates the two conditions by a description of healing and regeneration. An understanding of the histological picture of chronic inflammation is certainly assisted by knowledge of the process involved in repair, but the separation from acute inflammation leaves the impression that these two varieties of inflammation are distinct from each other, although sharing the term inflammation in their name. In general, the term acute, as applied to inflammation, refers to a response with a rapid onset and relatively short duration characterized by particular vascular phenomena. The common sequel is resolution of the inflammatory exudate and restoration of normal structure and function. In some cases the acute events lose their intensity, so that the process continues as a smoldering chronic condition. As long as irritation persists, the lesion should be considered inflammatory and once the irritation abates or is removed, the process becomes reparative.

Acute Inflammation

The inflammatory process has been described in terms of the immediate reaction to injury which gives rise to acute inflammation. The physiological features of acute inflammation have been recognised since Celsus described them in the first century A.D. as the cardinal signs. These are redness, oedema, pain and heat. The fifth sign *i.e.*, loss of function was added later by Galen (1978). Infact acute inflammation is not a disease, but a defensive response to injury, however, it is not always beneficial. In human beings inflammation occurs in vascular tissues, and is primarily a local vascular response where protein rich fluid and cells are brought to the site of injury to neutralize the damaging agent. The damaging agent can be due to a variety of causes *viz.*, mechanical damage (cuts, blows etc.), chemical damage (acids, alkalies, toxins etc.), radiation, burns, scalds, viruses, bacteria, parasites, insect bites, stings, and local pathology such as antigen-

antibody reactions. Early events in inflammation are obviously vascular in nature and these can be demonstrated on the skin by stricking it with a ruler. Initially a white line is produced because of mechanical stimulus to local capillaries, which have responded with vasoconstriction when blood is pushed out of the vessels. This is a transitory phenomenon, but if sufficient pressure is applied a red line results which is because of vasodilation where the new dilated capillaries fill with blood and produce the red color. The sharpness of the red line fades in about thirty seconds to one min and the area of pressure takes on a dull red, blotchy appearance. This is known as flare and is caused by arteriolar dilation. If sufficient force was applied in the initial trauma a wheal will develop due to the loss of fluid through the vascular wall, resulting in the formation of oedema. As the oedema developed some of the redness may be lost where pressure from the collected fluid close down the capillaries. Vasodilation, in particular, arteriolar dilation increases the local blood supply which, being at a higher temperature than surface tissue, gives a feeling of warmth.

These vascular changes are due to chemical mediators being released at the site of injury. The early changes in the blood vessel wall and the changes in the blood flow are known as "stasis". As the fluid leaves the blood with the result it becomes more viscous and the flow slows down, the erythrocytes tend to stick together in the form of rouleaux and push the white cells towards the vessel walls. The collision of white cells with the vessel endothelium lining stimulates them and they increasingly adhere to the vessel wall and this adherence of cells to the vessel wall is known as pavementing of cell. Changes also occur in the endothelial cells of the vessel wall. A sticky film containing fibrin, which entraps polymorphonuclear leukocytes and other blood cells such as platelets, covers their surface.

The passage of fluid through the blood vessel walls is due to changes in the hydrostatic and osmotic pressure. In acute inflammation the arteriolar dilation increases the flow of blood in the inflamed region and the pressure on small blood vessels. Eventually when the pressure in the capillaries exceeds the osmotic pressure of the proteins, fluid will leave the vessels and infiltrate into the tissues and form an exudate. Normal interstitial fluid has a low protein content and low specific gravity. In acute inflammation there are changes in the permeability of blood vessel wall, which allow both proteins and cells to pass through it. Although the passage of plasma proteins through the vessel wall is a passive process but cells leave by a different mechanism. The first cells to leave the blood vessels are the PMNLs, but if inflammation persists, monocytes follow and after some time become the dominant cells infiltrating into the inflamed tissue. Erythrocytes can also leave the blood vessels by a passive movement known as diapedesis.

After they leave blood vessels, white cells show directional movement towards the injured tissue. About 90% of PMNLs are neutrophils. Infact the neutrophils are the typical cells of acute inflammation. *In vitro* neutrophils move faster than the monocytes but the neturophils are short lived, whilst monocytes and macrophages are comparatively long-lived cells. These factors may explain why neutrophils are predominant in acute inflammation, but monocytes, and macrophages and other cells such as plasma cells are predominant in established or chronic inflammations.

Chronic Inflammation

Chronic inflammmation may arise following acute inflammation or the response may be chronic almost from the onset. Chronic inflammation usually occurs when acute inflammation remains unresolved due to either the persistence of the injurious agent or to some interference in the normal healing process. Chronic inflammation is associated with a range of inflammatory diseases such as rheumatoid arthritis, hepatitis, chronic obstructive pulmonary disease, various inflammatory diseases of gastrointestinal tract and chronic inflammatory periodonal disease. Chronic inflammatory disease shows a pathological immune response probably due to the presence of a perpetuating antigenic determination.

Cells derived from three lines *viz.*, mononuclear phagocyte, lymphoid cells and fibroblasts infiltrate the tissues. In addition there may be granulocytic leukocytes, particularly eosinophils. Macrophages, epitheloid cells, or multinucleated giant cells represent mononuclear phagocytes. Lymphoid cells are present as lymphocytes, plasma cells, or immunoblasts. Fibroblasts produce collagen and ground substance and proliferate to form more fibroblasts. At varying stages during the course of the lesion, lymphocytes, fibroblasts, or granulocytes may predominate, but the most important unit of chronic inflammation is almost always the macrophage and its derivatives, the epitheloid cells and the giant cells. Necrosis is also common in chronic inflammatory lesions. The overall participation of the dividing cells and their efforts in producing new tissue have resulted in chronic inflammation, being described as proliferative, in contrast to the exudative features of acute inflammation. In acute inflammation, macrophages disappear in a few days because of death of the cells, their removal by lymphocytes or migration elsewhere. In chronic inflammation the persistence of macrophages may result from their local proliferation, from longevity of the cells in the tissue, or from their continuing mobilization from the bone marrow.

Although the mechanism of inflammation has been studied extensively, it is only recently that major attention has been paid to the target areas available for the pharmacological attack. Only a few drugs help to cure the disease. Many drugs assist the body's own healing process by holding a disease at bay, thereby allowing the natural defenses to build

up or recover after the initial onslaught of disease. Other drugs such as non-steroidal antiinflammatory drugs merely relieve the symptoms of disease such as the pain and swelling in arthritis, without actually affecting the disease process. The major target areas identified for the pharmacological attack are as followed.

1. Factors responsible for the onset of inflammation.
2. Mediators released during initial injury
3. Non specific endogenous antiflammatory factors evoked by the injured tissue.
4. The processes attempting to restore normal function.

These target areas are inter-related as a chain reaction and the interruption of this chain at any point helps and lead into the success.

Biochemical Events

A series of characteristic changes occur in the small blood vessels and the related tissues within the damaged area, when the living tissues are injured. The reaction in the first few hrs. is more or less independent of the nature of the injurious agent. The biochemical events that form the basis of acute inflammation are triggered by a number of compounds. The biochemical changes occur simultaneously and aid numerous changes. The different compounds with diverse chemical structure and actions aiding to inflammation are called inflammatory mediators, which are derived both from tissues and vascular components of the damaged area. Most of the cells release histamine and eosinophil chemotactic factors from their granules and also form and release prostaglandins, leukotrienes (Brain *et al.*, 1985) and platelet activating factor. Serotonin, a vasodilator in man, does not produce significant change in permeability in rats, however, it appears to be a potent inflammatory agent. (Declerk & Harman, 1983). Neutrophil release LTB_4, a potent chemotactic agent for stimulating and recruiting more neturophils (Bray *et al.*, 1981) may also release several neutral and acid proteases as well as cationic protein that stimulates the release of the contents of the mast cell granules (Safayhi *et al.*, 1992). The lymphocytes release a variety of peptides called lymphokines that inhibit the migration of macrophages, neutrophils, basophils, and eosinophils, exert cytotoxic actions, induce lymphocyte proliferation or induce vascular permeability. Their peptides include interleukins and interferans (Camp *et al.*, 1986).

The kinin system, the complement system and the clotting and fibrinolytic system of plasma provide more mediators than influence inflammation. These mediators enhance the vascular symptoms of inflammation. Stimulation of factor XII or Hageman's factor in the clotting system activates pre–kallikrin to kallikrin, which gets converted into

kininogen and then to bradykinin, as well as activates more factor XII. The latter converts plasminogen proactivators to plasminogen activator that converts plasminogen to plasmin. This, in turn activates the complement system that mediates a series of peptides and cytotoxic agents.

MEDIATORS OF INFLAMMATION

It is recognised that inflammation differs from species to species. In same species from one tissue to another and also in the same tissue according to the type of traumas. Yet the early inflammatory events induced by various trauma in different species and tissues have much in common, as far as sign and symptoms are concerned (Ferreira & Vane, 1979). Several highly active substances are liberated locally in tissue during inflammatory reactions. In different types of inflammation, some mediators may have more prominent role than others. The sequence of mediator release may also be important. For instance, in anaphylactic shock there is an explosive and simultaneous release of histamine, SRS-A, PGE_2 and $PGF_{2\alpha}$ and rabbit aorta contracting substance (Piper & Vane, 1971). However, in the inflammatory response to subcutaneous injection of carrageenan in rat, there is sequential release of the mediators (Willis, 1969) at first; there is an output of histamine followed by the detection of bradykinin. After 3 h little PG activity (<5 ng/mL) was observed but this concentration gradually rose to an average plateau of 80 ng/mL between 18 h to 24 h.

Once the initial reaction to injury has developed, the subsequent changes within the damaged area depend upon the severity, nature and duration of action of the injurious agents. If this is of brief duration or is rapidly and successfully overcome by the defense mechanism of the host, the inflammatory changes will either resolve completely or subside leaving a variable amount of scar tissue with in the injured area. Some types of persistent stimuli lead to massive continued cell migration and to suppuration (pus formation). Cells and tissue changes caused by mediating substances that originate from various cells or plasma are the salient features of the inflammatory process. Stimuli, which trigger the inflammatory process, activate these mediators or lead to their release. Different groups of cells contain several potent mediators. The cells most studied in this respect are neutrophills (polymorphonuclear neutrophills leukocytes), basophills, mast cells and platelets and to a lesser extent macrophages and lymphocytes. These mediators are released in a sequential manner from the inflammatory site by the effected organelles in which histamine is the first to come followed by serotonin bradikinin, members of prostaglandin series and the other related mediators.

The onset of inflammation begins with the migration of various inflammatory cells at the site of inflammation. Once inflammatory cells arrive at the site of inflammation, they release mediators, which control

the later accumulation and activation of other cells. However, inflammatory reactions initiated by the immune system, the ultimate control are exerted by the antigen itself, in the same way as it controls the immune response. For this reason, the cellular accumulation at the site of chronic infection, or in autoimmune reactions, is quite different from that at sites where the antigenic stimulus is rapidly cleared.

Inflammatory mediators are soluble, diffuseable molecules that act locally at the site of tissue damage and infection and more distant sites. These mediators are

a) Exogenous mediators

b) Endogenous mediators

Exogenous Mediators

The bacterial products and toxins can act as exogenous mediator of inflammation. Notable among these is endotoxin or LPS of gram negative bacteria. The immune system of higher animals has probably evolved in a variable sea of endotoxin, so it is not surprising that this substance evokes powerful responses. For example, endotoxin can trigger complement activation, resulting in the form of anaphylatoxins C3a and C5a, which cause vasodilation and increase vascular permeability. Endotoxin also activates "Hageman factor", leading to activation of both the coagulation and fibrinolytic pathways as well as the kinin system. In addition, endotoxin elicits T cell proliferation as have been described as superantigen for T cells.

Endogenous Mediators

These mediators are produced from within the immune system itself, as well as other systems. For example they can be derived from molecules that are normally present in the plasma in an inactive form. Mediators of inflammation are also released at the site of injury by a number of cell types. These cell types either contain them as preformed molecules within storage granules, *e.g.*, histamine or rapidly switch on the machinery to synthesize the mediators when required *e.g.*, metabolites of aracidonic acid such as prostaglandins.

Further more the inflammatory mediators are early phase mediators and the late phase mediators. The early phase mediators are produced by the mast cells and platelets and are especially important in acute inflammation which includes mainly histamine the first to come at the site of inflammation followed by serotonin and other vasodilators like bradykinin. The chemoattractants like C5a and cytokines such as interleukine-1 (IL-1), IL-6 and tumor nacrotic factor-α are also early phase mediators. Late phase mediators are responsible for the regulation of vascular event occuring later after 6 to 12 h of the initiation of inflammation these include the products of arachidonic acid.

Histamine

Histamine is a multifunctional substance exerting influence on many types of cells and physiological processes through a distinct set of G protein-coupled receptors. Generally, histamine modulates inflammation and allergic responses via H_1 receptors (Ash & Schild, 1966), gastric acid secretions through H_2 receptors (Black *et al.*, 1972), and neurotransmitter release in the central nervous system via H_3 receptors (Arrang *et al.*, 1983). All of these histamine receptor types are members of the supper family of G protein coupled receptors (Lovenberg *et al.*, 1999). Recently cloning and characterization of a new class of histamine receptor, H_4 has been reported (Nakamura *et al.*, 2000; Liu *et al.*, 2001). These receptors clearly documented the expression of H_4 receptor mRNA on variety of cell types including peripheral blood mononuclear cells, neutrophils, eosinophils, mast cells and resting $CD4^+$ cells. These findings are highly suggestive of a role for the H_4 receptor in immune and /or inflammatory modulation.

Histamine is the most important vasoactive inflammatory mediator released at the site of inflammation by the inflammatory cells. It is one of the primary mediators released and plays an important and pivotal role in the development and further progression of inflammatory reaction. However, the contents of histamine dwindle within the first sixty min and antihistaminics have no effect on the delayed permeability response. Thus, histamine is mainly important in the early phase of inflammatory responses and in immediate IgE mediated hypersensitivity reaction. Being a biologically potent low molecular weight endogenous biogenic amine, it is distributed in mast cells and basophil granules. It is also present in human platelets. The release of histamine from platelets (platelet release reaction) is stimulated when platelets aggregate after contact with collagen, thrombin, ADP and antigen- antibody complexes. Platelet aggregation and release are also stimulated by platelet activating factor derived from mast cells during IgE-mediated reaction. It is stored in mast cells and basophils largely complexed to mucopolysaccharide such as heparin. It is synthesized from L-histidine by histidine decarboxylase.

Two forms of histamine have been described *viz.*, a storage or bound form in the mast cells and intrinsic or free form. Its wide distribution would appear to make it ideally suited as a mediator of acute inflammation and of acute anaphylactic reaction. Widely distributed in the inflammatory cells, histamine has diverse functions including primary, local dilation of small vessels, widespread arteriolar dilation, local increased vascular permeability by contracting endothelial cells, the contraction of non vascular smooth muscles, chemotaxis for eosinophils and blocking T-lymphocyte function. It acts mainly on the microcirculation through H_1 receptors (Buss, 1979). The H_2 receptors mediate a number of antiinflammatory effects, including the inhibition of eosinophil chemotaxis but cause the vasodilation.

It appears, therefore, that the role of histamine in the inflammatory response is transient and other mediators and mechanisms are required to explain the continued vascular and cellular changes characterizing the progression and regression of the overall response.

Serotonin

Serotonin (5-hydroxytryptamine) is a biogenic amine that was first discovered in the gut (Vialli & Erspamer, 1933). Serotonin was later shown to correspond to a vasotonic substance found in the serum and was therefore called serotonin (Rapport *et al.*, 1948) Serotonin is also found in brain (Twarog & Page, 1953) and is involved in a wide range behaviors such as sleep, appetite, inflammation and pain perception, locomotion, thermoregulation and sexual activity (Wilkinson & Dourish, 1991). Furthermore, serotonin drugs are used in the treatment of a number of pathological states such as migraine, depression, and anxiety (Sleight *et al.*, 1991).

The multiple action of serotonin is mediated by the specific interaction of this amine with several receptors. Pharmacological and physiological studies identified distinct receptors that were designated 5HT-1A, 1B, 1C, 1D, 2,3, and 4 (Saudou & Hen, 1994). However, some pharmacological studies suggested the existence of additional serotonin receptor subtypes. Further, the classification of 5HT receptor subtypes takes into account not only their pharmacological profile and coupling with second messenger but also their sequences (Saudou & Hen, 1994). Except for 5HT-3 receptors, which are ligand-gated ion channel receptors, all other 5HT receptors belonging to the large family of receptors that interact with G protein.

Serotonin is the most important vasoactive mediator that is stored in the mast cell and basophil granules in rodents. It is an endogenous biogenic amine, which, like histamine, has potent effect on small blood vessels and smooth muscles of certain mammalian species. The possible role of serotonin in acute inflammation has not yet been clearly described. In many animal species, mast cells and platelets contain considerably large amount of serotonin which is released along with histamine by many of the inflammatory agents *viz.*, dextran, turpentine and egg albumin (Singh, 1983). Serotonin increases vascular permeability when injected subcutaneouly in small amounts. Furthermore, in some of the studies it has been observed that animals depleted with serotonin showed poor response to local inflammatory reaction produced by different oedemogenic agents. Serotonin is also capable of dilating capillaries producing contraction of nonvascular smooth muscles. Most of it is stored in the gastrointestinal tract and central nervous system but a large amount is stored in the dense granules of platelets.

Bradykinin

Kinins are produced from precursor kininogen via the action of proteolytic enzymes. Two major biochemical cascades for the production of kinins have been described. One within the cardiovascular system activated in association with the blood clotting and another in many other tissue systems activated following tissue injury and inflammation. Bradykinin is the major kinin liberated in the plasma, while in tissues kallidin (Lysyl-bradykinin) is the major kinin formed. Both bradykinin and lysyl-bradykinin are rapidly degraded by kininases to generate the active metabolite des-Arg^9 bradykinin or des-Arg^{10} kallidin, respectively, which also contribute to inflammation and hyperalgesia (Dray & Perkins, 1993).

Bradykinin has been more extensively characterized than kallidin. It exerts several proinflammatory effects, including (a) plasma extravasation; (b) stimulation of free radical, prostaglandin, and cytokine production from a variety of cells; (c) stimulation of postganglionic sympathetic neurons to affect blood vessels and to release prostanoids; (d) degranulation of mast cells to release histamine and other proinflammatory mediators; (e) chemotaxis of lymphocytes to the sites of injury; and (f) mitogenic activity, possibly relating to tissue repair (Dray & Urban, 1996). In addition bradykinin is one of the most potent endogenous algogenic substance which induces pain by stimulating nociceptors directly (skin, joint, muscle) and by sensitizing them to other stimuli. Indeed there is a strong synergism between the actions of bradykinin and other algogenic substances such as PGs and 5-hydroxytryptamine.

The effects of kinins are mediated *via* two main classes of G protein–coupled receptors, B1 and B2. Additional evidences for B3 receptor have been provided by antagonistic studies through pharmacological and molecular techniques (Farmer, 1995). In humans, B1 and B2 receptors are composed of 353 and 364 amino acids respectively representing distinct gene products. Thus couple primarily through the $G_{q/11}$ family of GTP-binding proteins (Hall, 1997). The B2 receptor is expressed more commonly in many tissues and is the preferred site of action for bradykinin and kallidin. Studies with selective antagonists have shown that B2 receptors make a major contribution to inflammatory pain and hyperalgesia (Sterauka *et al.*, 1988; Heapy *et al.*, 1993; Perkins *et al.*, 1993). On the other hand, B1 receptor is encountered less frequently but can be constitutively expressed in certain tissues such as blood vessels. This receptor is preferentially activated by the metabolites des-Arg^9 bradykinin or des-Arg^{10} kallidin. B1 receptor expression markedly increases during inflammation or infection. The significance of this behavior is not entirely clear but under these circumstances, B1 receptor makes an important contribution to the hyperalgesia (Dray & Perkins, 1993).

Several potent B2 antagonists with analgesic and antiinflammatory activity have been made. These include the peptide analogues *viz.*, NPC567, Hoe 140, NPC16731, the dimeric peptide CP-0127 (bradycor) and a non peptide antagonist, WIN 64338 (Hock *et al.*, 1991; Cheronis *et al.*, 1992; Sawutz *et al.*, 1994). Less is known about the B1 receptor, but it has little homogeneity with the B2 receptor (Mauke *et al.*, 1994), and in keeping with current pharmacological studies, antagonists for this receptor will likely be different from those developed at B2 sites. However, both B1 and B2 receptor antagonists are likely to be useful analgesic and antiinflammatory agents.

The kinins, bradykinin and lysylbradykinin, are important mediators of inflammatory response. In asthma also they mediate inflammation with potent bronchoconstrictor actions. The endogenous release of nitric oxide may inhibit bradykinin-induced bronchoconstriction (Kharitonov *et al.*, 1999). They are liberated from precursor molecules, kininogens, by the action of various proteases, collectively known as kininogenases. Three types of kininogens have been identified *viz.*, high- and low molecular weight kininogen and T-kininogen. These molecules are synthesized by the hepatocytes and are released into the plasma, where in addition to releasing kinins, they function as cofactor in the cogulation pathway, inhibitors of cysteine protease enzymes and part of the acute phase response. Three different pathways may lead to kinin formation during inflammation *i.e.*, the generation of bradykinin as a result of activation of the Hageman factor and the production of plasma kallikrein, the production of lysylbradykinin by tissue kallikreins and the action of cellular proteases in kinin formation. As bradykinin is such a potent peptide, its activity and its formation must be carefully controlled. Activation of the pathway is controlled internally by the presence of inhibitors for each of the active components. Complement component C1 inhibitor controls the activity of the activated Hageman factor, while α2- macroglobulin and C1 inhibitor act as kallikrein inhibitor. There are a variety of enzymes in plasma that control bradykinin activity, including carboxypeptidase N, which removes the C-terminal arginine residue, thus inactivating the peptide.

Prostaglandins

Prostaglandins belong to a family of chemical messengers, which have been studied extensively over the last 20 to 25 years. Prostaglandins were first identified in semen, and received their name because at that time they were thought to origin from the prostate gland. However, it is now well known that the prostaglandins in semen are synthesized mainly by the seminal vesicles, and the prostate makes a small contribution. Further, it has been discovered that prostaglandins are produced in almost all tissues of the body, and are ubiquitous messenger molecules with a variety of actions. They are fatty acids synthesized from arachidonic acid, a long chain

polyunsaturated fatty acids, and have been classified into groups from prostaglandin A to prostaglandin I according to structural variation in the five-carbon ring at one end of the molecule. There is further subdivision based on the number of double bonds in the two side chains.

Prostaglandins are key mediators of inflammation and play critical physiological role in tissue homeostasis and function. Tissue injury and certain other stimuli such as inflammatory stimuli cause the local release of chemical mediators such as prostaglandins (Bolten, 1998). During an inflammatory reaction, there is a rapid activation and recruitment of inflammatory cells such as macrophages and neutrophils, release of numerous cytokines and enzymes, and production of pro-inflammatory mediators. Prostaglandins are synthesized from arachidonic acid, the initial step of which is catalyzed by the enzyme cyclooxygenase. They are implicated in the inflammatory response and in sensitizing nociceptors to the actions of other mediators. Occuring during the acute and chronic inflammatory illness, prostaglandins are produced at the site of inflammation where it is believed that they mediate many of the symptoms of inflammation, such as oedema and pain.

Arachidonic acid, precursor of prostaglandins, is released from cell membranes by phospholipases. Cyclooxygenase catalyzes the addition of molecular oxygen to arachidonic acid to form initially the endoperoxide intermediate prostaglandin G_2 (Peskar & Maricic, 1998). The same enzyme also possesses peroxidase activity that catalyzes the reduction of this prostaglandin to form PGH_2. PGH_2 may then react with a number of enzymes sometimes called isomerase to become one of the prostagladins or thromboxanes. The prostagladins are characterized by a five-membered ring which determines the type of prostaglandin. Two products of this reaction are PGD_2 and thromboxane A2. Two other prostaglandins are produced, *viz.*, PGE_2 and PGI_2, which act as mediators of the vascular phases of inflammation. They are both potent vasodilators. In addition, they act synergistically with certain of the vasoactive mediators, such as histamine and kinnis, to increase vascular permeability. They also stimulate osteoclastic bone resorption suggesting that bone erosion in chronic inflammatory disease may be mediated at least in part by prostaglandins.

Prostaglandins are found throughout the body in almost all tissues. The physiological actions of prostaglandins are medited through an interaction with specific receptors coupled to different secondary messenger system that ultimately produce an array of biologic effects. The same pathway used to generate prostaglandins for the inflammatory process is used to produce prostagladins in other tissues. Generally, the prostaglandin produced in a tissue is a function of specific cell type. For example, endothelial cells produce primarily the vasodilatory PGI_2, platelets generate

the vasoconstrictor TXA_2, renal prostaglandins are involved in regulation of renal blood flow and prostaglandins in the gastric mucosa have cytoprotective or, more appropriately termed, gastroprotective functions.

The major difference in the production of prostaglandins is the specific type of cyclooxygenase used in the pathway. Until recently, it was believed that there is only one type of cyclooxygenase throughout the body. However, Simons and colleagues (1989) identified a second form of cyclooxygenase, COX-2 distinguishing it from the originally identified enzyme (Bjorkman, 1998b). Subsequent work has shown that the two enzymes have a similar size, nearly identical enzyme kinetic, and have 75% amino acid homology, with complete preservation of the catalytic sites for cyclooxygenase and peroxidase activity. The genes for the two enzymes are found on different chromosomes.

The two enzymes are known as cyclooxygenase-1 (COX-1) and cyclooxygenase–2 (COX-2). Under normal homeostatic conditions, COX-1 is a constitutive enzyme –present in most cells and tissues-where it functions to produce physiologically important prostaglandins. COX-1 and COX-2 enzymes differ significantly both in protein structure and mRNA. The human cDNA for COX-2 has been cloned and expressed (Hla & Neilson, 1992). It encodes a 70 kD polypeptide of 604 amino acids that is 61% identical to human COX-1. The mRNA of COX-2 consists of 4.1 kilobases and is considerabely larger than the 2.8 kilobase mRNA of COX-1. This is consistent with its inducible nature. Cells use COX-1 to produce prostaglandins in the continuous response to stimulation by circulating hormones and other factors (DeWitt, 1995). This differs significantly from the role of COX-2 and enzyme that is rapidly induced at the site of inflammation and is responsible for the production of pro-inflammatory prostaglandins (Masferrer *et al.*, 1996; Peskar & Maricic, 1998). Under normal conditions, resting neutrophils, macrophages, endothelial cells, and fibroblasts show little or no COX activity but in response to an inflammatory stimulus there is increased activity due to synthesis of COX-2 (Richardson & Emcry, 1995) distribution studies indicate the presence of COX-1 in nearly all normal issue including kidney, spleen, stomach, liver, heart and brain. In contrast virtually no expression of COX-2 in normal tissues is detectable. COX-2 expression is readily induced in response to pro-inflammatory stimuli in cells *in vitro* and *in vivo*, including macrophages, monocytes, synovial cells and endothelial cells. COX-2 is not expressed to a significant degree in resting tissue, except in brain, in reproductive organs and kidneys (DeWitt, 1995). The function or activity of COX-2 in these tissues is not known yet.

In the gastrointestinal tract, prostaglandins PGE-2 and PGI-2 are derived from COX-1 activity and have been correlated to the long term protection of mucosal lining from damage (Frolich, 1997). COX-1 is found in the blood platelets where it is essential for the synthesis of thrombaxane

A-2. COX-1 activation leads to the production of prostacyclin which is ant-ithrombogenic when released from the endothelium and cytoprotective when released from the gastric mucosa. This cytoprotection of the gastric mucosa extends to both exogenous damaging substances as well as endogenously produced gastric juice. In the kidney, prostaglandins are synthesized mainly in the renal medula, the ascending loop of Henley, and the cortex. Renal prostaglandins influence several functions including, total renal blood flow, distribution of renal blood flow, sodium and water reabsorbtion and renin released. These functions with the possible exception of renin secretion seem to depend on COX-1 activity. Renin secretion has been linked to COX-2 activity because the presence of COX-2 has been demonstrated in the macula densa and found to up-regulate with sodium deprivation.

A close link between the inflammatory response and COX-2 induction is becoming evident (Frolich, 1997). On the basis of studies using pro-infalmmatory and anti-inflammatory cytokines to induce inflammation, COX-2 is induced in macrophages, while the anti-infalmmatory cytokine, IL-10 down-regulates COX-2 expression. Synovia obtained from patients suffering from osteoartritis and rheumatoid arthritis shows COX-2 in mononuclear cells, endothelial cells of blood vessels and synovial fibroblast cells.

The evidence for the involvement of prostaglandins in arthritic diseases is extensive (Williams & Higgs, 1988; Goodwin, 1991; Malmberg & Yaksh, 1992). The classical inflammation of rheumatoid arthritis consists of redness, oedema, pain and heat. Proataglandins are found at the sites of inflammation and prostaglandins I-2, E1, E2 and D2 cause vasodilation leading to redness (Robinson, 1991). Although prostaglandins I-2, E2, and F2 are only weakly active in producing oedema, they contribute synergestically to the tissue swelling induced by other inflammatory mediators such as bradykinin and histamine. The role of prostaglandins in fever is complex and involves interlukin-1 (Rothwell, 1992). The discovery by Vane in 1971 that NSAIDs such as aspirin exert their antiinflammatory activity by inhibiting the COX enzyme has found wide acceptance although part of the mechanism of action of these compounds probably does not involve prostaglandins (Abramson & Wissmann, 1989; Brooks & Day, 1991).

The effect of prostaglandins on the immune system is complex, incompletely understood and often controversial. Among the many actions of prostaglandins on the immune system, inhibition of B and T cell proliferation (Bray, 1987), inhibition of rheumatoid factor (Alvarellos *et al.*, 1988) and suppression of antigen presentation by macrophages, probably are the most relevant effects as far as arthritic diseases are concerned. Although it is generally accepted that prostaglandin E2 is largely immunosuppressive by virtue of its downregulation of B and T cell functions. It has been recently found that this compound also potentiates Ig class switching both indirectly by inhibiting interferon secretion from TH1 cells

and directly by enhancing IL-4 induced class switching to IgG and IgE (Phipps *et al.*, 1991). T cells, in particular CD4+ helper cells, play a central role in orchestrating the immune responses that underlie rheumatoid inflammation (Cush & Kavanaugh, 1995). Of the various subsets of helper T cells, the Th1 subset in particular is relevant (Miossec & van den Berg, 1997). Growing attention has been paid to the balance of Th1/Th2 subsets of helper cells. The Th1 subset of cells which functions primarily in cell-mediated immune reaction, seems to play the dominant role in rheumatoid arthritis (Miossec & van den Berg, 1997). Therefore, alteration of the Th1/Th2 balance has been suggested to be a practically valuable therapeutic approach.

Prostaglandin E_2 has been implicated in the angiogenesis required for the spread of the pannus in the synovial hyperplasia component of rheumatoid arthritis (Colville-Nash & Scott, 1992).

Cytokines

During the course of non-specific as well as immunologically mediated inflammatory reactions, many hormone-like mediators called cytokines are produced. They regulate local as well as systemic inflammatory host diffense. Unlike many hormones, cytokines are not normally present in plasma and nor are produced by glands, but are autocrine and paracrine mediators that are produced by inflammatory cells and many other tissues in response to invasive organisms, irritants or trauma. Although monocytes and lymphocytes are the major source of cytokines among blood cells yet evident that cytokines are generated by many other cell types (Cassatella, 1995 & 1999). The ability of polymorphonuclear leukocytes (PMNs) to produce such a variety of cytokines readily suggests that these cells might not only be inflammatory and immune responsives, but also other processes such as hematopoiesis, wound healing, angeogenesis, and antiviral defense. PMNs can express proinflammatory cytokines such as TNFα, (Djeu *et al.*, 1990), IL-1α and IL-1β (Lord *et al.*, 1991) and IL-12 (Cassone *et al.*, 1997), antiinflammatory cytokines such as IL-1ra (Fava *et al.*, 1991). Many cytokines have been identified those contribute either directly or indirectly to inflammatory processes. Cytokines along with some growth factors produce specific protein at the site of inflammation (Kujubu *et al.*, 1991).

In general the cytokines are soluble (glyco) proteins, nonimmunoglobulin in nature, released by living cells of the host, which act nonenzymetically in picomolar to nanomolar concentrations through specific receptors to regulate host cell function. They make up the fourth major class of soluble intercellular signaling molecules, alongside of neurotransmitters, endocrine hormones, and autacoids. They possess typical hormonal activities *i.e.*, they are secreted by a single cell type, react specifically with other cell types (target cells) and regulate specific vital functions that are controlled by feedback mechanism. They generally act at short range in a paracrine or autocrine (rather than endocrine) manner.

They interact first with high affinity cell surface receptors (distinct for each type or even sub type) and then regulate the transcription of a number of cellular genes by little understood second signals. This altered transcription, which can be an enhancement or inhibtion, results in changes in the cell behavior.

Target cells, on which cytokines transform their information signal, may be localised in any body compartment (sometimes a long distance from the sight of secretion). Other type of these molecules mostly on neighbouring cell in the microenviornment where they have been released. These are characterised as local hormones and their secretion is brought about by autocrine or paracrine mechanisms. During the paracrine secretion some cytokines may escape cell binding and may spill over into general circulation via lymph or plasma. This is important, especially for the products of lymphoid cells which are mobile after having picked up the message in the microenviornment through out the body. Therefore, their immunoregulatory products, (Lymphokines, monokines, interleukins and other cytokines), despite being of local hormone character, may infact act systemically.

Cytokines are synthesised, stored and transported by various cell types (Cassatella, 1995) not only inside the immune system (lymphokines, monokines, tumour necrotic factor, and interferons) but also by other which are mainly studied by haematology (colony stimulating factors), oncology (transforming growth factors), and cell biology (peptide growth factors, heat shock and other stress protiens). The central role of cytokines is to control the direction, amplitude, and duration of immune responses and to control the modelling and remodelling of the tissues, be it developmentally programmed, constitutive or unscheduled. Unscheduled remodelling accompanies inflammation, infection, wounding and repair. Individual cytokines can have pleiotropic, overlapping and sometimes contradictory functions depending on their concentration, the cell type they are acting on, and the presence of other cytokines and mediators thus the information which an individual cytokine conveys depend on the pattern of regulators to which a cell is exposed, and not on one single cytokine. It is supported that all cytokines form the specific system or net work of communication signals between cells of the immune system, and between the immune system and other organs. In this inter cell signalling network, the signal is usually transferred by means of specific set of cytokines.

Because of the potential and profound biological effects of cytokines, it is not surprising that their activities are tightly regulated, most notably at the level of secretion and receptor expression. Additional regulatory mechanisms are provided by the concomitant action of different cytokines and the presence in the biological fluids of specific inhibitory proteins, soluble cytokine binding factors and specific autoantibodies. The cytokine is a very potent force in homeostasis when activation of the net work is local and cytokines act vicinally in surface-bound or diffusible form, but

when cytokine production is sustained/or systemic, there is no doubt that they contribute to the signs, symptoms and pathology of inflammation, infections, autoimmune, and malignant diseases. TNFα is an excellent example of such duel action. Locally it has important regulatory and antitumour activity but when it circulates in higher concentrations beyond the organ of origin, it may be involved in the pathogenesis of endotoxic shock, cachexia and other serious diseases.

From the inflammatory point of view there are two main groups of cytokines *viz.*, pro-inflammatory and antiinflammatory. Pro-inflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reaction (Beauerle & Baltimore, 1996) whereas, antiinflammatory cytokines belong to the T cell-derived cytokines and are involved in the down-regulation of inflammatory reactions. The cytokines which play the central role in inflammation are IL-1 and TNFα (Arend & Dayer 1990), because the administration of their antagonists, such as IL-Ira (IL-1 receptor antagonist), soluble fragment of IL-1 receptor, or monoclonal antibodies of TNFα and soluble TNF receptor, all blocks various acute and chronic responses in animal models of inflammatory diseases (Dawson *et al.*, 1991). Some of these antagonists are beginning to utilize as antiinflammatory agents in diseases such as sepsis and rheumatoid arthritis. IL-1 and TNFα together with IL-6 serve as endogenous pyrogens. The up-regulation of inflammatory reaction is also performed by IL-11, IFNβ, IFNγ, and especially by the members of cytokines superfamily. On the other hand, antiinflammatory cytokines (IL-4, IL-10 and IL-13) are responsible for the down-regulation of inflammatory response. They are able to suppress the production of pro-inflammatory cytokines. Their strong antiinflammatory activity suggests possible utilization in management of many antiinflammatory diseases, including sepsis, rheumatoid arthritis, inflammatory bowl disease, psoriasis, T cell-mediated autoimmune diseases such as type-I diabeties, as well as in acute graft-versus-host disease. IL-10 is capable of effectively protecting mice from endotoxin-induced shock, a lethal inflammatory reaction mediated by TNFα and IL-I (Firstein & Zvaifler, 1997). The production of most of the lymphokines and monokines such as IL-I, IL-6 and TNFα is also inhibited by transforming growth factor (TGFa), but on the other hand, TGFα has a number of pro-inflammatory activities including chemoattractant effects on neutrophils, T lymphocytes, and unactivated monocytes. TGFα has been demonstrated to have *in vivo* immunosuppressive and antiinflammatory effects as well as pro-inflammatory and selected immunostimulatory activities. When administered systemically, TGFα acts as an inhibitor, but if given locally can promote inflammation. Generally, TGFα stimulates neurovascular iztation and the proliferation and activities of connective tissue cells and is a pivotal factor in scar formation and wound healing. But TGFα has antiproliferative effect on most other cell types including

epithelial cells, endothelial cells smooth muscle cells, fetal hepatocytes and myeloid, erythroid, and lymphoid cells. TGFα is a potent immunosuppressive cytokine that suppresses cell mediated as well as humoral immunity including tumor immunity.

Products of the Complement System

Complement is a complex system containing more than thirty various glycoproteins present in serum in the form of components, factors, or other regulators and/or on the surface of different cells in the form of receptors. These are present in blood serum in an inactive form and are activated by immune complexes (the classical pathway), by carbohydrates (the lectin pathway), or by other substances, mainly of bacterial origin (the alternate pathway). The components of the classical pathway are numbered 1 to 9 and prefixed by the letter C, *e.g.*, C1, C2 up to C9. C1 is composed of three subcomponents C1q, C1r and C1s. The early components of alternate pathway are known as factors and each molecule is named by a letter, *e.g.*, factor B, D, P. The lectin pathway is the same as the classical pathway, only C1q is omitted. All these pathways use in the later stages of activation the same terminal components C5-C9 that form membrane attack complex (MAC). C3 also participates in all pathways.

The complement system influences the activity of numerous cells, tissues and physiological mechanisms of the body. These effects may involve either the whole complement, or only individual components of fragments. Activation of the complement cascade, with the formation of the effector MAC unit, results in cytotoxic and cytolytic reactions. Target cells for MAC action may be heterologus erythrocytes, nucleated cells, bacteria (gram-negative, susceptible to serum), microscopic fungi, viruses with a surface envelope and viruse-infected cells. The results of the cytotoxic complement reaction may be beneficial for the body (elimination of the infectious agent or damaged cells) or harmful (damage to autologus normal cells by immunopathological reactions).

The complement system is a potent mechanism for the initiation and amplification of inflammation. This is mediated through fragments of complement components, which belongs to anaphylatoxins. Anaphylatoxins are proteolytic products of the serine proteases of the complement system C3a, C4a, and C5a. The production of anaphylatoxins follows not only from complete activation, but also from activation of other systems which may directly cleave C3, C4 and C5. Such enzymes include plasmin, kallikrine, tissue and leukocyte lysosomal enzymes, and bacterial proteases. C5a is extremely potent for stimulating neutrophils chemotaxis, adherence, respiratory burst generation and degranulation. C5a also stimulates neutrophils and endothelial cells to express more adhesion molecules. Ligation of the neutrophil C5a receptor is followed by mobilization of the membrane arachidonic acid which is mobilized to prostaglandins and

leukotrienes including LTB_4, another potent chemoattractant for neutrophils and monocytes. Following ligation of C5a receptor, IL-1 is released. Thus, the local synthesis of C5a at the site of inflammation has powerful pro-inflammatory properties. C3b and C4b fragments act as opsonins and enhance phagocytosis. In addition to inducing phagocytosis, ligation of complement receptor on neutrophils, monocytes and macrophages may also stimulate exocytosis of granules containing powerful proteolytic enzymes and free radical production through the respiratory burst which further act as pro-inflammatory.

The activation of complement by immune complexes is normally beneficial. Immune complexes bearing C3b are efficiently removed from tissues and from the circulation by monocytes and other phagocytes. However, there are circumstances in which immune complex production continues at a high level; complement complexes may then prove deleterious. Such complexes may form in tissues, *e.g.*, in glomeruli of patients with autoantibodies to glomerular basement membrane (Goodpasture's syndrome) or at motor end–plates in-patients with autoantibodies to acetylcholine receptors (*Myasthenia gravis*).

Chemotactic Factors

The term chemotaxis refers to the movement of leukocytes (or cells in general), induced by a chemotactic stimulus. Beside chemotaxis, which is the stimulated directional migration, leukocytes also possess two other types of movements *viz.*, random migration which is undirected spontaneous migration and chemokinesis *i.e.*, stimulated unidirectional migration. A chemotactic stimulus is provided by substances that can either attract or repulse the cells. Thus, chemotactic cell movements can be either negative or positive, *i.e.*, the cells may move towards the source of the chemotactic substance (towards an increasing concentration gradient) or in the opposite direction. The positive movement is typical for leukocytes. Substances possessing chemotactic activity are called chemotactic factors (chemotaxins, chemoattractants). Leukocyte chemotaxis is mainly responsible for their mobilization at the inflammatory site. Both exogenous and endogenous chemoattractants participate in this event. Exogenous chemotaxins include bacterial oligopeptides, lectins, denatured proteins, some lipids and lipopolysaccharides. Endogenous chemotaxis are produced by the host organism and are of humoral or cellular type. The humoral endogenous chemotaxins are complement fragment C5a, C5desArg, and Ba, fibrinopeptides, kallikrein and plasminogen activators whereas, the cellular component comprised of LTB_4, platelet activating factor, and chemotactic cytokines.

Interaction between the chemotactic factor and its corresponding receptor triggers a series of coordinated biochemical events, which include changes in the cell transmembrane potential, altered cyclic neucleotide

levels and ion flow across the cytoplasmic membrane and increased glucose utilization and oxygen consumption. The composition of membrane phospholipids is altered and arachidonic acid, released by phospholipases, is metabolized into a number of biologically active intermediates and products. Within a few min, the leukocyte changes from round to a triangular shape that is oriented along the direction of the chemotactic gradient. Reorganisation of cytoskeletal contractile elements, particularly active microfilaments and microtubular structures, contribute to this shape change. Activation of the contractile cell system not only results in the migration but also in other form of movement such as enhanced adherence, spreading, endocytosis and secretion of lysosomal enzymes resulting in the further stimulation of the inflammatory mediators.

The Acute Phase Reactants

Within the spectrum of systemic reaction to inflammation two physiological responses in particular are regarded as being associated with acute inflammation. The first involves the alteration of the temperature set-point in the hypothalamus and the generation of the febrile response. The second involves alteration in metabolism and gene regulation in liver. Three cytokines that are released from the site of injury *viz.*, IL-1, TNFα and IL-6 are considered to regulate the febrile response, possibly as a protective mechanism. It is important to consider the acute phase response (and inflammation) as a dynamic homeostatic process that involves all of the major systems of the body, in addition to the immune, cardiovascular and central nervous system. Normally, the acute phase response lasts only a few days; however, in case of chronic recurring inflammation, an aberrant continuation of some aspects the acute phase response may contribute to the underlying tissue damage that accompanies the disease, and may also lead to further complications.

The second important aspect of the acute phase response is the radically altered biosynthetic profile of liver. Under normal circumstances, the liver synthesizes a characteristic range of plasma proteins at steady state concentrations. Many of these proteins have important functions and higher plasma levels of these acute phase reactants or acute phase proteins are required during the acute phase response following an inflammatory stimulus. Although most of the acute phase reactants are synthesized by hepatocytes, some are produced by the other cell types, including monocytes, endothelial cells, fibroblasts and adipocytes. Acute phase reactants have a wide range of activity that contribute to host defense *i.e.*, they can directly neutralize inflammatory agents, help to immunize the extent of local tissue damage, as well as participate in tissue repair and regeneration. There is a rapid increase in the plasma concentration of many complement cascade components the activation of which ultimately results in the local accumulation of neutrophils, macrophages and plasma proteins. These

participate in the killing of infectious agents, the clearance of foreign and host cellular debris, and the repair of damaged tissue. Coagulation components, such as fibrinogen, play an essential role in promoting wound healing.

Proteinase inhibitors neutralize the lysosomal proteases released following the infiltration of neutrophils and macrophages, thus controlling the activity of the proinflammatory enzyme cascade. The major acute phase reactants in mammals include serum amyloid-A and C-reactive protein or serum amyloid-P component depending on the species. Generally, only one of these proteins is an acute phase reactant in a given mammalian species. In humans normal plasma serum amyloid P component levels remain constant during inflammation but C-reactive proteins can increase upto 1000-folds depending on the disease and its severity. Serum amyloid-A enhances the binding HDL3 to macrophages during inflammation, concomitant with a decrease in the binding capacity of HDL3 to hepatocytes.

The exquisite responsiveness of C-reactive protein to acute phase stimuli, along with its wide concentration range and ease of measurement, have led to plasma C-reactive levels being used to monitor accurately the severity of inflammation and the efficacy of the disease management during an infection. Conversely, some diseases are associated with relatively low plasma levels of C-reactive proteins. C-reactive protein and serum amyloid-P component are archetypal examples of plasma proteins that are likely to be beneficial during the transient acute phase response but these have detrimental effects in chronic inflammation. Acute phase reactant synthesis is under control performed by inflammatory mediators from which several cytokines and hormones specifically regulate the transcription of human acute phase reactants these include TNFα, IL-1, IL-6, IL-11, IFNγ, TGFα and glucocorticoids. In addition, insulin and akadaic acid have been shown to act as inhibitor of the cytokine-derived induction of some acute phase reactants. Most of the increase in acute phase reactant biosynthesis is due to increase (or decrease) gene transcription.

Caspases

Caspases are a family of intracellular cysteine proteases that clear their specific substrates at aspartic acid residues. These proteases have been implicated in two aspects of cytokine biology *viz.*, proteolytic processing and bioactivation of the proforms of certain interleukins such as pro-IL-1β and pro 1L-18 and signaling by apoptosis inducing members of the tumor necrosis factor receptor family, such as tumor necrosis factor receptor-1 (TNFR-1) (CD120a) and Fas (CD95). Abundant evidence, derived from molecular biological, genetic, and pharmacological studies, has demostrated a critical role for specific caspases in the bioactivation or bioactivity of particular cytokines. Moreover, these caspases to play nonredundant roles in a wide variety of cytokine mediated inflammatory and autoimmune

diseases. The involvement of caspases in cytokine biology is examined, with emphasis on relevance to infectious diseases, inflammation and autoimmunity.

Ten members of the caspase family of cysteine proteases in humans have been described (Alnemri, 1997; Salvesen & Dixit, 1997). These proteins are initially synthesized as single polypeptide zymogens that undergo proteolytic processing at specific aspartic acid residues to produce the active enzyme. The active, processed caspases are composed of heterotetramers, containing two large and two small subunits, which form two active sites per molecule. Caspases represent a unique family of intracellular cysteine proteases, with absolute specificity for aspartic acid in the P-1 position of substrates. This fact, together with the observation that procaspase zymogens are processed at aspartic acids to produce the active proteases, creates opportunities both for autoactivation of caspases and for cascades of caspase activation in which one active member cleaves and activates another. The N-terminal region of the zymogens is typically but not always removed from the protein during processing.

Caspases can be grouped into two broad categories (a) the caspase-1 like family and (b) the caspase-3 like family. The categorization of caspases into subfamilies is based on differences in their substrate preference, comparisons of their primary amino acids sequences, and depending on whether their primary function is most relevant to cytokine processing versus apoptosis regulation. The human caspase-1 subfamily consists of caspase-1, 4 and caspase-5. The murine caspase-II protein is probably the orthologue or a close homologue of human caspase-4 (Wang *et al.*, 1998). These caspases share extensive aminoacid sequence identity and are intimately in proinflammatory cytokine processing.

The human caspase-3 subfamily consists of caspase-2, caspase-3, caspase-6, caspase-8 caspase-9 and caspase-10. Compared to the caspases 1 subfamily, these caspases generally share greater primary amino acid sequence. The members of the caspase-3 subfamily are initimately involved in apoptosis, functioning either as upstream initiators or down stream effectors of this cell death process. Based mostly on differences in their prodomains, the caspase-3 subfamily can be further divided into three additional groups. Abundant evidence implicates caspases in inflammatory and autoimmune conditions mediated by cytokines. The production of bioactive interleukin" a critical mediator of endotoxin shock, is absolutely dependent on caspases-1 *in vivo*. Similarly, proteolytic processing of pro-interleukin-18, a major inducer of interferon-γ *in vivo*, appears to be mediated exclusively by caspase-1. Cytolytic T-cells and natural killer cells also induce caspase-1 activation. The central importance of caspase-1 as a mediator of inflammatory responses is further supported by the observation that some types of bacteria produce proteins that directly bind to and activate caspase-1, whereas some viruses express proteins that directly inhibit this protease.

Two forms of interleukin-1, IL-1α and 1L-1β bind to the same receptor(s) explaining their essentially superimpossible bioactivity profiles (Dinarello, 1994). Both IL-1α and 1L-1β are produced initially as intracellular proproteins which require proteolytic processing to result in secretion from cells. These cytokines are principally produced by activated macrophages, though some other cell types can also produce 1L-1β, including microglial cells, endothelial cells, vascular smooth muscle cells and epidermal langerhans cells. 1L-1β stimulates proinflammatory responses in neutrophils, endothelial cells, synovial cells, osteoclasts and other cell types (Dinarello & Wolff, 1993). 1L-1 has been implicated in a wide variety of inflammatory conditions *in vivo*. Multiple animal model experiments have demonstrated a critical role of 1L-1 for inflammatory conditions including arthritis, inflammatory bowel disease. Clinical studies in patients with rheumatoid arthritis suggest that soluble 1L-1R or 1L-1RA may reduce joint tenderness and inflammatory symptoms. (Campion *et al.*, 1996; Drevlow *et al.*, 1996).

The *in vivo* importance of caspase-1 for inflammatory diseases has also been confirmed by animal model experiments using peptide inhibitors of caspase-1 also significantly reduced collagen-induced arthritis in mice. Caspase-1 inhibitors are superior to indomethacin and prednisolne in this animal model of arthritis (Ku *et al.*, 1996). Similarly, the caspase-1 antagonist benzyloxy-valingy alanyl-3(s)-3-amino-4-oxo-5-(2,6-dichlorobenzoyl-oxy acid) ethyll ester, which inhibits LPS - induced IL-1β secretion from a monocyte cell line with an IC_{50} of 24 mm, suppressed carrageenan-induced paw oedema in rats by upto 60% when administered 1 h after the inflammatory agent (Elford *et al.*, 1995). Though overexpression of caspase-1 can induce apoptosis (Miura *et al.*, 1993), lack of caspase-1 does not interfere with the normal cell turnover required for tissue homeostasis, caspase-1 knockout mice develop normally and have no apparent increased incidence of tumors or other anomalies (Kuida *et al.*, 1995). Moreover, absence of caspase-1 does not interfere with any immune system functions. The T-cell proliferative responses to listeria monocytogenes and delayed type hypersensitivity reactions for example, are reportedly normal in caspase deficient mice, suggesting that the inflammatory cytokines generated by caspase-1 dependent processing may be more relevant to macrophage-mediated inflammatory responses than to T cell-dependent immune reactions (Gu *et al.*, 1997) Tumor necrosis factor-α (TNF-α) has been implicated at some level in most inflammatory and auto immune diseases, as well as in graft rejection, heart failure and other conditions LPS-induced production of TNF-α is some what reduced in caspase-1 and caspase-II knockout mice (Wang *et al.*, 1998) but probably not enough to be of physiological relevance. Interferon-γ, which is linked to the pathogenesis of inflammatory and other autoimmune diseases are elevated indirectly by caspase-1. The indirect mechanism of caspase-1 production of interferon-γ has been demonstrated by experiments

in caspase-1 knockout mice (Gu *el al.*, 1997). Hence caspase-1 represents an attractive target for treatment of autoimmune, inflammatory and other diseases when these cytokines are implicated.

It is quite evident that caspase are absolutely required for the production of several proinflammatory cytokines including IL-1α, IL-1β, IL-18 and probably interferon-γ. These cysteine proteases also play a requisite role in the signaling mechanisms. Human diseases involving these cytokines therefore, are potentially amenable to modulation by caspase inhibitors. Further investigations of the effects of caspase inhibitors are now required. From this analysis, clinical indications for caspase- inhibiting drugs can be deducted and appropriate clinical trials initiated to test their efficacy in humans.

Nitric Oxide

Nitric oxide (NO), a small hydrophobic inorganic gas molecule, highly versatile, potent, short lived and free radical which acts as a unique neuronal messenger molecule for both intra and intercellular messages involved in the regulation of diverse physiological processes including smooth muscle contractility, platelet reactivity, central and peripheral neurotransmission and the cytotoxic actions of immune cells. It is a relatively stable unchanged radical that readily crosses lipid membranes and interacts with a few specific targets. It also reacts with other species possessing unpaired electrons, to yield secondary products that are often more reactive. It has become a species of extreme biological interest due to the diversity of its physiological functions and general ubiquity. NO is crucial for many physiological functions, an inappropriate release of this mediator has been linked to a number of pathologies. Thus agents that modulate the activity of NO may be of considerable therapeutic value, in particular that reduce the formation of NO. It may be beneficial in pathophysiological states in which excessive production of NO is the contributory factor. These include diseases, such as septic shock, neurodegenerative diseases and inflammation.

In fact NO is a colorless gas at room temperature and pressure. Its maximum solubility in water is similar to that of molecular oxygen, 2-3 nM. It is a fairly nonpolar molecule which could be expected to freely diffuse through membranes. One of the most unique and outstanding chemical features of NO is that it is a paramagnetic species. Using the most basic bonding description, it is immediately evident that NO has an unpaired electron. Although much of the chemistry of NO is dominated by the fact that it is a radical, it does not possess the type of reactivity normally associated with other radicals. For example, unlike other, carbon, oxygen or nitrogen centered radicals, NO does not even have the tendency to dimerize *i.e.,* at standard temperature and pressure, NO tends to remain in monomeric form. This lack of dimerization has been attributed to the fact

that overall bonding does not increase when two NO molecules interacted (Palmer *et al.*, 1988) thus, the formal bond order in NO is 2.5, while the bond order for ONNO is 5.

N = O	O = N-N=O
Bond order 2.5	Bond order = 5 (still 2.5 bonds per NO)

NO which is one of the smallest biologically active messenger molecules has a wide range of physiological and pathological actions. It is synthesized from the terminal guanidino nitrogen atom of L-arginine by the action of cytosolic enzyme nitric oxide synthase (Hibbs *et al.*, 1987, Knowles *et al.*, 1989, and Bredt *et al.*, 1991). Nitric oxide synthases (NOS) constitute a family with atleast three distinct isoforms. Inorder of their molecular characterisation these include neuronal (cNOS) (Murphy *et al.*, 1993), inducible (iNOS) (Leone *et al.*, 1991) and endothelial (ecNOS) (Marletta, 1991) synthases. This nomenclature is based on the tissue of origin from which original protein and cDNA were first isolated.

Although the three isoforms have different molecular weights and variable cofactor requirements, all of them are dependent on NADPH, and show similarities with cytochrome P-450 reductase and also with the bacterial enzymes sulfite reductase and cytochrome P-450 BM3. The formation of NO by NOS is linked to incorporation of molecular oxygen into the molecule (Bredt & Snyder, 1990).

NOS has been proposed to form NO and L-citrallin in two steps, the first step being the formation of N-hydroxy-L arginine and in the second, its three electron oxidation. Both steps may utilize different heme-based oxidants *i.e.*, a perferryl species, $[FeOO]^+$, for the first step and a peroxoiron species $(FeO)^{3+}$ for the second step. Both of these are produced when heme reacts with molecular oxygen (Moncada *et al.*, 1991). All forms of NOS contain four prosthetic groups, flavin-adenine dinucleotide, flavin mononucleotide, tetrahydrobiopterin and a heme complex, iron protoporphyrin IX. All NOS isoforms are dependent on calmodulin. In the inducible isoform calmodulin is already present in a tightly bounded form. The role of calmodulin is to control the electron transfer from NADPH to the flavins, possibly by causing a reorientation of the reductase and oxygenase domains of NOS, perhaps moving them into a favourable position for electron transfer between them. The function of tetrahydrobiopterin is unclear, but may substitute for peroxy-heme to carry out nucleophilic attack on hydroxyarginine or perhaps may act as a stabilizer of the active structure of NOS (Moncada *et al.*, 1991).

cNOS and ecNOS are the constitutive isozymes and are calcium/ calmodulin dependent, whereas iNOS is absent from the mammalian cells but under physiological conditions is induced by proinflammatory stimuli,

such as bacterial lipopolysaccharide or the cytokines *viz.*, tumor necrosis factor (TNF), interleukin-1 (IL-1), or interferon-γ (IFN-γ), as well as their combination. cNOS and ecNOS constitutive isozymes, when activated, release short "Puffs" of NO from neurons and endothelial cells which is important in both intercellular and intracellular signalling (Charles *et al.*, 1993). iNOS, the inducible isozyme is induced in many cell types including vascular smooth muscle cells, endothielial cells, hepatocytes macrophages, neutrophils, chondrocytes and cynoviocyted (Stadler *et al.*, 1991; Stefanovic-Racic *et al.*, 1993) in response to inflammatory and immunologic simuli. iNOS generates much larger quantities of NO over longer period of time (nanomoles, rather than picomoles). The NO generated by macrophages activated by cytokine and endotoxin contributed to their cytotoxic and cytostatic properties against target cells (Palmer *et al.*, 1993). Although NO generated by constitutive NOS appears to be beneficial in many physiological processes, the excess of NO generated by iNOS has been implicated in the pathogenesis of various inflammatory and immunologically mediated diseases including graft-vs-host, diabetes, viral infections and arthritis (Ialenti *et al.*, 1993)

During inflammation profound physiological changes are often observed including vasodilation, fever and activation of the immune system. These changes are induced by a myriad of cellular mediators, such as endotoxin, various cytokines and eicosanoids. It has been established that NO plays an important role as one of these mediators in addition to it's described functions in cell-cell communications and neurotransmission. As the product of three isoforms of NO synthases, it is the iNOS which is expressed by a wide variety of cell types in response to stimulation (Nissler & Billiar, 1993) producing much large quantities of NO relative to the two other constitutive isoforms during sepsis and inflammation.

Cell type that expresses iNOS in response to stimulation by certain cytokines and microbial products includes hepatocytes, macrophages, Kupffer's cells and chandrocytes. The regulation of iNOS expression is quite complex, as it involves a variety of mechanisms within a wide range of cell type. For example, the factors that have been important in the rodent for regulating iNOS expression within hepatocytes, as compared to nonhepatocytes, include transcriptional, posttranscriptional as well as posttranslational mechanisms. Regulation of iNOS gene expression is helped by calmodulin molecules to be fully activated at basal levels of calcium allowing the transcription of enzyme. Thus, transcription with subsequent translation of the iNOS gene appears to play an important role in its regulation. Transcription of the iNOS gene is controlled, both positively and negatively, by a number of inflammatory mediators present during infection and inflammation such as interferon- γ, tumor necrosis factor α interleukin-1β, interleukin-2 and lipopolysaccharide (Xie *et al.*, 1992; Feldman *et al.*, 1993; Lorsbach *et al.*, 1993; Vodovotz *et al.*, 1993). The

expression of iNOS response to these agents varies among different cell types and there is often strong synergy among many of these agents. The specific mechanisms by which transcription of the iNOS gene is initiated appear to be quite complex and remain to be elucidated, however, the possible regulatory sequences within the promoter, regulatory region in both mouse and human iNOS has been identified. iNOS gene expression can also be regulated at posttranscriptional level, possibly by altering the stability of the newly transcribed mRNA.

As L-arginine is the sole physiological nitrogen donor for NO formation, it is clear that regulation of its availability could present one method of controlling cellular rates of NO systhesis. Intracellular arginine may be increased via one of three mechanisms, through uptake from the extracellular fluid, by intracellular protein degeneration, or by endogenous synthesis. There is no evidence that the availability of arginine as a substrate for NO formation is affected by change in the rate of cellular protein degradation. Hepatocytes, macrophages, and pulmonary artery endothelial cells have been shown to up-regulate arginine transport in response to inflammatory mediators that stimulate the production of NO (Bogle *et al.*, 1992; Inove *et al.*, 1993; Lind *et al.*, 1993).

NADPH is an essential cofactor in NO synthesis, as it functions as an electron donor (Stuehr & Griffith, 1992). One and a half NADPH molecules are required for each molecule of NO production. Correlation between the formation of NO and the activity of NADPH-generating pentose phosphate pathway, as well as the rate limiting enzyme glucose-6-phosphate dehydrogenase has been made (Blachier *et al.*, 1991; Corraliza *et al.*, 1993). These correlations suggest that NADPH may be up-regulated by the same inflammatory mediators that induce NO formation. Tetrahydrobiopterin, another essential cofactor in NO synthesis (Stuehr & Griffith, 1992) is thought to be involved in the binding of two inactive iNOS isomers into the active dimeric form.

During sepsis and inflammation a wide array of cell types are induced to express iNOS, which then produces large amounts of NO, that may then interact with various target molecules, such as oxygen, that groups and metals within the prosthetic groups of various enzymes resulting in their activation or inactivation. In addition, NO may interact directly with DNA as well as with toxic oxygen radicals, either potentiating their toxicity or neutralizing them. The cellular response to NO are dependent on the cell types producing NO as well as the local environment, as the effects of NO appear to be primarily limited to paracrine and autocrine activities as a result of the short biological halflife of NO and its interaction with oxyhemoglobin in the blood stream. Thus, it results in the production of inactive nitrate (NO_3^-) and methemoglobin. However, there have been suggestions that NO may exert some endocrine like activity by binding to albumin and glutathione, there by forming stable nitrosothiols that may

circulate and release active NO at distant sites (Gaston *et al.*, 1993; Keaney *et al.*, 1993).

There is evidence that NO can potentiate as well as ameliorate the toxicity of circulating oxygen radicals often seen during sepsis and inflammation as well as during the periods of reperfusion. Rubbo *et al.* (1995) implicated NO involvement in oxygen radical mediated membrane lipid peroxidation. In contrast, in a perfusion liver model exposed to endotoxin, the inhibition of NO synthesis increases superoxide (O^-_2) mediated injury (Bautista & Spitzer, 1994). It appears that NO can combine with O^-_2 to form the intermediate peroxynitrite, which is then protonated to peroxynitrous acid. Depending on the pH of the local environment, peroxynitrous acid degrades to either inactive metabolites or toxic radicals. Within an alkaline environment peroxynitrous acid adopts a cis configuration, with subsequent decay to the inactive metabolite nitrate (NO_3). However, under acidic conditions peroxynitrous acid adopts trans configuration and yields toxic products, hydroxy radical (OH) and nitrogen dioxide (NO_2). Thus, it appears that NO may behave either as a toxic free radical or as an oxygen radical scavenger, depending on the local conditions.

The roles of constitutive and inducible NOS isoforms in inflammation have been extensively studied (Ialenti *et al.*, 1993). It is likely that NO from constitutive eNOS plays a role in the early stages of inflammation as a mechanism to decrease and limit the process by inhibiting white cell activity and platelet aggregation (Moncada *et al.*, 1990) and by inducing vasodilation (Kajekar *et al.*, 1995). In contrast, NO from iNOS contributes too many aspects of chronic inflammation.

In models of acute and chronic inflammation, it has been shown that constitutive NOS accounts for majority of NO activity in early parts of the process, at this point, some polymorphonuclear cells and a few resident macrophages express iNOS. At the peak of chronic inflammations, there is an eightfold increase in total NOS activity, of which greater than 90% can be attributed to iNOS in activated macrophages. The NOS activity is substantially reduced after 14 days as the inflammation disappears.

The use of selective iNOS inhibitors may be of benefit in management of chronic inflammatory processes. Chondrocytes in culture express iNOS (Charles *et al.*, 1993) and decreases their proteoglycan synthesis when stimulated by inflammatory cytokines. Adjuvant arthritis in rats in exacerbated by L-arginine and reduced by NOS inhibitors (Ialenti *et al.*, 1992). In rheumatoid arthritis elevated concentrations of nitrite and nitrate have been found in synovial fluid of patients, which suggests an increased local release of NO (Farrell *et al.*, 1992). However, it is likely that NO plays a role in down-regulating the activity of osteoclasts. Furthermore, NOS inhibitors have been shown to potentate bone resorption and enhance bone loss in animals.

Psoriasis lesions which is a chronic inflammatory disease, contains high levels of iNOS, which is absent from healthy skin and there is increased expression of iNOS (Bruch *et al.*, 1996). Further inflammatory conditions in which excessive NO production by iNOS has been demonstrated include asthma and inflammatory bowel disease (Allcan & Kubes, 1996).

Furthermore, a consequence of the release of early mediators in inflammation is the modulation of threshold and sensitivity of nociceptors leading to inflammatory pain. Prostaglandins play a major role in this process. NO may also be involved but it is not clear at this stage whether NO enhances or decreases the pain threshold. The ability of NO to reduce inflammatory pain may also underlie the anti-nociceptive effects of endogenous compounds such as opiates (Ferreira *et al.*, 1991). Thus, NO may itself possess anti-nociceptive actions. In human studies involving pain in the hand vein, NO also appears to exert nociceptive effects. Following administration of bradykinin or hyperisomolar solution to isolated perfused hand vein, NOS inhibitors markedly reduced the discomfort (Kindgen & Arndet, 1996). As such, this profile of activity suggests a possible therapeutic role for cNOS inhibitors in reducing inflammatory pain.

Free Radicals

The role of free radicals have been implicated in causation of several diseases such as liver cirrhosis, atherosclerosis, cancer, diabetes, ageing and inflammatory disorders etc. and the drugs that can scavenge oxygen radical have great potential in ameliorating these disease processes. Although, the body has several mechanisms to protect the action of oxygen radicals but at times these protective mechanisms are not sufficient when compared to the insult caused to the body. Supplementation of non-toxic antioxidants may have a chemoprotective role in the body in these conditions. The superoxide anion ($O^{\cdot}$), H_2O_2 and hydroxyl radical ($OH^{\cdot}$) are the major ROS which induce cell degeneration by increasing lipid peroxidation of cell membrane, break DNA strand and denaturate cellular proteins resulting in further tissue damage.

The natural cellular antioxidant enzymes include superoxide dismutase (SOD), which scavenges superoxide radicals by speeding up their dismutation, Catalase (CAT) a haeme enzyme which removes H_2O_2 and glutathione peroxidases (GPX), selenium- containing enzyme which scavenges H_2O_2 and other peroxidases. Detoxification of the superoxide anion is not a terminating step in free radical scavenging, since the enzymes catalysed dismutation results in the production of H_2O_2 which accumulates in the mitochondria and cytosol. Unless the peroxide is scavenged by CAT and GPX, it in the presence of iron, may also lead to production of $OH^{\cdot}$. These ROS together with singlet molecular oxygen may attack lipids, proteins and DNA of cells following increased lipid peroxidation chain reactions resulting in wide spread cellular injury and ultimately pathophysiological conditions.

A significant understanding of the reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) in inflammatory diseases is underway. During last two decades interest in the role of free radicals in diseases with an inflammatory component, such as rheumatoid arthritis, inflammatory bowel disease, pulmonary emphysema, atherosclerosis, and neurodegenerative disorders, grew rapidly. Most research into the role of free radicals in inflammation was directed toward the putative role of ROI as damaging agent. Many studies centered on the oxidative modification of biomolecules such as α1- antitrypsin, cartilage proteoglycans and collagen, low-density lipoprotein, DNA, lipids, immunoglobulin G, and so on (Halliwell & Gutteridge, 1985; Merry *et al.*, 1989). The cytotoxicity of free radicals and their reaction products was also investigated, particularly in relation to enthothelial cell function (Kus *et al.*, 1995)

The free radicals were studied in context of a direct, destructive role in biology. In the course of the present decade researchers have increasingly recognized this cellular control aspect of free radical action. Endothelium-derived relaxing factor has been identified as nitric oxide (NO) (Snyder, 1995) and ROI can activate apoptosis, programmed form of cell death, at least under some circumstances (Jacobson, 1996). In these cases, ROI activity plays a critical role in regulating the expression of genes that encode either proteins with potential proinflammatory action, or proteins that may act in a protective fashion such as heat shock protein 32, also known as heme oxygenase (Willis *et al.*, 1996). Fibroblast proliferation and collagen gene expression, associated with the late wound-healing phase of inflammation, have also been linked to intracellular ROI activity (Houglum *et al.*, 1994).

Perhaps the key to future antioxidant drug development for the treatment of inflammatory diseases lies in our ability to modulate the previously mentioned ROI-mediated control pathways when they become perturbed as part of the inflammatory process. The inappropriate activation of the responses referred to earlier, such as overexpression of the particular genes, excess NO production, and apoptosis, may be involved in disease progression. Nevertheless, it should be remembered that these processes, when properly controlled, are physiological. Therefore, ROI/RNI can no longer be regarded solely as damaging species whose complete elimination by antioxidants is bound to have beneficial effects on human health. Indeed, it can be argued that the converse may be the case.

In case of treatment of inflammatory diseases, it may be necessary to define the stage of the disease at which a particular antioxidant therapy may most effectively be used, *e.g.*, during acute inflammatory episodes, or in chronic inflammation, or prophylaxis. Another factor that must be taken into account is the individual variation in sensitivity within the population

(Harris *et al.*, 1994), as well as the different susceptibilities to oxidative stress of different organs (Fraga *et al.*, 1990), tissues (Kristal *et al.*, 1994) and subcellular locations (Richter *et al.*, 1988). An increased susceptibility to the actions of ROI on target lymphocytes of patients with autoimmune disorders could thus be an important mechanism in the pathogenesis of disease such as rheumatoid arthritis by inducing excessive inflammatory responses and increased clonal proliferation of autoreactive lymphocytes via the activation of transcription factors like NF-κB.

These considerations pose a challenge in the search for appropriate antioxidant therapies in human inflammatory diseases. It is suggested that the successful therapeutic manipulation of cellular responses by antioxidant drugs will necessitate the maintenance of a critical balance between free radical activity and antioxidant selectively to specific cell types, or even defined subcellular locations, and within a narrow "concentration window" that blocks "inappropriate" cellular responses, but leaves the physiological levels of free radical activity necessary for normal cell function.

Adhesion Molecule

Leukocytes are the principal actors in the body's defense system against invading microorganisms (Wissman & Cooper, 1993). This defense system has a nonspecific branch consisting of granulocytes and macrophages and a specific branch of lymphocytes. Granulocytes (neutrophils, eosinophils and basophils) release cytotoxic compounds from their intercellular granules to their local environment when they encounter microorganisms. This random destruction happens rapidly but it may also harm healthy tissue of the body. Macrophages, the other class of defense cells from the nonspecific immune system, can ingest and destroy microorganisms by phagocytosis or, in a similar way to granulocytes by the secretion of cytotoxic compounds. However, macrophages can also act more specifically by collaborating with lymphocytes and their products.

The lymphoid system comprises the cellular components responsible for antigen specific immune defense. B-lymphocytes produce antibodies that bind to foreign organisms and facilitate their destruction, either by activating the complement system which in turn can perforate the membrane, or by opsonizing the microorganisms, *i.e.*, trigger phagocytosis due to receptors for antibodies and the macrophage surface. T-lymphocytes act mainly by cell-to-cell contact. One subpopulation of T-lymphocytes recognizes and kills cells which bear foreign antigen; the second subpopulation helps activity of other hemopoietic cells in the immune response or helps to multiply effector cells.

All of these leukocytes patrol the body by circulating through the blood and lymphatic system ensuring a continuous surveillance which is a prerequisite for efficient defense (Anderson *et al.*, 1982). Upon tissue damage

and inflammation, leukocytes are recruited from the blood to sites of injury, and this trafficking displays exquisite specificity (Butcher, 1991; Dunon *et al.*, 1993; Springer, 1994). For instance, neutrophils selectively enter sites of acute inflammation or tissue damage, while eosinophils extravasate into sites of allergic reactions and parasitic infestations. The migration of lymphocytes is even more selective and includes a complex pattern of recirculation that relates to differentiation and activation.

To efficiently protect the body from infectious organisms, the cells of the immune system circulate as nonadherent cells in the blood and lymph, and migrate as adherent cells into tissues when necessary. Rapid transition between adherent and nonadherent states is the key to the dual function of immune surveillance and responsiveness. Circulating lymphocytes in the blood have first to adhere to and then to cross the endothelial lining in order to enter the various lymphoid tissues which are involved in recirculation. One exception is the spleen where there is unhampered access of blood leukocytes to parenchyma (Fawcell, 1994).

Three classes of adhesion molecules are involved in leukocytes endothelial interactions. These are the selectins, integrins, and members of immunoglobulin (Ig like) superfamily.

Selectins

The selectins are a family of adhesive receptors found on leukocytes (L), platelets and endothelial cells (P) or endothelial cells alone (E) (McEver, 1994). They are designated as L-selectin (CD 62L), P-selectin (CD-62P) and E-selectin (CD 62E). They mediate the initial, low-affinity adherence of leukocytes to endothelium, manifested by rolling along the endothelial cell surface under conditions of flow. These receptors belong to a family because they share a common mosaic structure consisting of an aminoterminal C-type lecting domain, a single epidermal growth factor like domain several short consensus repeats similar to those found in regulatory proteins that bind complement, a transmembrane domain, and a short C-terminal cyto plasmic domain. Selectins are known for their binding to carbohydrate ligands via the lectin domain (Rosen & Bertozzi, 1994).

L-selectin was first identified as a homing receptor for lymphocyte migration to peripheral lymph nodes (Watson & Bradlley, 1998) and was found to mediate the binding of lymphocytes to high endothelial venules in peripheral lymph nodes. It has since been found to be constitutively expressed on most leukocytes, including lymphocytes, monocytes, neutrophils (PMN) and eosinophils. Leukocytes can be induced to shed L-selectin from their surface by a variety of activating agents such as formylpeptide, phorbol ester, and lipopolysaccharide. P-selectin is stored in the Weibel-Palade bodies of endothelial cells and the α granules of platelets (Hartwell *et al.*, 1998). Its expression on endothelial cells can be induced by a variety of stimuli, including thrombin, histamine, and phorbol

esters. In response to this stimulation, P-selectin is rapidly redistributed to the cell surface, resulting in expression within min. In some endothelial cells P-selectin is also induced by cytokines or LPS.

E-selectin is expressed on the surface of endothelial cells only following stimulation. Tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), Substance P, LPS, and several other stimuli have been found to induce the expression of E-selectin (Vestweber & Blank, 1999). E-selectin expression requires *de novo* protein synthesis with transcription and translation resulting in peak surface expression in 4-6 h following stimulation *in vitro* with a gradual decrease to basal levels by 24 h.

Integrins

The second class of adhesion molecules involved in leukocyte traffic is the integrin family involved in extracellular material/cell and cell to cell interactions (Hynes, 1992). The term integrin refers to the ability of these molecules to integrate the intracellular media with the extracellular environment. Currently, integrins are considered as a wide family of cell-adhesion receptors, with a broad pattern of cellular expression that exerts a key role as modulator of major cellular events, such as proliferation differentiation and death. Integrins are transmembrane heterodimers consisting of non-covalently linked α and β chains. Integrins are sub-classified based on the specific β sub unit. The sub classes that have been found to be involved in the leukocyte endothelial cell interactions are the β2 integrins, the β1-integrin, and the β7-integrin. The three β2 integrins are leukocyte restricted and are composed of a common β2 sub-unit (CD18) that is noncovalently linked to one of three α subunits. Cell surface expression of β2 integrins is not sufficient for adhesion of leukocytes to endothelial cells. Activation of the β2-integrins due to a change in conformation or postreceptor events is required before adhesion occurs (Aplin *et al.*, 1998). This activation is induced by soluble agents like cytokines, chemotactic factors and coagulation factors etc. and by ligation of other cell surface receptors. The β1 integrin consists of a common β1 subunit (CD 29) noncovalently linked to a α subunit and are found on nearly all cell types. However, one β1–integrin, VLA-4, is found primarily on leukocytes (Werr *et al.,* 1998). It is constitutively expressed on all circulating leukocytes except PMN and like β2 integrin requires activation in order to bind. The β7 integrin is expressed on some B and T lymphocytes and functions as the homing receptor for intestinal lymphoid tissue (Carlos & Harlan, 1994).

Immunoglobulin Superfamily

Members of the immunoglobulin superfamily constitute the third class of adhesion molecules. The superfamily consists of cell surface proteins that

are involved in antigen recognition and complement binding, as well as cell adhesion. Endothelial members of this superfamily involved in leukocyte endothelial cell adhesion.

Adhesion Cascade and Inflammation

The sequence of events involved in leukocyte adherence to vascular endothelium and transmigration into tissue is complex and has been termed the adhesion cascade (Springer, 1994). The first step involves a random contact of the leukocytes with the endothelial cell surface. After initial contact, relatively weak binding of L, P, and/or E selectin with their carbohydrate ligands occurs. Under conditions of flow, these multiple weak interactions are sufficient to slow the leukocyte down, and the leukocyte is seen to roll along the surface of the endothelial cells. The next involves further activation of the endothelial cells and leukocytes by cytokines, chemoattractants, and chemokines that are produced locally (Berlin *et al.*, 1995). This leads to the upregulation and/or activation of the leukocyte integrins and the endothelial Ig-like molecules. The interaction of these two classes of adhesion molecules mediates the firm adherence of leukocytes to the endothelial cell surface (Huber *et al.*, 1991).

The firmly adherent leukocytes migrate across the endothelial cell surface via integrin-Ig like interactions, diapedesis between endothelial cells and then migrate to the sub endothelial matrix via integrin binding to matrix components. (Feng *et al.*, 1998). While the adherence cascade functions normally to recruit leukocytes to extravascular sites for host defense and repair, under some circumstances leukocytes are firmly adhere to the endothelium, a protected microenvironment is found. In this microenvironment, inflammatory mediators produced by the leukocytes may potentially reach high concentrations and overcome local and systemic antiinflammatory protective mechanisms, thereby allowing endothelial cell injury to occur resulting in an increase in microvascular permeability and hemorrhage. This series of events may initiate and sustain a cycle of inflammation, leading to further leukocyte recruitment and endothelial cell injury. The continued recruitment of leukocytes can lead to occlusion of the microvasculature by leukocyte aggregates, causing local ischemia. The leukocytes may also diapede between endothelial cells, gaining access to the extravascular space, where they can mediate further tissue damage, producing organ dysfunctions. This cascade of events leading to vascular and tissue injury dependent on leukocyte-endothelial adhesive interactions, and thus, interrupting the interactions at the level of rolling, firm adherence, or diapedesis should decrease leukocyte mediated injury, in other words inflammation. In addition to inflammation, integrins and selectins are also involved in other abnormal conditions, such as septic shock, thrombosis, reperfusion damage or metastasis (Frenette & Wagner, 1996; Mojcik & Shevach, 1997; Gonzalez-Amaro *et al.*, 1998).

Phagocytosis and Inflammation

When inflammation remains unresolved, stimulated phagocytes can inflict damage on tissues and produce superoxide anion (O^-_2) which in turn forms extremely toxic metabolites such as the hydroxyl radical (O^-H), peroxy radical (O^-OH) hypochlorous acid, hydrogen peroxide, singlet oxygen and hydroperoxide (Rowe *et al.*, 1983). All these oxygen species derived from superoxide are damaging either by a powerful, direct oxidizing action or indirectly as with the hydroxyl and perhydroxyl radical which initiates lipid per oxidation and consequently results in membrane destruction (Mead, 1976). Tissue damage provokes inflammatory responses by the production of mediators and chemotactic factors. Nature has programmed phagocytes such as the neutrophil to use these toxic oxygen metabolites to kill invading organisms. Phagocytic cell attaches itself to the foreign particle at certain specific sites. These sites also have an adjacent enzyme system known as NADPH-oxidase (Roos *et al.*, 1976) which converts oxygen to superoxide anion which in turn is converted to other toxic oxygen species. Moreover, phagocytes are not particularly sensitive in identifying microorganisms.

In general, they will attach to any surface which they are unable to recognize as 'self'. In rheumatoid arthritis, the invading neutrophils attach themselves to collagen fibrils in cartilage and other joint tissues (Bromley *et al.*, 1984; Crisp *et al.*, 1984). These fibrils have been made 'non self' to the neutrophils by considerable biochemical damage inflicted during the inflammatory process. The phagocyte is physically incapable of engulfing these large structures but attempts to do so. A vesicle is opened up on the surface of the phagocyte but the massive size of the collagen prevents the vesicle from closing and it remains open to the extracellular compartment. However, the cell is stimulated and lysosomes fuse with the vesicle and superoxide anion and other oxygen products are produced. These products bathe the collagen fibrils and other structures with active oxygen species and hydrolytic enzymes. More tissue is rendered 'non-self' by the damage inflicted. Consequently more phagocytes are attracted by chemotaxis in an attempt to remove it and a cascade of destruction is initiated. The non-self material is also the source of auto-antigens, characteristic of chronic inflammation (Scher *et al.*, 1980). Therefore, the blood of patients with rheumatoid arthritis contains large number of antibodies which have been formed to act against auto-antigens produced by enzymic or oxygen damage. A characteristic antibody is rheumatoid factor is formed which is an antibody formed against altered immunoglobulins (IgG). Another common antibody formed is antinuclear antibody (ANA), formed against antigens produced from cells disrupted in the disease process. The phenomenon of frustrated phagocytosis is a key pathological event in the transformation of acute inflammation to chronic inflammation.

CONCLUSIONS

The process of inflammation is very complex; different scientists have given different views regarding inflammation and its propagation. To understand the complexity of this process we tried to explain some of the mediator mechanisms of this protective defence of the body. We feel that this review shell give some understanding to the readers about the basis of inflammation and its mediators responsible for its initiation and activation yet this is not the ultimate according to our views but some of the achievements of the science towards inflammation. Although there are still so many things left unwound to know completely about inflammation yet a sincere attempt has been made to explain the phenomenon of inflammation and its mediators.

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7

Recommendations for Reporting Randomized Controlled Trials of Herbal Interventions: CONSORT for Herbal Medicine Trials

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ABSTRACT

Controlled trials that utilize randomized allocation are the best tool to control for bias and confounding in trials testing clinical interventions. Investigators must be sure to include in reports of these trials information that is required by the reader to judge the validity and implications of the findings. In part, complete reporting of trials will allow clinician's to modify their clinical practice to reflect current evidence towards the improvement of clinical outcomes. The CONSORT statement was developed to assist investigators, authors, reviewers and editors on the necessary information to be included in reports of controlled clinical trials. The CONSORT statement is applicable to any intervention, including herbal medicinal products. Controlled trials of herbal interventions do not adequately report the information suggested in CONSORT. Recently, reporting recommendations were developed in which several CONSORT items were elaborated to become relevant and complete for randomized controlled trials of herbal medicines. We expect

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that these recommendations will lead to more complete and accurate reporting of herbal trials. We wrote this explanatory document to outline the rationale for each recommendation and to assist authors in using them by providing the CONSORT items and the associated elaboration, together with examples of good reporting and empirical evidence, where available, for each. These recommendations for the reporting of herbal medicinal products presented here are open to revision as more evidence accumulates and critical comments are collected.

Key words : Herbal interventions, randomized controlled trials, CONSORT

INTRODUCTION

Randomized controlled trials (RCTs) provide the best evidence for efficacy of health-care interventions (Altman *et al.*, 2001). Carefully planned and well-executed RCTs give us the best estimates of treatment effect and can thusly guide clinical decision making (Mulrow *et al.*, 1998; Sackett *et al.*, 1998). Although, trials which lack methodological rigor cause over- or underestimations of treatment effect sizes due to bias or confounding factors (Schulz *et al.*, 1995; Moher *et al.*, 1996; Jadad *et al.*, 1996; Begg *et al.*, 1996; Moher *et al.*, 2001; The CONSORT Group). Hence, efforts have been undertaken towards improving the design and reporting of RCTs (Altman *et al.*, 2001; Mulrow *et al.*, 1998; Sackett *et al.*, 1998; Schulz *et al.*, 1995; Moher *et al.*, 1996; Jadad *et al.*, 1996; Ioannidis *et al.*, 2004; MacPherson *et al.*, 2001).

Current research suggests that reporting quality of complementary and alternative medicine (CAM) trials is poor (Linde *et al.*, 2001a; Moher *et al.*, 2002). Linde *et al.* (2001a) found that most CAM trials do not describe the generation of the random sequence, an adequate method of allocation concealment, and the number and reasons for drop outs and withdrawals. Moher *et al.* (2002) (The CONSORT Group, 2004) reported that a sample of pediatric CAM RCTs reported less than 40% of the CONSORT checklist items with a 24% increase in the number of checklist items included in reports over time. That is, less than half of all information necessary in the reporting of these trials appeared in their reports. Specifically, only 50% of trials reported how random numbers were generated and 25% if allocation concealment was done (Moher *et al.*, 2002). The results suggest that a large proportion of CAM trials have poor reporting quality resulting in difficulties with assessment of internal and external validity (Linde *et al.*, 2001a; Moher *et al.*, 2002).

Linde *et al.* (2001a) showed that reporting quality may vary across different types of complementary therapies with herbal medicine[1] trials

being somewhat superior to homeopathy and acupuncture trials. Although, several systematic reviews state that trials of botanical medicine still fail to report information necessary to judge internal validity, external validity, and reproducibility (Gagnier *et al.*, 2003a; Gagnier, 2003b; Little & Parsons, 2000). A study examining the quality of reports of a sample of 206 English language herbal medicine RCTs found that less than 45% of the information suggested within the CONSORT statement was reported (Gagnier *et al.*, 2006a). For example, approximately 28% of trials described if the person administering the intervention was blinded to group assignment or not, only 22% described the methods for implementing the allocation sequence and 21% the method for generating the allocation sequence. Also, reporting quality differed between individual botanical medicines and improved across decades from the 1980s to the 2000s (Gagnier *et al.*, 2006a). Furthermore, it has been suggested that trials often do not include detailed information on the herbal product itself (Gagnier *et al.*, 2003a; Gagnier, 2003b).

It is known that herbal medicines may vary by part of plant used, time of harvest, active constituent levels, type of extract (aqueous, alcoholic, glycerin) and delivery form. Therefore, results of clinical trials on heterogeneous products may vary considerably even if they are using the same botanical species. Variation in herbal products between trials precludes pooling in systematic reviews of herbal medicines since invalid inferences may result from the combined data (Gagnier *et al.*, 2003a; Gagnier, 2003b; Linde *et al.*, 2001b). It is clear that readers, editors and reviewers require increased transparency in the reporting of RCTs of botanical medicines. Reporting guidelines for controlled clinical trials have been developed.

The consolidated standards of reporting trials (CONSORT) statement was first published in 1996 and revised in 2001 (Begg *et al.*, 1996; Moher *et al.*, 2001). This statement consists of a checklist and flow diagram to guide writers and reviewers on the information that should be available from published reports of two-group parallel randomized controlled trials (RCTs) (Begg *et al.*, 1996; Moher *et al.*, 2001). The CONSORT statement has been endorsed by many leading medical journals, editorial associations, professional societies and funding agencies (The CONSORT Group, 2004). Since its inception, several extensions of the CONSORT statement have been developed (Campbell *et al.*, 2004; Ioannidis *et al.*, 2004). Recently CONSORT was extended to cluster randomized trials (Campbell *et al.*, 2004) and for trials of harms (Ioannidis *et al.*, 2004). Also, an international group of acupuncture researchers developed a set of recommendations for

improving reporting of the interventions in parallel group trials of acupuncture: the Standards for Reporting Interventions in Controlled Trials of Acupuncture or STRICTA (MacPherson *et al.*, 2001). Though not a formal extension of CONSORT, MacPherson *et al.* (2001) describe STRICTA as a elaboration of item 4 in CONSORT and suggest STRICTA be used together with CONSORT in reporting acupuncture trials.

In June 2004 an international group of trialists', methodologists, pharmacologists and pharmacognosists met for a consensus meeting in Toronto, Canada that led to the development of recommendations for the reporting of herbal medicine trials (Gagnier *et al.*, 2006b; See Tables 1 & 2). The resulting guidelines amounted to a set of elaborations of current CONSORT items that will aid editors and reviewers in assessing the internal/ external validity and reproducibility of herbal medicine trials, allowing an accurate assessment of safety and efficacy.

During the development of the elaborations it became clear that an explanation of the concepts within and underlying the elaborations would aid researchers in planning, conducting and writing reports of RCTs of herbal medicines. In the current paper we discuss the rationale and scientific background for each elaboration, provide examples of good reporting for each. Where possible, we discuss empirical evidence for each. It should be noted that each elaboration is an addition to existing CONSORT recommendations. Thus all CONSORT items are first listed in Table 1, and elaborations in Tables 2.

Table 1. The CONSORT checklist (1,8)

Standard CONSORT checklist: Paper Section and Topic	Standard CONSORT checklist: Item	Descriptor	Reported on Page number
Title & Abstract	1	How participants were allocated to interventions (*e.g.* "random allocation," "Randomized" or "randomly assigned").	
Introduction Background	2	Scientific background and explanation of the rationale.	
Methods Participants	3	Eligibility criteria for participants and the settings and locations where the data were collected.	
Interventions	4	Precise details of the interventions intended for each group and how and when they were actually administered.	
Objectives	5	Specific objectives and hypotheses.	
Outcomes	6	Clearly defined primary and secondary outcome measures and, when applicable, any methods used to enhance the quality of measurements (*e.g.* multiple observations, training of assessors).	

Table 1. *Contd.*

Table 1. *Contd.*

Standard CONSORT checklist: Paper Section and Topic	Standard CONSORT checklist: Item	Descriptor	Reported on Page number
Sample size	7	How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules.	
Randomization Sequence allocation	8	Method used to generate the random allocation sequence, including details of any restriction (*e.g.*, blocking, stratification).	
Allocation concealment	9	Method used to implement the random allocation sequence (*e.g.* numbered containers or central telephone), clarifying whether the sequence was concealed until interventions were assigned.	
Implementation	10	Who generated the allocation sequence, who enrolled participants, and who assigned participants to their groups.	
Blinding (Masking)	11	Whether or not participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment. When relevant, how the success of blinding was evaluated.	
Statistical methods	12	Statistical methods used to compare groups for primary outcome(s); Methods for additional analyses, such as subgroup analyses and adjusted analyses.	
Results Participant flow	13	Flow of participants through each stage (a diagram is strongly recommended). Specifically, for each group report the numbers of participants randomly assigned, receiving intended treatment, completing the study protocol, and analyzed for the primary outcome. Describe protocol deviations from study as planned, together with reasons.	
Recruitment	14	Dates defining the periods of recruitment and follow-up.	
Baseline data	15	Baseline demographic and clinical characteristics of each group.	
Numbers analyzed	16	Number of participants (denominator) in each group included in each analysis and whether the analysis was by "intention-to-treat". State the results in absolute numbers when feasible (*e.g.* 10/20, not 50%).	
Outcomes and Estimation	17	For each primary and secondary outcome, a summary of results for each group, and the estimated effect size and its precision (*e.g* 95% confidence interval).	
Ancillary analyses	18	Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those pre-specified and those exploratory.	

Table 1. *Contd.*

Table 1. *Contd.*

Standard CONSORT checklist: Paper Section and Topic	Standard CONSORT checklist: Item	Descriptor	Reported on Page number
Adverse events	19	All important adverse events or side effects in each intervention group.	
Discussion Interpretation	20	Interpretation of results, taking into account study hypotheses, sources of potential bias or imprecision, and the dangers associated with multiplicity of analyses and outcomes.	
Generalisability	21	Generalizability (external validity) of trial results.	
Overall evidence	22	General interpretation of the results in the context of current evidence.	

Table 2. Proposed elaborations of CONSORT items for randomized controlled trials of herbal medicine interventions*(20)

Standard CONSORT checklist: Paper Section and Topic	Standard CONSORT checklist: Item	Descriptor	Reported on Page number
Title & Abstract	1	How participants were allocated to interventions (*e.g.* "random allocation," "Randomized" or "randomly assigned"). *Either the title or abstract, or both should state the herbal medicinal product's Latin binomial, the part of the plant used, and the type of preparation.*	
Introduction Background	2	Scientific background and explanation of the rationale. *Including a brief statement of reasons for the trial with reference to the specific herbal medicinal product being tested and, if applicable, whether new or traditional indications are being investigated*	
Methods Participants	3	Eligibility criteria for participants and the settings and locations where the data were collected. *If a traditional indication is being tested, a description of how the traditional theories and concepts were maintained. For example, participant inclusion criteria should reflect the theories and concepts underlying the traditional indication.*	

Table 1. *Contd.*

Table 2. *Contd.*

Standard CONSORT checklist: Paper Section and Topic	Standard CONSORT checklist: Item	Descriptor	Reported on Page number
Interventions	4	Precise details of the interventions intended for each group and how and when they were actually administered.	
	4.A. Herbal medicinal product name	*1. The Latin binomial name together with botanical authority and family name for each herbal ingredient; common name(s) should also be included* *2. The proprietary product name (i.e. brand name) or the extract name (e.g. EGb-761) and the name of the manufacturer of the product* *3 Whether the product used is authorized (licensed, registered) in the country in which the study was conducted*	
	4.B. Characteristics of the herbal product	*1. The part(s) of plant used to produce the product or extract.* *2.The type of product used [e.g raw (fresh or dry), extract]* *3.The type and concentration of extraction solvent used (e.g., 80% ethanol, H_2O 100%, 90% glycerine etc.) and the herbal drug to extract ratio (drug:extract; e.g. 2:1)* *4 The method of authentication of raw material (i e. how done and by whom) and the lot number of the raw material. State if a voucher specimen (i.e. retention sample) was retained and, if so, where it is kept or deposited, and the reference number.*	
	4.C. Dosage regimen and quantitative description	*1. The dosage of the product, the duration of administration and how these were determined.* *2.The content (e.g. as weight, concentration; may be given as range where appropriate) of all quantified herbal product constituents, both native and added, per dosage unit form. Added materials, such as binders, fillers, and other excipients; e.g. 17% maltodextrin, 3% silicon dioxide per capsule, should also be listed)* *3. For standardized products, the quantity of active/ marker constituents per dosage unit form*	
	4.D. Qualitative testing	*1. Product's chemical fingerprint and methods used (equipment and chemical reference standards) and who performed it (e.g. the name of the laboratory used). Whether or not a sample of the product (i.e retention sample) was retained and if so, where it is kept or deposited.*	

Table 1. *Contd.*

Table 2. *Contd.*

Standard CONSORT checklist: Paper Section and Topic	Standard CONSORT checklist: Item	Descriptor	Reported on Page number
		2. Description of any special testing/purity testing (e.g. heavy metal or other contaminant testing) undertaken. Which unwanted components were removed and how (i.e. methods). *3. Standardization · what to (e.g. which chemical component(s) of the product) and how (e g. chemical processes, or biological/functional measures of activity)*	
	4.E. Placebo/ control group	*The rationale for the type of control/placebo used.*	
	4.F. Practitioner	*A description of the practitioners (e.g. training and practice experience) that are a part of the intervention.*	
Objectives	5	Specific objectives and hypotheses.	
Outcomes	6	Clearly defined primary and secondary outcome measures and, when applicable, any methods used to enhance the quality of measurements (e.g. multiple observations, training of assessors). *Outcome measures should reflect the intervention and indications tested considering, where applicable, underlying theories and concepts.*	
Sample size	7	How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules.	
Randomization Sequence allocation	8	Method used to generate the random allocation sequence, including details of any restriction (*e.g.* blocking, stratification).	
Allocation concealment	9	Method used to implement the random allocation sequence (*e.g.* numbered containers or central telephone), clarifying whether the sequence was concealed until interventions were assigned.	
Implementation	10	Who generated the allocation sequence, who enrolled participants, and who assigned participants to their groups.	
Blinding (Masking)	11	Whether or not participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment. When relevant, how the success of blinding was evaluated.	
Statistical methods	12	Statistical methods used to compare groups for primary outcome(s); Methods for additional analyses, such as subgroup analyses and adjusted analyses.	

Table 1. *Contd.*

Table 2. *Contd.*

Standard CONSORT checklist: Paper Section and Topic	Standard CONSORT checklist: Item	Descriptor	Reported on Page number
Results Participant flow	13	Flow of participants through each stage (a diagram is strongly recommended). Specifically, for each group report the numbers of participants randomly assigned, receiving intended treatment, completing the study protocol, and analyzed for the primary outcome. Describe protocol deviations from study as planned, together with reasons.	
Recruitment	14	Dates defining the periods of recruitment and follow-up.	
Baseline data	15	Baseline demographic and clinical characteristics of each group.*Including concomitant medication, herbal and complementary medicine use.*	
Numbers analyzed	16	Number of participants (denominator) in each group included in each analysis and whether the analysis was by "intention-to-treat". State the results in absolute numbers when feasible (*e.g.* 10/20, not 50%).	
Outcomes and Estimation	17	For each primary and secondary outcome, a summary of results for each group, and the estimated effect size and its precision (*e.g.* 95% confidence interval).	
Ancillary analyses	18	Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those pre-specified and those exploratory.	
Adverse events	19	All important adverse events or side effects in each intervention group.	
Discussion Interpretation	20	Interpretation of results, taking into account study hypotheses, sources of potential bias or imprecision, and the dangers associated with multiplicity of analyses and outcomes. *Interpretation of the results in light of the product and dosage regimen used.*	
Generalisability	21	Generalizability (external validity) of trial results. *Where possible, discuss how the herbal product and dosage regimen used relate to what is used in self-care and/or practice.*	
Overall evidence	22	General interpretation of the results in the context of current evidence. *Discussion of the trial results in relation to trials of other available products.*	

*CONSORT items (1,17) are listed in normal text. Proposed recommendations for reports of herbal medicine RCTs are listed in italicized text.

CONSORT ELABORATIONS FOR TRIALS OF HERBAL INTERVENTIONS

Where necessary, we have elaborated items, to describe information suggested in reports of RCTs of herbal medicinal product interventions. When reporting an RCT of an herbal medicine, authors should consider reporting the information outlined in the CONSORT statement in addition to the information suggested in these elaborations. Below we list original CONSORT items (in normal type) followed by elaborations (in *italicized* text) for RCTs of herbal interventions. Excerpts from trials include information suggested by the CONSORT items and the developed elaborations.

TITLE AND ABSTRACT

Item 1. How participants were allocated to interventions (*e.g.* "random allocation," "Randomized" or "randomly assigned"). *Either the title or abstract, or both should state the herbal medicinal product's Latin binomial, the part of the plant used, and the type of preparation.*

Examples

Title: "A double-blind, placebo-controlled, randomized trial of Ginkgo biloba extract EGb 761 in a sample of cognitively intact older adults: neuropsychological findings" (Mix & Crews, 2002)

Abstract: "This was a randomized, double-blind placebo controlled...The active treatment group received tablets containing 300 mg of Garlic Powder (Kwai).....This is equivalent to approximately 2.7 g or approximately 1 clove of fresh Garlic per day." (Isaacshon *et al.*, 1998).

Explanation

CONSORT item #1 is meant to aid in the indexing and identification of reports of randomized controlled trials (RCT) using electronic databases (Altman *et al.*, 2001). Hence the use of the words "randomized", "randomly" or "random allocation" is suggested in the CONSORT statement. Additional language is required in the title and abstracts of trials of herbal medicinal products.

The practice of evidence-based herbal medicine requires access to the herbal scientific literature. The identification of RCTs of herbal medicinal products requires that the product's Latin binomial, the part of the plant used and the type of preparation be reported in the title and/or abstract. This information would allow increased specificity in the indexing and identification of RCTs of particular herbal medicine products or preparations. Some herbal medicinal products have a specific trade name or commercial name. Where applicable, this name should be listed, together with the Latin binomial of the ingredient herb. When the herbal medicinal product used in the trial is a combination herbal product, we suggest listing

the product name in the title and the separate herbal medicinal species contained within this product in the abstract. In this way, the title of the trial will not be prohibitively long by listing all separate herbal species' Latin binomials.

Studies indicate that searching for complementary and alternative medicine (CAM) related topics is challenging due to diversity of use of controlled vocabulary and indexing procedures between different databases (Murphy *et al.*, 2003). It has been suggested that if authors of CAM trials (*e.g.* botanical medicine trials) report abstracts or titles without reference to standard controlled vocabulary, indexers may not assign appropriate indexing terms for particular studies (Murphy *et al.*, 2003; McGregor, 2002). A further problem arises from indexers not having a sufficient number and variety of descriptors for CAM interventions (Kronenberg *et al.*, 2001). When reporting RCTs of herbal medicinal products, the use of the information suggested for titles and abstracts above will likely lead to improved indexing and retrieval.

INTRODUCTION

Background

Item 2. Scientific background and explanation of the rationale. *Including a brief statement of reasons for the trial with reference to the specific herbal medicinal product being used, and if applicable, whether new or traditional indications are being tested.*

Example[A]

The extract of *Ginkgo biloba* leaves entitled EGb 761 is a complex mixture that is standardized with respect to its flavonol glycoside (24%) and terpene lactone (6%) content [1,2]. These two classes of compounds have been implicated in the benefcial effects of EGb 761 in treating peripheral and cerebral vascular insuffciency, age-associated cerebral impairment, and hypoxic or ischemic syndromes [1,3]. Electron spin resonance (ESR) studies conducted *in vitro* have shown that EGb 761 is an efficient scavenger of various reactive oxygen species, including superoxide anion radical (O282) and hydroxyl radical (HO8), and that it also exhibits superoxide dismutase–like activity [4]. Recent *in vitro* studies with animal models have revealed that the extract may exert an anti–free radical action in myocardial ischemia–reperfusion injury. In these studies [5,6], inclusion of 200 mg/l of EGb 761 in the medium that was used to perfuse isolated ischemic rat hearts signifcantly improved postischemic recovery, reduced ventricular arrhythmias and enzyme leakage, and lowered the content of

[A] Please note, all references listed as numbers in all quotes provided refer to the reference number in the original publication from which this excerpt was taken.

spin-trapped oxyradicals in the coronary effluents. Interestingly, antiarrhythmic effects were also observed when animals were treated orally with EGb 761 prior to heart perfusion, but a significant reduction in ventricular arrhythmias could be achieved only with high dosages (.100 mg/kg for 10 days) [5]. In addition to these studies conducted with EGb 761 [5,6], numerous other studies with experimental animals have indicated that active reduced forms of molecular oxygen, including O282, HO8, and hydrogen peroxide (H2O2), are involved in the pathogenesis of tissue injury that follows myocardial ischemia–reperfusion [7–10] (Pietri *et al.*, 1997) ..... In the present double-blind study, we tested the cardioprotective efficacy of oral treatment with EGb 761, which is known to have *in vitro* antioxidant properties [4–6], in patients undergoing CPB surgery by manual palpation (Pietri *et al.*, 1997).

Explanation

The background of reports of controlled clinical trials partially serve to lay-out the rationale of the trial (Altman *et al.*, 2001) with special reference to the specific intervention under study. There is great heterogeneity in the types of herbal medicinal products available. Two different preparations/ products of the same herbal species can have different phytochemical profiles, differing pharmacokinetic properties, etc. Given the variability in products, the rationale should clearly overview the scientific data concerning the specific herbal medicinal product under study (*e.g.* in the above example EGb 761). Where no clinical trials are available for review, extrapolation from pre-clinical work (*i.e.* animal studies, observational studies, case reports, known mechanisms) is acceptable. Where no data on the product is available, previous research on similar products to that being tested in the current trial should be reviewed. This information should be clearly stated and ideally include a description of a systematic review of previous studies utilizing the herbal product (Altman *et al.*, 2001; Savulescu *et al.*, 1996).

Also, if the authors are testing a traditional use, a review of the theory and concepts underlying this indication should be reported. Readers with some relevant knowledge of the area should be able to determine the reasoning for the indication. For example, trials of traditional Chinese medicine (TCM) may choose to test a TCM diagnosis (*e.g.* Liver Blood Deficiency) and not a western diagnosis (Hepatitis). If this is so, the authors should be explicit in their description of why the particular intervention being tested is indicated. For example,

In traditional Chinese medicine, the "Nei-Kuan" acupoint (EH-6, where EH denotes equilibrium envelope of the heart meridian) has been believed to correlate with the function of the heart (Chuang, 1977). Recently, some investigators (Mah *et al.*, 1992; Hsu *et al.*, 1989) observed that

acupuncture at Nei-Kuan can improve left ventricular function in patients with coronary heart disease (Ho *et al.*, 1999).

Additionally, other data (*i.e.* clinical trials, animal studies, observational studies, case reports, known/proposed mechanisms) that would aid in creating a rationale, even for this traditional indication, can be described in the background. The assumption is that a rationale can be clearly and explicitly reported and that it may be derived from scientific, empirical, historical[4] or traditional[5] sources.

METHODS

Participants

Item 3. Eligibility criteria for participants and the settings and locations where the data were collected.

If a traditional indication is being tested, a description of how the traditional theories and concepts were maintained. For example, participant inclusion criteria should reflect the theories and concepts underlying the traditional indication.

Example

There were altogether 118 cases, which were randomly divided into two groups: The QTG *(Qingluo Tongbi Granules: A Chinese herbal medicine)* treated group and the control group treated with tripteryguim glycosides. In the treated group (n=63), there were 18 males and 45 females, aged from 18 to 65 years with an average of 39.5 $\pm$ 16.6 and the disease course ranging 2-22 years averaging 7.5 $\pm$ 3.6 years. The cases were graded by x-ray according to the criteria set by the American Association of Rheumatoid Arthritis (ARA), USA: 7cases were grade I, 30 grade II, and 26 grade III. In the control group (n=55), there were 10 males and 45 females, aged from 18 to 65 years with an average of 38.3 $\pm$ 16.7 and the disease course ranging 1-21 years, averaging 6.9 $\pm$ 3.1 years. Among them, 15 cases were grade I, 21 grade II, and 19 grade III. The cases in the two groups were comparable in sex, age, disease course and X-ray grading ($p>0.05$).

Diagnostic criteria and TCM differentiation criteria: Diagnosis of RA was made according to the ARA criteria revised in 1987. Criteria for TCM differentiation of the type of yin-deficiency and heat in collaterals: burning pain in joints, local swelling, or deformity and rigidity, reddened skin with a hot sensation, low fever, dry mouth, yellow urine, red or dark red tongue with ecchymosis and petechia, thin or yellow and greasy or scanty fur with fissures, fine, rapid and slippery or fine and rapid pulse.

Included in the study were inpatients and outpatients who were diagnosed to have RA of the type of yin-deficiency and heat in the collaterals *(Italicized words added from a previous passage in the manuscript)* (Xueping *et al.*, 2004).

Explanation

The external validity, generalizability, of a trial is partially dependent on the eligibility criteria for participants (Altman *et al.*, 2001). Reporting of eligibility criteria in trials of herbal medicine interventions is often poor. One study found that less than 75% of RCTs of herbal interventions adequately reported eligibility criteria (Gagnier *et al.*, 2006a). As a result, determination of generalizability of one quarter of these trials would not be possible from reading the published reports. Trials of herbal medicines that aim to test traditional indications must be sure to report any eligibility criteria which reflect this.

On a related note, authors may choose to exclude participants with previous use of the specific herbal medicinal product itself. It has been suggested that use of herbal medicinal products prior to trial commencement can lead to increased amounts of adverse effects. A trial of *Tanacetum parthenium* (Feverfew) (Johnson, 1984) against placebo for migraine prophylaxis included current use of feverfew as an eligibility criterion. Those in the placebo group experienced more side effects, these were attributed to a "post-feverfew syndrome" which is the equivalent of withdrawal effects. Such symptomatic worsening following cessation of long-term feverfew consumption has been reported elsewhere (Johnson, 1984). To date there is no empirical evidence to suggest that use of an herbal medicine prior to a controlled clinical trial of that same herbal medication biases estimates of treatment effect. Although, the use and reporting of eligibility criteria to exclude trial participants with recent use is suggested.

Example

Anyone with a prior adequate trial of St John's wort (at least 450 mg/d) for the treatment of depression or those who had taken St John's wort for any reason in the last month were excluded. To reduce the potential for including a treatment nonresponsive sample, participants who had failed to respond to a trial of an antidepressant (fluoxetine hydrochloride, 20 mg/d, for at least 4 weeks or the equivalent) in the current episode or who had failed to respond to more than 1 adequate trial of antidepressant in a previous episode also were excluded (Shelton *et al.*, 2001).

Explanation

It is important that trials of herbal medicines report the settings and locations where the data where collected (Altman *et al.*, 2001). The location highlights physical factors (*e.g.* climate, food sources), economics, geography and social and cultural factors that may affect the generalizability of a study. As well, research settings may vary greatly in their organization, resources, experience, baselines risk, and physical appearances (Altman *et al.*, 2001). One study found that less than 40% of herbal medicine trial reports adequately reported the setting and location of the trial (Gagnier

et al., 2006a). External generalizability of trial results partially rests on complete reporting of this information.

Item 4: Precise details of the interventions intended for each group and how and when they were actually administered. *Where applicable, the description of a herbal intervention should include:*

4.A. Herbal medicinal product name	*1. The Latin binomial name together with botanical authority and family name for each herbal ingredient; common name(s) should also be included* *2. The proprietary product name (i.e. brand name) or the extract name (e.g. EGb-761) and the name of the manufacturer of the product* *3. Whether the product used is authorized (licensed, registered) in the country in which the study was conducted*
4.B. Characteristics of the herbal product	*1. The part(s) of plant used to produce the product or extract.* *2. The type of product used [e.g. raw (fresh or dry), extract]* *3.The type and concentration of extraction solvent used (e.g. 80% ethanol, H_20 100%, 90% glycerine etc.) and the herbal drug to extract ratio (drug:extract; e.g. 2:1)* *4. The method of authentication of raw material (i.e. how done and by whom) and the lot number of the raw material. State if a voucher specimen (i.e. retention sample) was retained and, if so, where it is kept or deposited, and the reference number.*
4.C. Dosage regimen and quantitative description	*1. The dosage of the product, the duration of administration and how these were determined.* *2.The content (e.g. as weight, concentration; may be given as range where appropriate) of all quantified herbal product constituents, both native and added, per dosage unit form. Added materials, such as binders, fillers, and other excipients; e.g. 17% maltodextrin, 3% silicon dioxide per capsule, should also be listed)* *3. For standardized products, the quantity of active / marker constituents per dosage unit form*
4.D. Qualitative testing	*1. Product's chemical fingerprint and methods used (equipment and chemical reference standards) and who performed it (e.g. the name of the laboratory used). Whether or not a sample of the product (i.e. retention sample) was retained and if so, where it is kept or deposited.* *2. Description of any special testing / purity testing (e.g. heavy metal or other contaminant testing) undertaken. Which unwanted components were removed and how (i.e. methods).* *3. Standardization: what to (e.g. which chemical component(s) of the product) and how (e.g. chemical processes, or biological / functional measures of activity)*
4.E. Placebo/control group	*The rationale for the type of control / placebo used.*
4.F. Practitioner	*A description of the practitioners (e.g., training and practice experience) that are a part of the intervention.*

The type of information that is required for a complete description of any intervention is relative to the type of intervention being tested. For trials of surgical interventions, for example, a complete description of the individual performing the surgery may be required (Altman *et al.*, 2001; Roberts, 1999). For herbal medicines, the above information is required to determine, with specificity, the key characteristics of the product that was used. A complete description of the product will allow determination of its' efficacy and safety relative to other products.

There are a wide variety of commercially available products containing herbal medicines (Dreikorn *et al.*, 1998; Dreikorn *et al.*, 2001; Harkey *et al.*, 2002). In addition there is great variability in the content of these products (Harkey *et al.*, 2002; Gurley *et al.*, 2000; Liberti & Der Marderosian, 1978; Groenewegen & Heptinstall 1986; Heptinstall *et al.*, 1992; Nelson *et al.*, 2002; Zhang *et al.*, 2002). Often products do not contain the amount (weight, volume, proportion) of the individual constituents listed on their label (Manning & Roberts, 2003) or any of the constituents at all. Products containing the same botanical species [*e.g. Hypericum perforatum* (St. John's wort)] often contain varying amounts of the plant's marker/active[6] constituents. For example, research has shown that commercial products of the following botanical species contain varying levels of their respective constituents: *Hypericum perforatum* (St. John's wort) (Garrara *et al.*, 2003; Schulte-Lobbert *et al.*, 2004), *Camellia sinensis* (Green tea) (Manning & Roberts, 2003), *Tanacetum parthenium* (Feverfew) (Gronewegen and Heptinstall, 1986; Heptinstall *et al.*, 1992; Nelson *et al.*, 2002), *Eleutherococcus senticosis* (Siberian ginseng), *Panax quinquefolius* (American/Canadian ginseng) (Harkey *et al.*, 2002), *Hydrastis canadensis* (Goldenseal) (Weber *et al.*, 2003), and *Paullinia cupana* (Guarana) (Carlson & Thompson, 1998). As a result, the pharmacological properties and *in vitro* activities may vary between different products (*e.g.* Dreikorn *et al.*, 2001; Lefebvre *et al.*, 2004). Also, some studies have shown that botanical products not only contain varying beneficial constituents, but also varying harmful ones (Haller *et al.*, 2004; Oberlies *et al.*, 2004). Therefore, it is necessary that authors of trials of herbal interventions completely describe the product used.

Herbal medicinal product name

Example

The AG *(American Ginseng)* capsules contained 3-y-old Ontario grown, dried, ground AG root (*Panax quinqueefolius L.)* supplied by the same supplier, Chai-Na-Ta Corp., BC, Canada,.....This AG was the same commercially available product, but from a different batch than the original (Sievenpiper *et al.*, 2003).

Explanation

Reports should state the Latin binomial and common name/names together with the authority and family name. Reporting of the Latin binomial and common name were also suggested in item 1. The accepted international code of botanical nomenclature (Greuter *et al.*, 1999) indicates that the scientific naming of botanical species must include a Latin binomial (genus and specific epithet) and the authority name. For example, Genus: *Taraxicum,* Epithet: *officinale* Authority: Linnaeus. In full, this would result in: *Taraxicum officinale* L. (Linnaeus is abbreviated as L. here). The authority identifies who originally described the plant. Common names should also be listed here (*e.g.* Dandelion, Feverfew, St. John's wort). Alone, common names are not sufficient since different herbal species may have the same common name. For example, Echinacea is a common name used for E*chinacea angustifolia, Echinacea pallida,* and *Echinacea purpura.* These plants have heterogeneous biochemical profiles (Pellati *et al.*, 2004).

If relevant, the proprietary product[7] name (*i.e.* brand name, *e.g.* Kwai) or the extract[8] name (*e.g.* LI 160) and the manufacturer of the product should be reported. Such names are a quick means of identification of the specific herbal product including its contents and manufacturing or production. Alone, these names are not sufficient for the product description.

Authors should also report whether the product is licensed in the region where the trial took place. Specific regulatory bodies award licenses for herbal medicinal products. The regulations for attaining licensing are variable across jurisdictions. Though licensing does not ensure product quality or provide the reader with a sufficient description of the herbal product, it does allow the reader to determine the regulatory status and availability of a specific herbal medicine.

Physical characteristics of the herbal product

Example (raw herb)

The ginseng capsules contained 3-y-old Ontario dried and ground ginseng root (*P. quinquefolius* L.) ..... All ginseng and placebo capsules came from the same lot (Vuksan *et al.*, 2001).

Example (extract)

...all patients received 1 infusion/day with *Ginkgo* special extract Egb 761 (batch number: 5242) over 30 – 60 min (1 dry vial in 500 mL isotonic solution). The dry vials contained 200 mg of dry extract from *Ginkgo biloba* leaves (drug-extract ratio 50 : 1), ...:12 H_2O in 3 mL solution served as solvent (Morgenstern & Biermann, 2003).

Explanation

There must be a complete description of the physical characteristics of the herbal product including the part(s) of the plant contained in the product or

extract and the type of product [*e.g.* raw (fresh or dried) or extract]. The part(s) of the plant included in a product are related to the quantities and types of constituents present (Betz *et al.*, 1994). Also, the type of product used should be reported given that different product forms have different types and amounts of constituents (Patora *et al.*, 2003). If the product is an extract, the type and concentration of the extraction solvent should be reported (*e.g.* 80% Alcohol, H_20 100%, 90% glycerine etc.) as well as the plant to plant extract ratio (plant: plant extract; *e.g.* 2:1). This ratio tells the reader how much of the starting plant material (either by weight or volume) was required to produce a specific amount of the finished extract. The method of authentication of raw material (*i.e.* how done and by whom) describes how the original material or plant was identified and allows the reader to determine, to some degree, if the raw material for the herbal product was produced from the plant as reported. The lot number of the raw material provides the reader with key information as to where the raw material came from.

Dosage regimen and quantitative description

Example

The treatment was provided as 252 tablets containing 50 mg of either *Ginkgo biloba* standardized extract LI 1370 (containing 25% flavanoids, 3% ginkgolides, and 5% bilobalides) or placebo (both provided by Lichtwer Pharma). Participants were instructed to take three tablets daily for 12 weeks. The extract and dose of *Ginkgo biloba* were chosen on the basis of the results of previous trials in which this dose of this extract had been reported to be effective in treating cerebral insufficiency[5] (Drew & Davies, 2001).

Explanation

Authors of trials of herbal medicines should report the dosage regimen and provide a quantitative description of the herbal product. Information regarding the dosage and duration of the trial are of great importance to replicating trials, establishing efficacy or harm for specific dosages and durations, and for external generalizibility (Altman *et al.*, 2001). The rationale for dosage and duration of the trial should be clear as unclear reasoning questions the methods of a trial and possibly raises some ethical issues as to why the trial was carried out at all.

The weight or amount of all herbal product constituents, both native and added per dosage unit form (*i.e.* added materials such as binders, fillers, excipients, etc; *e.g.* 17% maltodextrin, 3% silicium dioxide per capsule) and the percentage of active/marker constituents per dosage unit form (*e.g.* 0.3% Hypericin per capsule) should also be reported. This provides the reader with a profile of the quantity of the botanical product constituents.

Qualitative testing

Example

The content of various ginsenosides (Rg_1, Re, Rf, Rb, Rc, Rb_2, and Rd), which are dammarane saponin molecules found among *Panax* species, was determined in the laboratory of Dr. John T. Arnason at the Department of Biology, Faculty of Science, University of Ottawa, Ontario, Canada, using high-performance liquid chromatography (HPLC) analyses, a method similar to the one developed for the American Botanical Council Ginseng Evaluation Program [27]. A Bechham HPLC system with a reverse-phase Beckham ultrasphere C-18, 5um octadecylsilane, 250 X 4.6mm column was employed for the analyses. The ginsenoside standards used for comparison were provided by two sources. Rg_1 and Re were provided by Dr. H. Fong, University of Illinois and Rf, Rb_1, Rc, Rb_2, Rd were provided by Indofine Chemical Co., Somerville N.J. (Vuksan *et al.*, 2000)

Table 1. Energy. nutrient, and ginsenodide profile of the American ginseng *(Panax quinquefolius* L.*)* and placebo capsules

Constituent (per g)	Placebo[t]	Ginseng[t]
Energy[2]		
(kJ) 14.68	14.39	
(kcal)	3.51	3.44
Macronutrients[2]		
Carbohydrate (g)	0.73	0.57
Fat (g)	0.039	0.013
Protein (g)	0.069	0.26
Ginsenosides		
(20*S*)-Protopanaxadiols (%)		
Rb_1	—	1.53
Rb_2	—	0.06
Rc	—	0.24
Rd	—	0.44
(20*S*)-Protopanaxatriols (%)		
Rg_1	—	0.100
Re	—	0.83
Rf	—	0
Total (%)	—	3.21

[1]To equate energy and macronutrient values to 1, 2, or 3 g American ginseng, multiply by 1, 2, or 3, respectively. To determine values for placebo, multiply by 2.
[2]Determined by the Association of Official Analytical Chemists methods for macrountrients (18).
[3]Determined by HPLC analyses (20).
Adopted from: Vuksan *et al.*, 2001.

Explanation

Trials should report the product's chemical fingerprint[9] and methods used (machinery and chemical reference standards) and who performed it (the

name of the laboratory used). The fingerprint can be reported in a graph or a table describing the key constituents of the herbal medical product. Chemical profiling, using the proper techniques is essential to providing a clear and accurate report of a product's constituents, and provides both qualitative and quantitative information (Sawnson, 2002; Phytomedicine: International Journal of Phytotherapy and Phytopharmacology; German Federal Institute for Drugs and Medical Device, 1999). Bauer and Tittel (1996) have provided some guidelines for the characterization and standardization of plant material used for pharmacological, clinical and toxicological studies. Also the Association of Analytical Communities (AOAC) has outlined standards for analyzing specific herbal medicinal products (AOAC, 2004) as has the American Herbal Pharmacopoeia produced by the American Botanical Council (American Herbal Pharmacopoeia, 2004) and the United States Pharmacopoeia (United states Pharmacopoeia, 2004). Reports might also describe if a voucher specimen[10] (*i.e.* retention sample) was retained and if so, where it is kept or deposited, so that independent sources can verify the chemical profile. Herbal medicines are often contaminated (Chan, 2003). Thus, a complete description of any special testing/purity testing (*e.g.* heavy metal or other contaminant testing) and the removal of unwanted components (which and how (the methods)) should be included in reports where relevant. All such methods are relevant given that they may alter the composition of the herbal product.

Standardization has been hotly debated in the literature (*e.g.* Swanson, 2002). Often, companies or researchers attempt to standardize botanical products to specific chemical "marker" constituents. These "marker" constituents may be considered to be the primary "active" constituents or merely serve an index of the product's chemical profile (Swanson, 2002). Though products on the commercial market may not be standardized and often an "active" constituent is not known, if standardization was done in a clinical trial of an herbal product, it should be reported. Authors should report, what the product was standardized to (*e.g.* which chemical component(s)), how this was done (*i.e.* chemical processes, or biological/ functional measures[11] of activity) (*e.g.* Murphy *et al.*, 1988) and the percentage of this particular constituent per dosage unit form.

The rationale for the type of control/placebo used.

Example

The placebo, on the other hand, consisted of identical capsules containing corn flour. The energy, carbohydrate content and appearance of the placebo were designed to match that of the AG *(American Ginseng capsules).* (Italicized words added). (Sievenpiper *et al.*, 2003)

Explanation

In botanical medicine trials, as in other trials, it is important to have a complete description of the characteristics of the control group and the way in which it was disguised (Altman *et al.*, 2001). If a placebo control was used, the placebo should be closely matched to the control intervention (Vickers & de Craen, 2000). For trials of herbal interventions the rationale for the type of control/placebo used should be described. There have been some trials that have reported using placebos that are matched to color and smell, but that contain ingredients that are active (*e.g.* Palevitch *et al.*, 1997). If a control group is active, comparisons between it and the experimental group will be affected. While it may be a challenge to construct matched placebos for certain herbal product interventions, it is not impossible.

A description of the practitioners that are a part of the intervention.

Example

In order to participate in the study, physicians had to (i) be a medical specialist with a degree in internal medicine and general medicine, (ii) have a certified degree in TCM by a German society for medical acupuncture, and (iii) have at least 5 years of practical experience in TCM (according to the German Acupuncture Societies Working Group standard)..... The herbal formulations for the TCM group were designed by a herbalist (Carl-Hermann Hempen) and prepared by a pharmacist who both specialise in Chinese herbal medicine (S. Dietz, Franz-Joseph-Pharmacy, Munich, Germany). In addition to the basic formula, every patient received a second additional formula tailored to his or her individual TCM diagnosis (Table 1). (Brinkhaus *et al.*, 2004)

Explanation

On occasion an herbal intervention trial may include a health-care practitioner as part of the intervention. Practitioners have varying levels of training, years of experience, theoretical orientations and work in different environments. Similar to surgical trials, such trials should provide a description of the practitioners (*e.g.* training and practice experience) that are a part of the intervention (Altman *et al.*, 2001).

Outcomes

Item 6: Clearly defined primary and secondary outcome measures and, when applicable, any methods used to enhance the quality of measurements (*e.g.*, multiple observations, training of assessors). *Outcome measures should reflect the intervention and indications tested considering, where applicable, underlying theories and concepts.*

Example

All outcome measures were assessed at baseline and after 30 days of treatment at the follow-up visit. The primary outcome measures were changes in quality of life as measured by the Physical and Mental Component Summary scales of the 12-Item Short Form Health Survey (SF-12). The SF-12 is widely used in measuring health and quality of life and has been shown to have a high level of agreement with scores from the original 36-Item Short Form Health Survey (SF-36)[11]. The SF-36 has been validated in several Chinese studies, whereas evaluation of the SF-12 is ongoing [11]. Secondary outcome measures included assessments of physical performance, memory, sexual function, and *qi*..... The *qi* scale is a 17-item instrument (14 items on an interviewer-administered questionnaire and three physical examination items) that was developed through an international collaboration of clinical investigators with expertise in scale development and traditional Chinese medicine. Questionnaire items address symptoms commonly included in a traditional Chinese medical interview, including breathing, energy level, appetite, heartburn, sweating, bowel patterns, pain, temperature sensations, sleep habits, and sexual ability. The physical examination items address tongue coating, tongue muscle quality, and pulse quality. The scale was developed for this study and has not been validated. The 14 questionnaire items are scored on a scale of 0 to 4 points, and the physical examination items are scored on a scale of 0 to 3. The total *qi* score is the sum of each score, ranging from 0 (best) to 65 (worst) (Bent *et al.*, 2003).

Explanation

As with any RCT, outcome measures, both primary and secondary, should be relevant to the indications being tested be fully reported and describe any methods used to enhance the quality of measurements (1,8)(Altman *et al.*, 2001; Moher *et al.*, 2001). When performing RCTs testing herbal interventions, concepts that go beyond western medical terminology and understanding may be relevant. For example, in the above trial the particular Chinese herbal remedy being tested is purported to increase longevity, quality of life, energy, memory, sexual function and *Qi,* a Chinese concept that is loosely translated as vital energy. Therefore, in addition to measures of health and quality of life, these investigators required a measure of *Qi* to test the change in vital energy during the course of this trial. Ultimately, to test the function of traditional herbal medicines we advise that the outcome measures reflect the underlying theories and concepts and therefore the indications for the specific herbal medicine intervention under investigation.

RESULTS

Baseline data

Item 15: Baseline demographic and clinical characteristics of each group. Including, *concomitant medication use or herbal medicinal product use.*

Example

Eight patients (mean age 44.9 (SEM 4.2) years) received feverfew and nine (mean age 51.2 (2.3) years) received placebo capsules. The patients in the active group had taken 2.44 (0.2) small leaves of feverfew daily for 3.38 (0.58) years before entry to the study, and those in the placebo group had taken 2.33 (0.48) small leaves daily for 4.18 (0.67) years. Thus the two groups did not differ in the amount of feverfew consumed daily or the duration of consumption.....

One patient in each group was taking conjugated equine estrogens (Premarin); the patient in the placebo group was also taking pizotifen. One patient given feverfew was taking the combined oral contraceptive Orlest 21. On patient in each group was taking a diuretic: the patient given feverfew was taking clorazepate and the patient given placebo was also taking a product containing tranylcypromine and trifluorperazine. In addition, two people in the placebo group were taking vitamin preparations and one prochlorperazine (Johnson *et al.*, 1985).

Explanation

A complete description of participants who entered a trial allows readers and clinician's to assess how relevant the trial is to a specific patient. As a part of the baseline assessments in trials of herbal medicinal products, authors should clearly assess and describe any current medication or herbal product use. Differences between groups on medication or herbal product use may confound results (Piscitelli & Burstein, 2002).

DISCUSSION

Interpretation

Item 20: Interpretation of results, taking into account study hypotheses, sources of potential bias or imprecision, and the dangers associated with multiplicity of analyses and outcomes. *Interpretation of the results in light of the product and dosage regimen used.*

Example

Although EGb 761 is generally used at a dose of 120 mg/day in treating chronic disease states, we chose to administer the extract at more than twice its usual dose, but for only 5 days before the operation, to cope with the enhanced generation of oxidant species that was expected to follow

post-unclamping procedures. Measurements of DMSO/AFR concentrations indicated that EGb 761 treatment significantly protected plasma ascorbate levels in all sampling sites during the initial 5–10 min. of reperfusion (see Table 3), a period during which free radical processes are considered to be critical. Analyses of plasma TBArs concentrations revealed that EGb 761 treatment also suppressed (or substantially attenuated) the transcardiac release of MDA, indicating protection against free radical–induced lipid peroxidation. These two findings offer some clues regarding the mechanisms that underlie the protective action of EGb 761 in open-heart surgery. It has been reported that EGb 761 protects ischemic rat hearts against reperfusion-induced decreases in ascorbate [6]. We did not observe any *in vitro* chemical interaction between EGb 761 and AFR or DMSO/AFR that would have maintained ascorbate levels. Therefore, in performing its antioxidant role, it seems that the extract competes effectively with circulating ascorbate (*e.g.* by directly quenching oxyradicals once they have been formed)..... (Pietri *et al.*, 1997).

Explanation

The discussion section of a clinical trial report provides the reader with information to determine the pros and cons of the study and how this study relates to other research in the area. Understanding how the results with this product and dosage regimen relate to the results of other trials with similar or different products or dosage regimens allows the reader to establish a context from which to determine strengths or drawbacks of the specific intervention used. Therefore, authors should clearly state specific aspects of the product or dosage regimen that could have resulted in trial findings.

This concept is discussed here under Item 20, to highlight the necessity of authors of reports of RCTs of herbal products to explicitly consider the product and dosage regimen as potential strengths or drawbacks of the study. Though we have formally separated this from the elaboration on Item 22, discussion of the trial results in the context of the current evidence (Item 22), we admit that these aspects of the discussion section are closely related, and may be written together in the body of the manuscript.

Generalizability

Item 21: Generalizability (external validity) of trial results. *Where possible, discuss how the herbal product used relates to what is used in self-care and/or practice.*

Example

G115 is a standardized ginseng extract which is often complexed with various other substances and marketed commercially. Ginsana®, Gericomplex®, Geriatric Pharaton®, and ARM229 are several commercial standardized

ginseng products which have been studied, and may include some or all of the following substances in addition to G115: vitamins, minerals, trace elements, dimethylaminoethanol bitarate.

A word of caution for the consumer. As noted previously, the FDA classifies ginseng as a food supplement, so it is marketed rather extensively in health food stores. An estimated 5-6 million Americans use ginseng products (6). However Chong and Oberholzer (11) note that there are problems with quality control, and indeed a recent report (12) indicated that of 50 commercial Ginseng preparations assayed, 44 contained concentrations of ginsenosides ranging from 1.9% to 9.0%, while six of the products had no detectable ginsenosides (Dowling *et al.*, 1996).

Explanation

Generalizability, or external validity is the extent to which the results of a study hold true in other circumstances (Fletcher *et al.*, 1996). The word "circumstances" here can mean other individuals or groups of individuals, other similar interventions, dosages, timing, administration routes, and other settings for starters. Given the wide variability in herbal medicinal products available on the market, and their variable quality and content, a review of how the products used in the current trial relate to what is available and/or used by consumers and practitioners is quite valuable. This information would allow the reader to determine the availability of products that may act similarly to the one used in the trial. Application of clinical trial results partly relies on the availability of the intervention or a similar intervention.

Item 22: General interpretation of the results in the context of current evidence. General *discussion of the trial results in relation to trials of other available products.*

Example

The majority of published studies to date have used a powdered garlic preparation, similar to the preparation method used in this study. Considerable variability in outcomes exists between these studies. For example, Adler *et al.* [13], using a commercial dehydrated garlic tablet, reported a significant net drop of 13.1% in LDL-C levels relative to the placebo group in 12 weeks, and Jain *et al.* [15], using the same product and a similar design, reported a significant net decrease of 8% in LDL-C levels in moderately hypercholesterolemic adults. However, three other studies [19,20,22], using the same dosage of the same commercial dehydrated garlic powder product (Kwai®, Lichtwer Pharmaceuticals) reported no significant effect. The dose of powdered garlic tablets used in the five studies just cited, 900 mg:day, was similar to the full dose of 1000 mg:day used in this study. The allicin content of the tablets used in this study, 1500 mg:day in the full dose, was lower than the amount used in other studies with powdered

garlic preparations. Other types of garlic preparations used in lipid lowering trials have included aged garlic extract and teamed garlic oil. Steiner *et al.* [14] used a large dose, 9 tablets: day, of aged-garlic extract, and reported a statistically significant 4.6% lowering of plasma LDL-C levels. In contrast, a recent study using steamed garlic oil supplementation reported no significant effect on cholesterol levels in hypercholesterolemic adults after 12 weeks [18]. One explanation could be that the oil is not as effective as dehydrated garlic powder because it contains different sulfur-containing phytochemicals [30]. Some of the discrepancies reported in these studies can be explained by the heterogeneity that exists among them in terms of study design, duration, subject characteristics, adherence, or confounders such as weight, diet, and exercise (Gardner *et al.*, 2001).

Explanation

Discussing trial results in the context of relevant studies is important to put the trial results in the context of existing empirical evidence (Altman *et al.*, 2001; Annals of Internal Medicine, 2001). Some trials fail to provide the reader with sufficient information to determine how the current results relate to other research. For example, Drew and Davies (2001) report that "*Ginkgo biloba* extract LI 1370 had no greater therapeutic effect than placebo in treating tinnitus. In addition, other symptoms of cerebral insufficiency were not significantly affected by the treatment (Table 3). The results from this trial are similar to some reports and contrast with others.[2] This study differs from other trials in many ways." (p.5). This provides the reader with little information from which to judge the efficacy of the product currently tested relative to other products that have been tested. Different botanical products can have different constituents and therefore differing therapeutic effects (Harkey *et al.*, 2002; Gurley *et al.*, 2000; Liberti & Der Marderosian, 1978; Groenewegen & Heptinstall 1986; Heptinstall *et al.*, 1992; Nelson *et al.*, 2002; Zhang *et al.*, 2002).

It is suggested here that discussion sections of trials of botanical interventions include a discussion of the trial results in the context of previous research while offering explicit consideration of the similarities or differences between products used therein. It is inappropriate to support or refute a trial's results by referring to literature that has tested a different product. Authors should be careful to clearly report when they are drawing inferences between heterogeneous products. When clinical trials on the specific product tested do not exist, pre-clinical data should be discussed. This includes animal, *in vitro* and other data.

COMMENTS

Randomized allocation is the best tool to control for bias and confounding in controlled trials testing clinical interventions. Investigators must be sure to include in reports of these trials information that is required by the

reader to judge the validity and implications of the findings. In part, complete reporting of trials will allow clinician's to accurately appraise studies so as to modify their clinical practice to reflect current evidence. The CONSORT statement was developed to assist investigators, authors, reviewers and editors on the necessary information to be included in reports of controlled clinical trials. The CONSORT statement is applicable to any intervention, including herbal medicinal products.

Controlled trials of herbal medicines interventions do not adequately report the information suggested in CONSORT. Recently, several CONSORT items were elaborated to become relevant and complete for controlled trials of herbal medicines (Gagnier *et al.*, 2006b). We expect that these recommendations will lead to more complete and accurate reporting of herbal medicine trials.

We wrote this explanatory document to further explain the suggested elaborations and to assist authors in using them. We provide the CONSORT items and the associated elaborations, together with examples of good reporting and empirical evidence, where available. These recommendations for the reporting of RCTs of herbal medicine are open to change and revision as more evidence accumulates and critical comments are collected.

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8

Anxiolytic Activity Screening Studies on Extracts of a Few Medicinal Plants

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ABSTRACT

Aethusa cynapium, Artemisia abrotanum, Cimicifuga racemosa and Clematis erecta have been used traditionally in anxiety and sleep disorders but these plants have not been investigated so far to substantiate these therapeutic claims. The present study was aimed at screening the anxiolytic potential of the four plants. Petroleum ether, chloroform, methanol and water extracts of the plants have been evaluated for their anxiolytic activity using the elevated plus-maze model in mice. The methanol extract of Aethusa cynapium has demonstrated significant anxiolytic activity as compared to the standard drug diazepam. These results indicate that it is worth undertaking in depth investigation on Aethusa cynapium with a view to develop a potentially useful anxiolytic drug.

Key words : *Aethusa cynapium, Artemisia abrotanum, Cimicifuga racemosa, Clematis erecta,* anxiolytic activity

INTRODUCTION

Anxiety is a normal emotional response to real or potential danger. If anxiety becomes excessive, out of proportion and interferes with everyday life, management becomes necessary. The conventional treatment of anxiety involves use of medication like benzodiazepines, selective serotonin reuptake inhibitors (SSRIs) etc or psychological therapy or a combination

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of the two. Common limitations of antianxiety drug therapy include comorbid psychiatric disorders and increase in dose leading to intolerable side effects (Cates *et al.,* 1996). This has led researchers worldwide to investigate plants which are commonly employed in traditional and alternative systems of medicine for sleep disorders and related diseases (Farnsworth, 1980).

Various plants have been investigated for their anxiolytic effects (Carlini, 2003). *Valeriana* species such as *V. walliichii, V. officinalis, V. eduli, V. thalictroides* have demonstrated antianxiety effect in human beings (Handa, 1992), *Piper methysticum* is employed clinically for the treatment of anxiety (Pepping, 1999; Reeder & Cupp, 2000; Billia *et al.,* 2002). Numerous plants like *Nardostachys jatamansi* (Handa, 1992), *Passiflora* species like *P. incarnata* (Dhawan *et al.,* 2001, 2001a), *P.alata* (Petry *et al.,* 2001), *Citrus aurantium* (Carvalho-Frietas *et al.,* 2002), *Scutellaria lateriflora* (Awad *et al.,* 2003), *Stachys lavandulifolia* (Rabbani *et al.,* 2003), *Ginkgo biloba* (Kuribara *et al.,* 2003), *Coriandrum sativum* (Emamghoreishi *et al.,* 2005), *Turnera aphrodisiaca* (Kumar & Sharma, 2005, 2005a), *Centella asiatica* (Wijeweera *et al.,* 2006), *Adiantum tetraphyllum* (Bourbonnais-Spear *et al.,* 2007), *Apocynum venetum* (Grundmann *et al.,* 2007), have shown antianxiety activity in experimental animals.

In the present study four plants, used in the traditional and complementary medicine have been investigated for their anxiolytic potential.

Aethusa cynapium Linn. (Umbelliferae) commonly called Fool's Parsley, has been used traditionally for gastrointestinal complaints in children, infantile cholera, summer diarrhoea, convulsions, sleep disorders and delirium (Flemming, 2000). In the homeopathic system of medicine, *A. cynapium* is used for milk intolerance in children, pylorus cramps and acute diarrhoea with vomiting (Vikramaditya & Joshi, 1997). *A. cynapium* contains alkaloid (Vikramaditya & Joshi, 1997), polyacetylenes (Andreev *et al.,* 2001), essential oil, flavone glycosides and ascorbic acid (Flemming, 2000).

Artemisia abrotanum Linn. (Compositae) commonly called Southernwood (Good, 1974), has been used traditionally for the treatment of digestive ailments, worms, menstrual disorders, marasmus, diarrhoea (www.herbs2000.com, 2000) and to relieve mental tension (www.herbs for life, 2003). *A. abrotanum* contains antimalarial sesquiterpene isofraxidin coumarin ethers (Greger *et al.,* 1983; Cubukcu *et al.,* 1990), essential oil (Mucciarelli *et al.,* 1995), hydroxy coumarins (Elzbieta *et al.,* 1984), spasmolytic flavonoid glycosides (Bergendorff & Sterner, 1995), peroxysemiketals (Ruecker *et al.,* 1993) and choleretic phenolic acids (Swiatek *et al.,* 1998).

Cimicifuga racemosa Linn. (Ranunculaceae; synonym *Actea racemosa*) commonly known as Black Cohosh (Gleason & Cronquist, 1991), has been traditionally used to treat diarrhoea, sore throat, rheumatism, painful menstrual periods, menopausal disorders (Lieberman, 1998) dysmenorrhea, dyspepsia and snakebites (Bisset, 1989). The herb is available commercially in combination with St. John's Wort for depressive moods associated with premenstrual and menopausal symptoms (Fleming, 2000). The tincture of *C. racemosa* is used as anxiolytic, sedative emmenagogue and antitussive (Vikramaditya & Joshi, 1997). Remifemin® is a standardized extract of the plant available in Germany for management of menopause syndrome (Bisset, 1989). The plant is a hormone-free phytomedicine for treatment of climacteric symptoms related to menopause (Freudenstein *et al.,* 2000; Liu *et al.,* 2001; Kennelly, 2002). *C. racemosa* is rich in triterpene glycosides (Bedir & Khan, 2000; Wende *et al.,* 2001; Chen *et al.,* 2002, 2002a), 15,16-seco-cycloartane glycosides and tetra nor cycloartane glycosides (Nishida *et al.,* 2003, 2003a), phenylpropanoid esters, aromatic acids, isoflavone and metals (Akihisa, 1999; Panossian *et al.,* 2004).

Clematis erecta Linn. (Ranunculaceae; synonym *Clematis recta)* is employed traditionally for disorders of skin, lymphatic system and genito-urinary organs especially for gonorrhea. The tincture of *C. erecta*, an official preparation in the Pharmacopoeia Homeopathica Polyglottia 1872, is employed for the symptomatic relief of anxiety, nervous tension, insomnia, stress, fear, panic, colds, flu, fever and minor injury (Llyod & Lloyd, 1887; Felter & Llyod, 1898). *C.erecta* is reported to contain quaternary aporphine alkaloid (Slavik *et al.,* 1987, 1995).

MATERIALS AND METHODS

Plants

Ariel parts of *Aethusa cynapium, Artemisia abrotanum, Cimicifuga racemosa* and *Clematis erecta* were collected from a cultivated source (Rati Ram Nursery) at village Khurrammpur via Kalsia, district Saharanpur (U.P., India) in March 2005. The identities of the plants were confirmed at the Museum-cum-Herbarium of the University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh.

Tested material

Petroleum ether, chloroform, methanol and water successive Soxhlet extracts of the dried aeriel parts of the four plants *viz. Aethusa cynapium, Artemisia abrotanum, Cimicifuga racemosa* and *Clematis erecta* were prepared. Yields (as %, dry weight) are detailed in Table 1.

Table 1. Percentage yields of extracts of the four investigated plants

Plant extract	Yields (%, dried weight)			
	Aethusa cynapium	*Artemisia abrotanum*	*Clematis erecta*	*Cimicifuga racemosa*
Pet.Ether	2.2	1.3	0.5	2.5
Chloroform	1.8	1.3	3.6	2.8
Methanol	5.8	9.1	12.1	12.8
Water	2.9	3.9	10.3	15.6

Studied Activity

Anxiolytic activity was evaluated using modified elevated plus-maze (Montgomery, 1958; Pellow *et al.,* 1985; Kulkarni & Reddy, 1996). Vehicle [5% Tween 80 in Simple Syrup I.P. (66.7% w/w sucrose in water)], test extracts and diazepam (reference drug), both suspended in vehicle, were administered orally 45 min prior to the antianxiety test, and the behavior of animals on the elevated plus-maze was recorded for 5 min in terms of (i) number of entries into open or closed arms (ii) average time spent by the mouse in open or closed arms.

Animals

Swiss albino mice of either sex, weighing 20-24 g were procured from the Central Animal House of the Panjab University, Chandigarh. The mice were allowed standard laboratory feed and water *ad libitum*. Groups of five mice were used in all sets of experiments. Animals were fasted overnight before use. All animal studies were carried out as per the guidelines of the Institute Ethical Committee, Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala.

Statistical Analysis

Analysis of variance (ANOVA) and the Tukey's multiple range tests was employed to determine statistical significance. Difference were considered significant at $p<0.05$.

RESULTS AND CONCLUSIONS

Aethusa cynapium, Artemisia abrotanum, Cimicifuga racemosa and *Clematis erecta* have been used traditionally in anxiety and sleep disorders but these plants have not been investigated so far to substantiate these therapeutic claims. The present study was carried out to validate the traditional claims.

Different extracts of the four plants have been prepared using solvents in increasing order of polarity. This is done in order to extract completely non polar to completely polar constituents from the plant material. The anti anxiety activity of these extracts were compared with

the activity of the standard drug diazepam and the control using the elevated plus-maze model.

The elevated plus-maze, is a novel test for the selective identification of anxiolytic drug effects in rodents (Vogel, 2002). The elevated plus-maze model is effective, cheap and simple, requires no preliminary training for the mice, and does not cause much discomfort to them while handling. The fear due to height (acrophobia) induces anxiety in mice when placed on the elevated plus-maze. The ultimate manifestation of anxiety and fear then is exhibited by decrease in motor activity, which is measured by the time spent by mice in the open arms. Anxiolytic compounds, by decreasing anxiety, increase the open arm exploration time, anxiogenic compounds have the opposite effect (Kulkarni, 2002).

The mean time spent by the mice in open arms of the EPM after oral administration of various doses of the different extracts of the four plants is reported in Table 2.

Table 2. Anxiolytic effects of extracts of the four investigated plants using the Elevated Plus Maze model

Comparison with	Average Time spent in open arms (seconds) [a]
Control (Vehicle, 0.25 mL)	3.2 ± 1.3
Standard (Diazepam, 2 mg/kg)	12.2 ± 1.2

Treatment	**Dose (mg/kg), *p.o***	**Average Time spent in open arms (seconds)[a]**			
		Aethusa cynapium	***Artemisia abrotanum***	***Cimicifuga racemosa***	***Clematis erecta***
Pet.Ether Extract	50	5.7 ± 1.0*,**	4.5 ± 0.5 *,**	1.7 ± 0.1*,**	2.3 ± 0.2*
	100	6.8 ± 0.4*,**	3.2 ± 0.4*	1.1 ± 0.1*,**	2.7 ± 0.2*
	200	7.9 ± 0.8*,**	7.9 ± 0.9*,**	1.6 ± 0.2*,**	3.3 ± 0.4*
	400	5.9 ± 0.9*,**	4.9 ± 0.5*,**	3.2 ± 0.4*	2.6 ± 0.2*
Chloroform extract	50	5.1 ± 1.2*,**	2.9 ± 0.2*	1.0 ± 0.1*,**	1.7 ± 0.1*,**
	100	8.1 ± 1.2*,**	4.3 ± 0.5*	1.6 ± 0.2*,**	2.6 ± 0.2*
	200	7.6 ± 1.0*,**	2.6 ± 0.2*	1.4 ± 0.2*,**	0.8 ± 0.2*,**
	400	7.0 ± 1.3*,**	2 ± 0.2*,**	2.1 ± 0.2*,**	3.3 ± 0.4*
Methanol extract	50	7.7 ± 0.9*,**	0.9 ± 0.1*,**	0.6 ± 0.1*	2.6 ± 0.2*
	100	8.2 ± 0.9*,**	1.6 ± 0.1*,**	1.0 ± 0.1*	2.5 ± 0.2*
	200	9.5 ± 1.1*,**	1.1 ± 0.1*,**	1.9 ± 0.1*	4.7 ± 0.5*,**
	400	12.7 ± 1.5**	6.9 ± 0.7*,**	5.6 ± 0.6*	1.2 ± 0.1*,**
Water extract	50	5.2 ± 0.6*,**	0.8 ± 0.1*,**	2.2 ± 0.3*	0.5 ± 0.1*,**
	100	8.2 ± 1.7*,**	0.7 ± 0.1*,**	1.5 ± 1.8*,**	0.9 ± 0.1*,**
	200	8.4 ± 1.5*,**	0 ± 0.0*,**	2.7 ± 0.3*	1.2 ± 0.1*,**
	400	7.9 ± 1.4*,**	8.3 ± 1.0*,**	1.15± 0.2*,**	0.7 ± 0.1*,**

a:Values are expressed as mean ± S.D. (n = 5). ANOVA followed by Tukey's multiple range test. $p<0.05$.

* =significant with respect to standard, ** = significant with respect to control.

Animals treated with diazepam (2 mg/kg, p.o.) spent 12.2 sec in open arms of the plus maze and the control group animals spent 3.2 sec. Of the four extracts of *Aethusa cynapium,* the methanol extract shows a dose dependent increase in motor activity, with the animals spending 12.7 seconds at 400 mg/kg in the open arms of the maze. This activity is statistically comparable to that of diazepam. The *Artemisia abrotanum* extracts, although significant at some doses when compared to the control, were not significant in comparison with diazepam. The four extracts of *Clematis erecta* and *Cimicifuga racemosa* do not show anxiolytic activity at any of the doses administered.

The results of the present study show that of the four plants tested only *Aethusa cynapium* demonstrated significant anxiolytic activity, and indicate that it is worth undertaking in depth investigation on *Aethusa cynapium* with a view to develop a potentially useful anxiolytic drug.

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9

Study of *Eugenia jambolana* and *Momordica charantia* in Respect to Safety and Antidiabetic Effect *In vivo* and *In vitro*

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ABSTRACT

Diabetes mellitus is composed of a myriad of derangements on carbohydrate, lipid and protein metabolism, which are associated to an absolute or relative deficiency of insulin secretion / action. Out of a large number of herbal drugs in the Ayurvedic system of medicine of India, Eugenia jambolana of family Myrtaceae and Momordica charantia from cucurbitaceae (Jamun) is being widely used to treat diabetes by the traditional practitioners over many years. They are prepared as an aqueous or ethanolic extract, by infusion or as a juice of the raw plant. MC has a long history of use as a folklore hypoglycaemic agent where the plant extract has been referred to as vegetable insulin Eugenia jambolina (EJ) is well known plant with identified antidiabetic, laxative, anti-inflammatory and antimicrobial properties in traditional literature. In the present study the plants have been explored for the following parameters like safety evaluation, antidiabetic properties and in vitro antidiabetic properties, individually as well as in combination (1:1) to find out additive of synergistic effect. Acute oral toxicity of water extract of Eugenia jambolana was evaluated in albino mice of both sexes. These results show that the water extract of Eugenia jambolana is toxicologically safe by oral administration. The effects of Eugenia jambolana bark extracts were studied with alloxan induced diabetes model for antidiabetic property. Albino Wistar rats weighing 150-200 g (N = 6) were fed E. jambolana bark extract and combination of EJ + MC (50:50) 500 mg/ kg of b.w. for 14 days. In another investigation, we have studied the effects

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of aqueous fruit extract of MC and aqueous bark extract of EJ on the transport of d-glucose, l-tyrosine across rat-everted intestine in vitro. Everted intestinal sacs from rats were mounted in an organ bath containing Krebs–Henseleit bicarbonate buffer. Graded concentrations (1.5–12 mg/mL) of MC and EJ extract were incubated in the mucosal solution. The serosal appearance and mucosal disappearance of d-glucose, and l-tyrosine were significantly inhibited with increasing graded concentrations of MC and EJ extracts. The aqueous extract of the plants was found to inhibit primarily the uptake of glucose in a dose-dependent manner. Uptake of tyrosine was affected at high substrate concentrations only. It is hypothesized that bioactive phytochemicals such as saponins in MC fruit extract and tannins from EJ inhibits the active transport of d-glucose, l-tyrosine across rat intestine It is revealed that MC and EJ can be a potential alternative drug therapy of postprandial hyperglycemia via inhibition of glucose uptake across the small intestine and could involve a washout of glucose from the blood stream.

Key words : Acute toxicity, intestinal uptake, *Eugenia jambolana, Momordica charantia*

INTRODUCTION

Diabetes mellitus is a group of metabolic disorders characterized by hyperglycemia. These metabolic disorders include alterations in the carbohydrate, fat and protein metabolisms associated with absolute or relative deficiencies in insulin secretion and/or insulin action. Insulin is the hormone produced in the β-cells of pancreas which enables the cells to absorb glucose from the blood and also help in the utilization of the glucose in the cells by glycolysis, tricarboxylic acid cycle, hexose monophosphate shunt, glycogenesis etc. In diabetes body fails to produce insulin and excess glucose accumulates in the blood instead of being utilized or stored. The characteristic symptoms of diabetes are polyuria, polydypsia, polyphagia, pruritus and unexpected weight loss etc. Diabetes can be classified into two types: (a) type 1 diabetes (insulin-dependent diabetes mellitus or IDDM) and (b) type 2 diabetes (non-insulin dependent diabetes mellitus or NIDDM) (Guyton, 2002). Exercise and having a controlled diet are recommended for the treatment of both types of diabetes. In addition, insulin is used to treat cases of type 1 diabetes. Oral hypoglycemic agents (OHA) such as sulfonylureas, biguanidines, thiazolidinediones and α-glucosidase inhibitors are often used to treat cases of type 2 diabetes. When therapy with oral hypo-glycemic agents is ineffective, insulin also can be used to treat type 2 diabetes (Liu & Wang, 1996; Zhao, 1999).

Management of diabetes without any side effects is still a challenge to the medical system. There is an increasing demand by patients to use the natural products with antidiabetic activity, because insulin and OHAs are having so many side effects. Medicinal plants continue to provide valuable

therapeutic agents, both in modern medicine and in traditional system. The doubts about the efficacy and safety of the oral hypoglycemic agents have prompted a search for safer and more effective drugs in the treatment of diabetes (Reaven, 1983). A wide variety of the traditional herbal remedies are used by diabetic patients, especially in the third world countries (Lin, 1992; Ospinag & Serrano, 1995; Day, 1998; Gray & Flatt, 1998; Shimada *et al.*, 1998) and may therefore, represent new avenues in the search for alternative hypoglycemic drugs. The folk medicine in India has described several kinds of ayurvedic herbs and plant crude drugs, belonging to various families to be concerned with the treatment of diabetes mellitus. Plant drugs are frequently considered to be less toxic and more free from side effects than synthetic ones (Pari & Umamaheswari, 2000).

In the traditional system of Indian medicine plant formulation and in several cases, combined extracts of plants are used as the drug of choice rather than individual. Presently several research laboratories are involved in isolating new herbal oral hypoglycemic agents or potent herbal formulation. The renounced OHA, Metformin which gained popularity in recent years got its shape from its ancestor, active ingredient Galegine or isoamylene guanidine from *Galega officinalis* (Cusi & DeFronzo, 1998). More than 100 medicinal plants are mentioned in the Indian system of medicines including folk medicines for the management of diabetes, which are effective either singly or in combinations. Many of these have shown promising effects (Kumari & Devi, 1993.). Various herbal formulations like D-400 (Mitra *et al.*, 1996) and Trasina (Bhattacharya *et al.*, 1997) are well known for their antidiabetic effects. While going through the previous works of different investigators, some variations in their experimental results have been observed. Incomplete extraction procedure, inadequate pharmacognosy and insensitive animal model may be the reasons for showing variable and even negative results. We have guarded against all these factors in our experiments and selected only two hypoglycaemic medicinal plants for our studies. Out of which one is *Momordica charantia*, traditionally and experimentally known for the antidiabetic activity (Grover *et al.*, 2002) and another crude drug is *Eugenia jambolana* L. (Bark) less know part of plant and hardly explored for its use in diabetes as compared to its other parts like leaves, fruit and pulp (Nadakarni, 1954).

Aim of present study is to confirm the ethnobotanical claim of antidiabetic property of *Eugenia jambolana* bark, and its use with *momordica charantia.* Folklore literature state that bark of EJ and fruit powder of MC exhibit the synergistic effect and control both blood glucose and body weight with short period of time as compared to individual drugs.

Sahyadri hills row (Pune-Kolhapur region, Maharashtra, India), which lie geographically in the Western Ghats, are known for the rich heritage of the flora. A number of plants with known and unknown medicinal values

are available here, which have to be explored for their use in the effective treatment of diabetes mellitus as described in the folk literature. But no systematic scientific studies were carried out on these plants by that mean.

MATERIALS AND METHODS

Plant Material

The bark of *Eugenia jambolana* and fruits of *Momordica charantia* were collected from local farm of Baramati (Maharashtra) and Pune region of Western Ghats of Maharashtra, respectively. Both the plant parts were authenticated by the scientists of BSI (Botanical Survey of India) Pune, Maharashtra.

After collection, Bark of *EJ* was dried under shade and then washed out debris and adhered matter and again dried to remove moistness of bark. Bark was applied to disintegrator to make the fine powder. Same way fruits of *MC* were shade dried after proper removal of seeds and washing with water and powdered to coarse size.

Preparation of Extracts

For entire experimentation cold macerated extract of both the plants were used. Powdered bark of EJ and fruits of MC were subjected for cold maceration up to 48 h. At the end of maceration syrupy liquid of both the drugs were obtained, to make it free flow powder extract were concentrated and dried under the reduced pressure with the help of rotary vacuum evaporator (Rotavap®). The yield of crude extracts from EJ and MC was 16.6% and 33.1% respectively. Extract of EJ was used individually and in combination with MC in the proportion of (EJ+MC-50:50). Alloxan was purchased from SD fine chemicals Company Inc., while all other chemicals used in this experiment were purchased from SD fine chemicals.

Acute Toxicity of EJ Bark

Swiss albino mice were used for oral acute toxicity study and divided into four groups, each containing five animals. The bark extract was administered orally at doses of 1000, 3000, and 5000 mg/kg body weight. Distilled water was administered to control group. The animals were evaluated at 0.5, 1, 2, 4, 24 and 48 h, after administration to assess possible clinical or toxicological symptoms. Signs of toxicity and mortality were observed daily for 13 days. At the end of 14th day after the last observation the mice were killed and the liver, lungs, heart, spleen and kidneys were withdrawn, weighed and carefully examined macroscopically for any abnormal, pathological signs of toxicity.

Experimental Animals for Antidiabetic activity

Male wistar rats weighing 180-200 g were obtained from the Haffkins' Research Institute Mumbai. The rats were housed at a regulated

temperature (22°C) and humidity (55%) controlled room with a 12 h light: 12 h dark cycle. A water and standard pellet diet were available *ad libitum* throughout the experimental period.

Antidiabetic activity

Effect of the crude extracts on serum glucose level in alloxan-induced diabetic rats

Diabetes was induced by a single intravenous injection of 60 mg/kg of alloxan monohydrate (dissolved just before use in 0.9% NaCl) to overnight fasted rats (Silva *et al.*, 2002 with modification). A serum glucose range of 400–550 mg/dl was used for the experiment. Animals in which the development of hyperglycemia was confirmed (around 90%), 72 h after the alloxan injection, were randomly allocated into four groups of five rats to each treatment:

Group I, diabetic control received the saline solution

Group II, non-diabetic received distilled water

Group III, diabetic received the crude EJ extract 500 mg/kg b.w.

Group IV, diabetic received the combined extracts EJ+MC (50:50) 500 mg/kg b.w

Blood samples were collected from the retro-orbital plexus before the administration of extract or combination serum glucose levels were measured at zero time (before receiving the extract).

Effects of (MC+EJ) on transport of d-glucose and l-tyrosine across the intestinal sac - in vitro

Adult male Swiss albino rats weighing 200-250 g maintained on commercial feed were used for this study. After overnight fasting, rats were killed by a severe blow on the head against a hard surface. The abdomen was opened by a midline incision. The whole of the small intestine was removed by cutting across the upper end of the duodenum and the lower end of the ileum and manually stripping the mesentery. The small intestine was washed out with normal saline solution (0.9% w/v NaCl) using a syringe equipped with blunt end. Intestinal segments (1062 cm) were then everted according to the method described by Wilson and Wiseman. After weighing, the empty sac was filled with 1 mL of Krebs– Henseleit bicarbonate buffer (KHB). The composition of the buffer was (mm/l): $NaHCO_3$ 25; NaCl 118; KCl 4.7; $MgSO_4$ 1.2; NaH_2PO_4 1.2; $CaCl_2$ 1.2; and Na_2EDTA 9.7 mg/l. Glucose (2 g/l) was added to the medium just before the start of the appropriate experiments. The pH was maintained at 7.4. The sac was filled with a blunted-ended syringe and then slipped off the needle carefully and the loose ligature on the proximal end was tightened. After weighing, the

distended sac was placed inside an organ bath containing 50 mL of the same incubation medium. The organ bath was surrounded by a water jacket maintained at 37-40°C and placed in metabolic shaker at a frequency of 100-110 shakes/min. The incubation medium was continuously being bubbled with a mixture of 95% O_2 and 5% CO_2. At the end of the incubation period (30 min.), the sacs were removed from the organ bath, blotted by a standardized procedure and weighted again. The serosal fluid was drained through a small incision into a test tube. The emptied sac was shaken gently to remove the adhered fluid and the tissue was weighted again. The weight of the empty sac before and after the incubation did not differ significantly. The initial serosal volume was determined as the difference between the weights of the empty and filled everted sac prior to incubation. The final serosal volume was calculated by subtracting (after incubation) the weight of the empty sac from that of the filled sac (Mahomoodally *et al.*, 2005, 2006).

The aqueous EJ extract individually and with MC extracts (50:50) were incubated in the mucosal solution in the organ bath. The active transport of d-glucose and l-tyrosine were evaluated by measuring the increase in concentration of both compounds inside and outside the intestinal sacs after 30 min. of incubation. L-Tyrosine in the incubating buffer solution was determined as described by Lowry *et al.* (1951) with modifications. Glucose was measured using a commercially available glucose oxidase kit. The terms used for d-glucose and l-tyrosine transfer are mucosal glucose transfer, serosal glucose transfer and gut glucose uptake. Mucosal glucose transfer is the amount of glucose that disappeared from the mucosal fluid while serosal glucose transfer is the amount of glucose that entered the serosal fluid. Gut glucose uptake is the difference in glucose concentration between the mucosal and serosal fluid after incubation.

RESULTS AND DISCUSSION

Acute Oral Toxicity

Acute oral toxicity study of *Eugenia jambolana* extract showed that no mortality of mice occurred, at the doses of 1000, 3000, and 5000 mg/kg body weight given orally. This is an indication that the extract has negligible level of toxicity when administered orally. Besides, no signs of toxicity, such as convulsion, vomiting, diarrhoea, paralysis, breathing difficulties, bleeding, restless, irritation, and abnormal posture, were observed in *Eugenia jambolana* extract–treated mice.

Thus, it can be suggested that *Eugenia jambolana* extract is virtually nontoxic. In conclusion, *Eugenia jambolana* extract was found to be fairly nontoxic when oral acute toxicity study in mice was performed.

Effect of the crude extracts on serum glucose level in alloxan-induced diabetic rats

Blood sugar level increased as expected in alloxan-injected animals, since alloxan causes a massive reduction in insulin release, by the destruction of the beta-cells of the islets of Langerhans and inducing hyperglycemia (Goldner *et al.,* 1943). Oral administration of *EJ* (500 and mg/kg b.w.) and combined form of extract EJ+MC (500 mg/kg b.w.) resulted in a significant reduction in the blood glucose.

In our study we have found that *EJ* decreases blood glucose in alloxan diabetic rats. In this context a number of other plants have also been observed to have hypoglycaemic effects. But when the results compared with individually action of EJ and its combination (EJ+MC) shows remarkably reduction in elevated blood glucose (Fig 1). The possible mechanism for hypoglycemic action may be potentiating the insulin effect of plasma by increasing either the pancreatic secretion of insulin from the β-cells of islets of Langerhans or its release from bound insulin. As MC is suppose to known to posses insulin like compound and EJ posses tannins, triterpenes responsible for activity of reduction in plasma blood glucose.

We have demonstrated that the folk medicinal plant *EJ* possesses a hypoglycemic effect. Further, active research is underway in our laboratory to elucidate the mechanisms of action of this medicinally important plant.

Effects of (MC + EJ) on transport of d-glucose and l-tyrosine across the intestinal sac - in vitro

The effects of EJ crude aqueous extract individually and in combination with MC on d-glucose, l-tyrosine and transport (serosal appearance) across everted intestinal sacs of rat are summarized in (Tables 1 & 2). In order to find the lowest inhibitory concentration and any dose dependent relationship, graded concentrations of EJ and EJ+MC (from 1.5 to 12 mg/mL) were incubated with the intestinal segments in the mucosal solution. Data from the present investigation indicate that EJ inhibits significantly ($p<0.05$) the active transport of d-glucose, l-tyrosine. It was found that increasing graded concentrations of EJ+MC from 1.5 to 12.0 mg/mL in the mucosal solution decrease significantly the absorption as well as the transport of these compounds across rat intestine. The concentration of d-glucose accumulated or metabolized by the enterocytes (gut wall content) in EJ+MC was higher ($p<0.05$) than the control experiments and individual EJ. However, at higher concentration (12 mg/mL), EJ did not have a significant ($p>0.05$) dose-dependent inhibition of d-glucose absorption and transport compared to 6, 3 or 1.5 mg/mL EJ+MC.

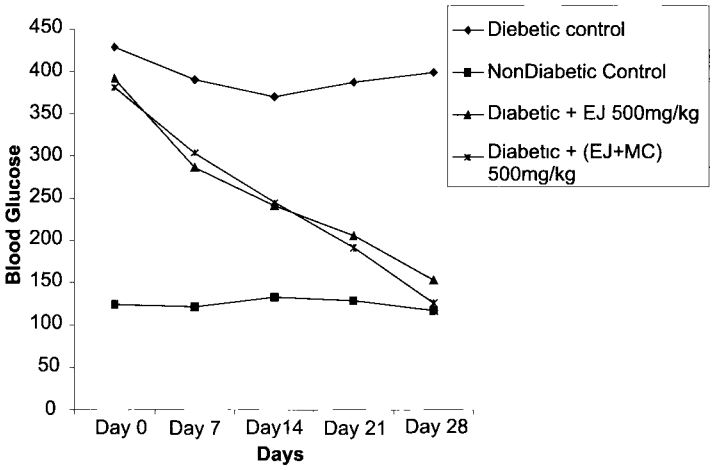

Fig 1. Effect of the crude extracts on serum glucose level in alloxan-induced diabetic rats

MC fruit and EJ bark has been found to possess a wide array of bioactive phytochemicals and several classes of biologically active saponins have been isolated (Khanna *et al.*, 1981; Murakami *et al.*, 2001; Matsuur *et al.*, 2002). Bioactive saponins, tannins constituents of natural medicines have been found to suppress the transfer of glucose from the stomach to the small intestine and inhibit glucose and fluid transport at the brush border membrane (Matsuda *et al.*, 2001; Matsuur *et al.*, 2002). Saponins constituents of herbs reduce the permeability barrier to Na^+ at enterocytes, thus, discharging the electrochemical gradient and removing the driving force for sugar and fluid transport. Thus, the observed increased inhibitory effect of EJ+MC in the present study might be due to the bioactive compounds such as saponins present in MC fruit (Johnson *et al.*, 1986).

The results of the study reveals that EJ bark extract inhibits the uptake of d-glucose, l-tyrosine across rat everted intestinal sacs but when the same drug given in combination with MC shows greater effect by means of increased inhibition of glucose uptake. It is likely that MC fruit and EJ bark possess bioactive phytoconstituents which in combination capable of lowering postprandial blood glucose levels by inhibiting the uptake of glucose across the intestine and at same time increasing metabolism of glucose in cells. As most challenging factor in management of diabetes is to keep PBG as close as to the normal level. Present study supports to corroborate the folklore claim of use of MC fruits with EJ bark in traditional medicines.

In the conclusion, study can be a confirmatory document to claim combination of EJ+MC (50:50) potential alternative drug therapy of, postprandial hyperglycaemia.

Table 1. Effects of graded concentrations of aqueous EJ bark extract (1.5–12 mg/mL) on transport of d-glucose and l-tyrosine across rat everted intestinal sacs

Concentration of *EJ fruit extract* *in medium (mg/mL)*	**d-Glucose transport (M/g tissue wet weight)**			**l-Tyrosine transport (M/g tissue wet weight)**		
	Mucosal disappearance	*Gut wall content*	*Serosal appearance*	*Mucosal disappearance*	*Gut wall content*	*Serosal appearance*
0	68.72 ± 1. 90	15.10 ± 1.21	54.10 ± 1.99	16.08 ± 0.76	1.39 ± 0.14	12.91 ± 1.42
1.5	62.32 ± 2.06	24.37 ± 1.73#	40.05 ± 2.40#	15.01 ± 1.41	1.89 ± 0.30#	11.82 ± 0.64
3	49.31 ± 1.40#	29.82 ± 1.23#	29.63 ± 0.03#,*	12.82 ± 0.83#	2.07 ± 0.30#	9.05 ± 0.56#,*
6	36.44 ± 2.83	24.84 ± 1.36	15.03 ± 0.86	10.03 ± 1.48	2.23 ± 0.24	8.70 ± 0.66
12	40.22 ± 1.80#	26.50 ± 0.93#	14.90 ± 1.46#	10.88 ± 0.68#	2.09 ± 0.28#	8.23 ± 0.55#

The results are expressed as means ± S.E.M of seven observations in each group, # $p<0.05$ from the control without EJ extract added to the mucosal solution. * $p<0.05$ from those values immediately above (between the different EJ concentrations).

Table 2. Effects of graded concentrations of extract MC + EJ (50:50) (1.5–12 mg/mL) on transport of d-glucose and l-tyrosine across rat everted intestinal sacs

Concentration of *EJ fruit extract* *in medium (mg/mL)*	**d-Glucose transport (M/g tissue wet weight)**			**l-Tyrosine transport (M/g tissue wet weight)**		
	Mucosal disappearance	*Gut wall content*	*Serosal appearance*	*Mucosal disappearance*	*Gut wall content*	*Serosal appearance*
0	79.56 ± 1.99	13.13 ± 2.02	66.43 ± 2.12	16.30 ± 0.86	1.32 ± 0.22	14.98 ± 1.37
1.5	70.61 ± 2.08	25.57 ± 1.73#	44.86 ± 2.51#	12.82 ± 1.24	1.66 ± 0.30#	11.16 ± 0.42
3	54.33 ± 1.29#	29.10 ± 1.18#	25.23 ± 0.79#	10.03 ± 0.54#	1.87 ± 0.41#	08.16 ± 0.35#
6	41.95 ± 2.58#	22.22 ± 1.36#	19.27 ± 0.93#	09.82 ± 1.27#	2.53 ± 0.33#	07.29 ± 0.81#
12	51.03 ± 1.87#	26.93 ± 0.61#	24.10 ± 2.09#	09.09 ± 0.84#	2.00 ± 0.20#	7.09 ± 0.28#

The results are expressed as means ± S.E.M of seven observations in each group, # $p<0.05$ from the control without EJ extract added to the mucosal solution. * $p<0.05$ from those values immediately above (between the different EJ concentrations).

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Larvicidal and Antimicrobial Activities of Seeds of *Annona cornifolia* A. St. -Hil. (Annonaceae)

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ABSTRACT

The hexane and ethanol extracts from Annona cornifolia A. St. -Hil seeds showed expressive activity in Artemia salina (brine shrimp) and antimicrobial tests. A fraction from hexane extract rich in fatty acids and the corresponding fatty acid methyl esters fraction showed antibacterial and antifungal activities. Methyl esters were prepared from saponifiable fraction of hexane extract with methanol / sulfuric acid and were identified by GC and GC-MS. A fraction obtained from chloroform extract, positive for acetogenins, was extremely active against Candida albicans.

Key words : *Annona cornifolia,* Annonaceae, antimicrobial activity, fatty acid methyl esters, larvicidal activity

INTRODUCTION

Plants from Annonaceae family are very important sources of edible fruits and they are used in folk medicine as antitumor, parasiticide, insecticide, antifever, antidysentery, antiedema (Cepleanu *et al.*, 1993), anti-influenza, anti-insomnia (Wang *et al.*, 2002) and antidiarrhoea agents (Correa *et al.*, 1984; Leboeuf *et al.*, 1982). *Annona* is far the greater genus in this family and species such as *A. crassiflora*, *A. squamosa* and *A. muricata* have a wide use in traditional medicine against diabetes, malaria and infections (Sousa *et al.*, 1991). These activities, from the phytochemical viewpoint, were initially associated only with the isolation of alkaloids, very common

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phytochemical constituents of this family. Nowadays, several activities associated to other classes of natural compounds isolated from this family, such as annonaceous acetogenins, have been reported (Alali *et al.*, 1999).

Annona cornifolia A. St. -Hil. is native from southeast of Brazil and its green fruits are popularly used to heal chronic ulcers (Correa, 1984). The only phytochemical study of this species, to our knowledge, has been done by our group and the isolation and characterization of three acetogenin were described (Santos *et al.*, 2006; Santos *et al.*, 2007).

Since most of the activities related to Annonaceae plants, including *Annona* species, concern to their anti-infectious potential, *A. cornifolia* was screened by larvicidal (Brine Shrimp Lethality test) and antibacterial/ antifungal protocols.

MATERIALS AND METHODS

General

IR spectra were recorded on a Shimadzu/IR-408 spectrophotometer. Fatty acids methyl esters analysis was carried out with a Varian CP-3380 Gas Chromatograph using hydrogen as the carrier gas. A GE Science BP1 column (12 m x 0.25 mm, 100% dimetylpolysiloxane, 0.25 mm) was used. The temperature of the GC oven was held at 150°C and programmed to 240°C with increase of 10°C/min; injection temperature was 250°C and detector, temperature 260°C. Split ratio was 1/80. Analysis GC-MS were performed in a Hewlett Packard 5890 Serie II Chromatograph coupled in a Hewlett Packard 5989A mass spectrometer, using electron ionization. Oven flow was 2 mL/min and mass range was 40–400 m/z. The fatty acids methyl esters were identified by comparison of their retention times with those of standards and by using the Wiley 138 Library database search.

Plant Collection, Identification and Extract Preparation

Fruits of *Annona cornifolia* A. St. -Hil. were collected in Curvelo city region, Minas Gerais State, Brazil, from January to March 1998. The species was identified by Dr. R. Mello-Silva and a voucher specimen (BHCB 68114) was deposited at the Instituto de Ciências Biológicas Herbarium, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.

The fruits were open, the seeds removed, washed, dried at 40°C, powdered (850.0 g) and extracted exhaustively with hexane to give extract F01 (79.5 g) and then with ethanol to give extract F02 (120.0 g), after solvent removal. The latter was dissolved in methanol/water (7:3) and extracted with chloroform to give F03 (7.3 g). Hexane extract (F01) was fractionated by column chromatography on silica gel using gradient systems of n-hexane/CH_2Cl_2/AcOEt/CH_3OH in increasing polarities; a fraction rich in fatty acids (F01-1, 53.2 g) was isolated. Part of this fraction (500 mg)

was refluxed with 1.0 mol/L methanolic sodium hydroxide solution for 30 min. and then extracted with ethyl ether. The aqueous phase was acidified with 1.0 mol/L HCl solution and extracted with ethyl ether. The organic phase was dissolved in hexane and then refluxed with H_2SO_4 methanolic solution 2% v/v for 1 h. 370 mg of a mixture of fatty acid methyl esters (F01-1Me) were obtained after extraction and solvent elimination.

Part of chloroform fraction (F03, 1.0 g) was submitted to medium pressure liquid chromatography using hexane/toluen/AcOEt/EtOH/MeOH in increasing polarities, to furnish several fractions, that were tested for acetogenins by TLC. Acetogenins containing fractions were expected to show pink color by Kedde's reagent and orange color by Dragendorff's reagent. Infrared profile were also obtained. Only fraction F03-7 was positive for acetogenins. Afterwards acetogenins were isolated from this fraction and were related elsewhere (Santos *et al.*, 2006; Santos *et al.*, 2007; Lima *et al.*, 2008).

Larvicidal Bioassay

The methodology used was described by Pimenta *et al.* (2003) and Stefanello *et al.* (2006). *Artemia salina* encysted eggs (10 mg) were incubated in 100 mL of seawater under artificial light at 28°C and pH 7-8. After incubation for 24 h, nauplii were collected with a Pasteur pipette and kept for additional 24 h under the same conditions to reach the metanauplii stage. The samples (triplicate) to be assayed were dissolved in dimethylsulfoxide (DMSO) (2 mg/400 μL or 2 mg/1000 μL) and diluted serially (10, 20, 30, 50 μL/5 mL) in seawater. About 10-20 nauplii were added to each set of tubes containing the samples. Controls with 50 μL of DMSO in seawater were included in each experiment. Lapachol dissolved in DMSO was used as positive control. 24 h later, the number of survivors was counted, recorded and the lethal concentration 50% (LC_{50} value) and 95% confidence intervals were calculated by probit analysis (Finney, 1974).

Antimicrobial Bioassay

The methodology used was described by Miguel *et al.* (1996); Bisagno *et al.* (2000); Almeida *et al.* (2006) and Smania *et al.* (2007). *Staphylococcus aureus* (ATCC 25985), *Streptococcus pyogenes* (ATCC 19615), both gram positive, *Salmonella typhimurium* (ATCC 13311), gram negative and the yeast *Candida albicans* were used for this test. The minimum inhibitory concentration (MIC) was evaluated by the macro dilution test using standard inoculums of 10^{-5} CFU mL^{-1}. Serial dilutions of the test compounds, previously dissolved in DMSO, were prepared to final concentrations of 550.00, 275.00, 137.50, 68.75, 34.38, 17.18, 8.59, 4.30, 2.15 and 1.07 μg/mL. To each tube were added 100 μL of a 24 h old inoculum. The MIC, defined as the lowest concentration of the test compound, which inhibits the visible growth after 18 h, was determined visually after

incubation for 18 h at 37°C. Tests using DMSO as negative control and chloramphenicol and ketoconazol as positive controls were carried out in parallel. All tests were performed in duplicate with full agreement between both results.

RESULTS AND DISCUSSION

Phytochemical analysis of extracts and fractions obtained from *A. cornifolia* seeds led to the identification of a fraction positive for acetogenins (F03-7), obtained from chloroform extract (F03). Both F03 and F03-7, as well as F01 (hexane extract), F01-1 (fatty acids fraction), F01-1Me (fatty acid methyl esters fraction) and F02 (ethanol extract) were tested for cytotoxicity by brine shrimp lethality (*A. salina*) test.

The results obtained from this bioassay are shown in Table 1. LD_{50} values, with 95% confidence range, in μg/mL, were determined using probits method (Finney, 1974). Hexane extract (F01), fraction rich in fatty acid (F01-1), and fatty acid methyl ester fraction (F01-1Me) showed good cytotoxic activity (LD_{50} = 10.00, 9.60 and 8.77 μg/mL, respectively), even higher than the activity presented by the standard, lapachol LD_{50} = 70 mg/mL ($61 < LD_{50} < 81$). Fraction F01-1Me was analysed by gas chromatography and the following fatty acid methyl esters were identified: miristate (C-14); palmitate (C-16); stearate (C-18); oleate (C-18, Δ^9); linoleate (C-18, $\Delta^{9,12}$) and linolenate (C-18, $\Delta^{9,12,15}$), in 0.17, 16.92, 5.56, 51.46, 19.12 and 0.81%, respectively.

Table 1. Larvicidal (brine shrimp lethality bioassay) and antimicrobial (minimum inhibitory concentration, MIC) activities for extracts and fractions from seeds of *Annona cornifolia*

Extracts and fractions	Larvicidal activity LD_{50} [a](μg/mL)	Minimum inhibitory concentration (mg/mL) (MIC)			
		Salmonella typhimurium	*Staphyloccoccus aureus*	*Streptococcus pyogenes*	*Candida albicans*
F01	10.00	137.50	68.75	137.50	34.38
F02	0.20	137.50	137.50	137.50	68.75
F01-1	9.60	275.00	137.50	137.50	68.75
F01-1Me	8.77	550.00	275.00	275.00	nd
F03	0.13	137.50	137.50	137.50	2.15
F03-7	0.16	—	—	—	—
Lapachol [b]	68.09	—	—	—	—
Chloranphenicol [c]	—	8.40	33.60	4.20	—
Ketoconazol [d]	—	—	—	—	40.60

[a] Dosis that kills 50% of the individuals of a population; [b] control used in the brine shrimp larvicidal bioassay; [c] control used in the MIC test for *S. aureus, S. pyogenes, Salmonella typhimurium*; [d] control used in the MIC test for *C. albicans*; nd = not detected.

The saponifiable part of the fraction F01-1 showed 6 fatty acids identified as methyl ester. The total of unsaturated fatty acids methyl esters found in F01-1Me was 71.39% (Table 2). Polyunsaturated fatty acids, when screened by the brine shrimp *A. salina* test, are reported to have higher toxicity compared to satured fatty acids (Ikawa, 2004). According to this report, the toxic activity of such fatty acids hypothetically arises from the corresponding oxidation products, derived from photooxidation or metabolic processes.

Table 2. Fatty acid composition of the F01-1 hexane fraction of *A. cornifolia* seeds in its methyl ester form

Compund	Relative percentage
Methyl myristate	0.17
Methyl palmitate	16.92
Methyl stearate	5.56
Methyl oleate	51.46
Methyl linoleate	19.12
Methyl linolenate	0.81

F02, F03 and F03-7 were highly active (LD_{50} = 0.20 μg/mL, 0.13 and 0.16 μg/mL, respectively), in brine shrimp (*A. salina*) test, presenting results compatible with literature values found for mixtures of acetogenins (Pimenta *et al.*, 2003).

All fractions were also tested for their antimicrobial activity. Minimum inhibitory concentration (MIC) was determined by macrodilution method for ten test concentrations ranging from 550 to 1.07 μg/mL. MIC values were determined after incubation at 37°C, for 14 h. All extracts and fractions were active (Table 1). F03-7 was not tested, due the little amount available. F01, constituted by a mixture of fatty acids, showed activity, against *S. aureus*, close to the control value. It is already known that even traces of certain long chain fatty acids inhibit microorganisms growth, specially Gram-positive bacteria, by affecting cell permeability and transport of nutrients (Wang & Johnson, 1992). Fatty acid monoesters of glycerol have strong bacteriostatic activity against *B. subtilis* and *S. aureus* (Kato & Shibasaki, 1975). According to Kabara (1987), esterification of polyhydroxyl compounds, as glycerol, with fatty acids generally leads to more active derivatives, whereas the same reaction with monohydroxyl compound cause deactivation of the fatty acid. In agreement with this information, esterified fraction (F01-1Me) showed less activity than the original fraction containing the fatty acids. F03, rich in acetogenins, showed good activity against *Salmonella typhimurium*, *Staphylococcus aureus* and *Streptococcus pyogenes*. Acetogenins isolated from *Uvaria hookeri* and *Uvaria narum* are reported to possess antibacterial and antifungal activities (Padmaja *et al.*, 1993).

The present study shows that traditional use of *A. cornifolia* fruits, such as against ulcers, can be associated to the cytotoxic and antibacterial activities found in their extracts. *A. cornifolia* seems to be extremely interesting as source of novel antifungal agents. Both hexane and ethanol extracts (F01 and F02) were highly active against *Candida albicans*, F01 being even more active than the control used (ketoconazol). F03, the chloroform fraction obtained from F02, presented a MIC = 2.15 μg/mL, being almost 20 times more active than the control. From the fraction F03 five acetogenins were isolated that have showed great activity in Brine Shrimp Lethality test (Santos *et al.*, 2006; Santos *et al.*, 2007; Pimenta *et al.*, 2008).

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Antidepressant Activity of the Extracts of Three *Hypericum* species Native to China

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ABSTRACT

The objective of this study was to evaluate three Chinese species of Hypericum genus (Hypericaceae): Hypericum erectum subsp. longisepalum, H. hubeiense and H. seniavinii, as the new sources of antidepressant phytomedicine. The hydroalcoholic extracts of the three Hypericum plants were evaluated for their antidepressant effects by forced swimming test (FST) and tail suspension test (TST) in mice. The mechanisms of action were also investigated by 5-HT-induced head twitches, potentiation of NA toxicity, and elevated plus-maze in mice. All the three extracts (at the doses of 250 or 500 mg/kg, or both) significantly shortened the immobility time in FST and TST. These extracts potentiated the head twitches induced by 5-HT, and H. hubeiense extract (at the dose of 500 mg/kg) also showed an increase of noradrenaline toxicity. In addition, H. erectum and H. hubeiense extracts showed anxiolytic action in the elevated plus-maze test. The results suggest that these three Hypericum extracts possess antidepressant activity, which may be mediated by serotonergic system, and noradrenergic system may also be involved for H. hubeiense. The three Chinese Hypericum species possess activity similar to H. perforatum and have the potential to be used as new sources of antidepressant products.

Key words : Antidepressant, forced swimming test, *hypericum*, tail suspension test

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INTRODUCTION

Hypericum perforatum L. (St. John's wort) is herbaceous plant used for the treatment of the mild-to-moderate depression (Linde *et al.*, 1996; Kasper *et al.*, 2001). It is used in traditional Chinese medicine for the treatment of burns, bruises, swelling, inflammation, and skin diseases (Wu *et al.*, 2002). It has been demonstrated that hypericins, hyperforin and flavonoids are the main active components (Jiang *et al.*, 2004; Sanchez-Mateo *et al.*, 2005). Recent reports have raised the concerns on its interference effect on the metabolism of some pharmaceutical drugs which may limit its future application for some patients (Jiang *et al.*, 2004). Therefore, there is a need to improve the safety profile of St. John's wort and extracts from other species of *Hypericum* which may offer the alternative (Sanchez-Mateo *et al.*, 2005).

The current study aims to identify new source of *Hypericum* of antidepressant activity from Chinese *Hypericum* species. There are over 60 *Hypericum* species distributing in China, but only a few of them have been studied for their chemical constituents and pharmacological activities (Wu *et al.*, 2002; Fu *et al.*, 2006). Recently new species of *Hypericum* were collected and identified by our laboratory (Wu *et al.*, 2004). In the current study, we reported the antidepressant activity and the possible mechanisms of three Chinese *Hypericum*: *Hypericum erectum* subsp. *longisepalum* L. H. Wu (*H. erectum*), *H. hubeiense* L. H. Wu et D. P. Yang, and *H. seniavinii* Maxim, using five well established animal models.

MATERIALS AND METHODS

Plant Materials

The aerial parts of *H. erectum*, *H. hubeiense* and *H. seniavinii* were collected in Badong (Hubei Province, China) during the flowering period in August 2001, and dried at room temperature avoiding direct sunlight. The voucher specimens (No. 01020, No. 01035, No. 01034, respectively) were deposited at Biological Museum of Sun Yat-sen University, Guangzhou, China.

The aerial parts of these plant samples were powdered for extraction. The hydroalcoholic extracts were obtained by maceration of the powdered samples with 6-fold (w/v) amount of 90% aqueous ethanol for overnight at room temperature, and this procedure was repeated twice. The extracts were filtered and dried under reduced pressure below 45°C. The contents of hypericins (hypericin and pseudohypericin) were determined by HPLC and the total flavonoids determined by UV using published methods (Zheng *et al.*, 2003).

Drugs

The following drugs were used as control in the study: imipramine hydrochloride (Jiufu, China), amitriptyline hydrochloride (Shizhou, China), 5-hydroxytryptophan (Acros, USA), N-methyl-N-propargyl-benzylamine (Pargyline) (Acros, USA), norepinephrine (Changji, China), and diazepam (Siyao, China).

Animals and Drug Administration

NIH male mice (18-22 g) were provided by the Medical Animal Center, Guangzhou University of Chinese Medicine (China). The naive animals were kept in a 12 h light/dark cycle (light on started at 07:00) at ambient temperature of 25 ± 1°C. Plant extracts and control drugs were suspended in a 0.5% carboxymethyl cellulose aqueous solution (vehicle) and adjusted to various concentrations so that the final volume given orally was 15 mL/kg, and the final dosage in mice were 250, and 500 mg/kg body weight. They were given orally by gavage 1 h before the tests. Control animals received vehicle under the same conditions. Behavioral observations took place between 8:00 and 15:00 o'clock. All studies were performed in accordance with the Experimental Animal Management Regulations of National Science and Technology Commission, China (November 14th 1988, Article No. 2). In each experimental paradigm, the observers were blind of the treatment protocol.

Imipramine and amitriptyline (20 mg/kg) were given by intraperitoneal route (i.p.) 10 min before FST, and TST respectively. In the elevated plus-maze test, diazepam (3 mg/kg) or vehicle was given orally 1 h before the tests were performed. Experiments were conducted between 8:00-15:00 o'clock. The mice were used just for one time. Each group of behavioral experiments consisted of 12 mice, unless otherwise stated.

Preliminary Acute Toxicity

Limit test was performed to observe the acute toxicity of the three *Hypericum* extracts. There were six mice in each group. Animals received decreasing doses of *H. erectum*, *H. hubeiense* and *H. seniavinii* extracts with the initial dose of 5 g/kg (p.o.), and were placed in empty cages to observe signs of toxicity. Observation was carried out until 7 days after administration.

Forced Swimming Test (FST) in Mice

The forced swimming test was performed according to the methods described by Porsolt *et al.* (1977). Mice were individually placed in a cylindrical glass swim vessel (25 × 12 cm i.d.) filled with 12 cm of water maintaining at 23-25°C. The total immobility time was observed during the last 4 min of a single 6 min test session by unaided eyes of a trained observer, who was blind to the experimental conditions. Mice were

considered immobile when they showed no further attempts to escape except the necessary movements to keep their heads above the water.

Tail Suspension Test (TST) in Mice

After 1 h following withdrawal, mice were individually taken out from the vivarium to an adjoining room and immediately suspended by the tail to a horizontal ring-stand bar (The distance from the Table top was 60 cm) using adhesive tape affixed 1 cm from the tip of the tail. The testing room was brightly lit. The mouse was 15 cm away from the nearest object and both acoustically and visually isolated. The cumulative period of immobility during an observation period of 6 min was recorded by a video camera. The video tapes were scored by a trained observer who was blind to the experimental conditions. Mice were considered immobile only when they hung passively and were completely motionless (Steru *et al.*, 1985).

5-HT-induced Head Switches in Mice

Test drugs were orally administered 30 min before 100 mg/kg i.p. pargyline, and then 1 h later, i.p. 20 mg/kg 5-HT. After administration, the mice were placed in empty cages and the number of head twitches was counted during 15-35 min after 5-HT injection (Goodwin *et al.*, 1984).

Potentiation of NA Toxicity in Mice

Eight groups of mice, ten in each group, were injected NA (3 mg/kg) subcutaneously 60 min after being administrated the extracts (250 and 500 mg/kg, p.o.), vehicle (p.o.), and amitriptyline (20 mg/kg, p.o.), respectively. The number of dead mice was counted 48 h after the administration of test drug (Alpermann *et al.*, 1992).

Elevated Plus-Maze in Mice

The mouse plus-maze consisted of two opposite open arms (30 × 5 cm) crossed with two closed arms of the same dimension with 15 cm height. The arms were connected with a central square (5 × 5 cm) elevated 45 cm above floor level. The animal was placed individually in the center of the maze facing a closed arm. The number of entries into open and closed arms, and the time spent in each arm during the next 5 min were evaluated by an observer inside the room (Willner *et al.*, 1984; Lister *et al.*, 1987). During the experiment, when each test of 5 min was completed, the maze was carefully cleaned with 20% ethanol solution and then thoroughly dried.

Statistical Analysis

Data analysis was performed by one-way analysis of variance (ANOVA) with the Dunnett post-hoc test for multiple comparisons by SPSS 10.0 software. All data were expressed as means ± S.E.M. and the level of statistical significance was set at $p<0.05$.

RESULTS

Preliminary Acute Toxicity

In limit test, *H. hubeiense* extract did not cause any toxicity manifestations until a dose of 5 g/kg (p.o.), while *H. erectum* and *H. seniavinii* extracts induced mortality at lower doses. *H. seniavinii* and *H. erectum* extracts showed no toxicity with doses up to 3.5 g/kg, and 1.75 g/kg, respectively. It was observed that the body weight of the mice treated with *H. seniavinii* extract was significantly lower than those of control group 3 days after administration at a dose of 3.5 g/kg, and then recovered to normal body weight 7 days later. No body weight reduction was observed in *H. erectum* or *H. hubeiense* treated groups.

Behavioral Despair Test

The activities of the three *Hypericum* extracts on the forced swimming test are shown in Fig 1. The tricyclic antidepressant imipramine (20 mg/kg) reduced the immobility time significantly (69.4 ± 8.5 seconds from control 105.9 ± 19.2 seconds, $p<0.05$). At 250 and 500 mg/kg, both *H. hubeiense* and *H. erectum* extracts, shortened the immobility time of mice significantly in FST ($p<0.05$ or $p<0.01$ vs control). The immobility time were *H. hubeiense* 63.7 ± 8.9 and 56.7 ± 22.8 s, *H. erectum* extracts 67.1 ± 14.1 and 63.0 ± 19.5 s. *H. seniavinii* at 250 mg/kg also shortened the immobility time significantly (63.7 ± 13.2 s), but not significantly at 500 mg/kg (81.8 ± 20.7 s).

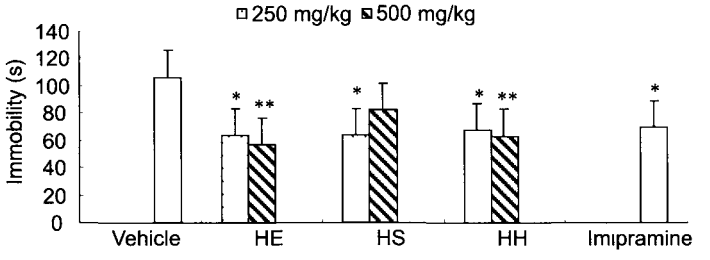

Fig 1. Effect of the extracts of three *Hypericum* species on the total duration of immobility in the forced swimming test (FST) in mice. Results are means ± S.E.M., n=12, *$p<0.05$, **$p<0.01$ compared with control. HE, *Hypericum erectum* subsp. *longisepalum*, HH, *H. hubeiense*, and HS, *H. seniavinii*

The activities of the three *Hypericum* extracts on the tail suspension test are shown in Fig 2. The tricyclic antidepressant imipramine (20 mg/kg) reduced the immobility time significantly (43.2 ± 10.6 seconds from control 107.9 ± 10.4 seconds, $p<0.05$). *H. erectum* extract shortened the immobility time not significantly at 250 mg/kg (86.0 ± 16.4 s), and significantly at 500 mg/kg (73.0 ± 8.9 s). *H. hubeiense* extract shortened the immobility time significantly at 250 mg/kg (74.2 ± 9.0 s), but not

significantly at 500 mg/kg (78.5 ± 12.5 s). *H. seniavinii* at 250 and 500 mg/kg shortened the immobility time significantly (71.7 ± 11.2, 53.0 ± 9.3 s respectively).

Fig 2. Effects of the extracts of three *Hypericum* species on the total duration of immobility in the tail suspension test (TST) in mice. Results are means ± S.E.M., n=12, *p<0.05, **p<0.01 compared with control. HE, *Hypericum erectum* subsp. *longisepalum*, HH, *H. hubeiense*, and HS, *H. seniavinii*

5-HT - Induced Head Twitches in Mice

In order to understand the mechanism of actions of antidepressant effects, the 5-HT - induced head twitches and potentiation of NA toxicity models were performed. The effects of the extracts of three *Hypericum* species on 5-HT induced head twitches are shown in Table 1, the extracts of *H. erectum* (250 and 500 mg/kg), *H. hubeiense* (250 mg/kg) and *H. seniavinii* (500 mg/kg) produced significant potentiation of the head twitches, while imipramine did not produce significant effect at the dose of 20 mg/kg. In the control group there was no mice died, but in all the extract groups and imipramine group, the mice died at different death rates. The number of head twitches was counted from the survival mice.

Table 1. Effects of the extracts of three *Hypericum* species on 5-HT induced head twitches (means ± S.E.M)

Treatment	Dose (mg/kg)	No. of head twitches	Rate of death
Vehicle	——	2.0 ± 0.4	0/12
H. erectum	250	30.7 ± 10.1**	4/10
	500	10.8 ± 0.7**	1/10
H. seniavinii	250	3.8 ± 1.9	3/10
	500	7.1 ± 0.8*	2/10
H. hubeiense	250	6.9 ± 0.7*	1/10
	500	2.5 ± 0.9	2/10
Imipramine	20	3.3 ± 1.8	2/10

*p<0.05,
**p<0.01 compared with control

Potentiation of NA Toxicity

No death of mice was observed in control group at the sub-lethal dose (3 mg/kg, sc.) of NA. The results, as shown in Table 2, demonstrated that only *H. hubeiense* extract (500 mg/kg) produced the potentiation of norepinephrine toxicity, similar to the control amitriptyline, whereas *H. seniavini* and *H. erectum* (250 and 500 mg/kg) did not have the potentiation effect after acute administration.

Table 2. Potentiation of NA (3 mg/kg, sc.) toxicity in mice (means ± S.E.M, n=12)

Treatment	Dose (mg/kg)	Rate of death
Vehicle	-	0
H. erectum	250	0
	500	0
H. seniavinii	250	0
	500	0
H. hubeiense	250	0
	500	3/12**
Amitriptyline	20	5/12**

*p<0.05,
**p<0.01 compared with control

Elevated Plus-Maze

The results of the anxiolytic activity of the extracts were shown in Table 3. *H. erectum*, *H. hubeiense* extracts (250 and 500 mg/kg) and the anxiolytic benzodiazepine drug, diazepam significantly increased residence time of animals in open arms and the number of entries to open arms. At the dosage

Table 3. Effects of the extracts of three *Hypericum* species in the elevated plus-maze (EPM) test in mice (means ± S.E.M, n=12)

Treatment	Dose (mg/kg)	Open arms		Close arms	
		Entries (N)	Time spent (s)	Entries (N)	Time spent (s)
Vehicle	-	5.2 ± 0.7	27.2 ± 5.6	11.3 ± 2.1	233.5 ± 15.8
H. erectum	250	8.7 ± 1.3*	40.7 ± 12.3*	12.8 ± 3.1	199.2 ± 19.5
	500	11.6 ± 3.1**	43.3 ± 19.1*	11.4 ± 3.7	128.0 ± 13.0**
H. seniavinii	250	7.0 ± 3.4	35.8 ± 6.9	9.7 ± 2.1	190.6 ± 20.2
	500	6.8 ± 1.8	35.7 ± 7.0	14.8 ± 3.1	206.7 ± 34.1
H. hubeiense	250	10.2 ± 2.1**	67.7 ± 16.6**	10.1 ± 2.5	130.7 ± 10.6**
	500	9.3 ± 1.3*	40.3 ± 18.9*	10.2 ± 4.1	129.9 ± 9.5**
Diazepam	3	9.6 ± 2.0*	62.5 ± 11.0**	12.8 ± 2.6	133.1 ± 8.0**

*p<0.05,
**p<0.01 compared with control

of 500 mg/kg the two extracts decreased residence time in enclosed arms significantly. However, there was no significant change observed in the number of entries to the closed arm for the control and all extracts. The other species *H. seniavinii* extract (250 and 500 mg/kg) did not significantly affect the residence time and the number of entries in open arms and closed arms.

DISCUSSION

The forced swimming test (FST) (Porsolt *et al.*, 1977) and the tail suspension test (TST) (Steru *et al.*, 1985) are the typical behavioral models commonly used in rodent for screening the antidepressant drugs. The immobility displayed in rodents, subjected to an unavoidable and inescapable stress, is known to reflect the behavioral despair, which may in turn reflect depressive disorders in humans (Goodwin *et al.*, 1984; Alpermann *et al.*, 1992). We studied the hydroalcoholic extracts of three *Hypericum* species endemic to China using these behavioral despair test models in mice. The results indicated that all the extracts (at the doses of 250 or 500 mg/kg, or both), and the conventional TCA antidepressants imipramine and amitriptyline, reduced the immobility time significantly.

Serotonin and norepinephrine are known to play key roles in depression occurrences. Drugs that inhibit the reuptake of serotonin and norepinephrine are known to be effective in the treatment of major depression. *H. hubeiense*, *H. seniavinii* and *H. erectum* extracts could all significantly potentiate the 5-HT-induced head twitches compared with the control group. Additionally, all the extracts and imipramine induced partial mice death at different rates, acompanying with simultaneously drooling and frequent head twitches. These results indicate the possible actions of 5-HT reuptake inhibition (Shank *et al.*, 1987) and potentiation of 5-HT toxicity in the serotoninergic system. It has been reported that some antidepressant drugs, such as, fluoxetine, dexfenfluramine, may cause weight loss, along with their activities in increasing 5-HT availability in the synaptic clefts (McTavish *et al.*, 1992; Goldstein *et al.*, 1994). The weight-losing effect of *H. seniavinii* extract was observed in Limit Test, which also indicates the possibility of serotoninergic system involvement.

The potentiation of norepinephrine toxicity in mice was examined to investigate the performance of the extracts in the noradrenergic system. In the experiment, 50% of the animals treated with amitriptyline died. However, only *H. hubeiense* extract could potentiate the norepinephrine toxicity at the dose of 500 mg/kg with 30% induced-mortality. *H. erectum* and *H. seniavinii* extracts showed no action in the noradrenergic system at the tested doses. No mortality occurred in the control group, neither. It suggested that the antidepressant activity of *H. hubeiense* extract maybe related to its possible effect in noradrenergic uptake inhibition.

Human depression is often accompanied by enhanced anxiety symptoms (Zimmernan *et al.*, 2002). There are reports showed that *Hypericum* also has anxiety activities besides its antidepressant effect. For example, Nicolas' experiments showed that *Hypericum* was active in preventing the anxiety effect of Mg-depletion animal model (Nicolas *et al.*, 2004). Therefore, in current study, we also tested the potential anxiolytic effects of the hydroalcoholic extracts of these three *Hypericum* species in established anxiety test model of the elevated plus-maze. As demonstrated in Table 3, both *H. hubeiense* and *H. erectum* extracts exerted the anxiolytic activity in the elevated plus-maze similar to that caused by benzodiazepine drug diazepam. However, *H. seniavinii* did not show significant effect in anxiety improvement. The results indicate the possible involvement of *H. hubeiense* and *H. erectum* with benzodiazepine receptor.

As the results indicate that the hydroalcoholic extracts of these three *Hypericum* species have antidepressant activities in mice models with different pattern of activities. *H. hubeiense* extract at the doses of 250 and 500 mg/kg significantly shortened the immobility time in FST, and significantly shortened the immobility time in TST at the dose of 500 mg/kg. It potentiated the head twitches induced by 5-HT, showed an increase of noradrenaline (NA) toxicity, and also showed anxiolytic activity in the elevated plus-maze test. These activities indicate that the possible mechanisms of action are inhibition of serotonine, and noradrenaline re-uptake and binding of GABA receptor. It has the lowest toxicity among the three plants. The multiple mechanism of actions of *H. hubeiense* is resemble to *H. perforatum,* which has been shown to shorten the immobility behaviour, inhibit noradrenaline and serotonin re-uptake, inhibit monoamine oxidase, and interact with GABA receptor at a similar dosage (Butterweck *et al.*, 2003).

H. erectum extract significantly shortened the immobility time in FST, and TST. It potentiated the head twitches induced by 5-HT, showed anxiolytic activity in the elevated plus-maze test, but did not increase noradrenaline (NA) toxicity. These activities indicate that the possible mechanisms of action are inhibition of serotonine re-uptake, and binding of benzodiazepine receptor, but not noradrenaline re-uptake. It has relatively high toxicity among the three plants.

H. seniavinii extract significantly shortened the immobility time in FST, and TST. It potentiated the head twitches induced by 5-HT, but did not increase noradrenaline (NA) toxicity, or show anxiolytic activity in the elevated plus-maze test. These activities indicate the possible mechanism of action is inhibition of serotonine re-uptake, but not noradrenaline re-uptake and binding of benzodiazepine receptor.

The dose responses at 250 and 500 mg/kg were not observed for the TST activity of *H. erectum* extract, and potentiation of head twitches by *H.*

hubeiense, while the activities of the two extracts in FST and elevated plus-maze test were dose-dependent. These phenomena may be due to the complexicity in chemical composition and pharmacological activities of the herbal extracts. The inverted U-shaped dose-dependent curve has been observed in shortening of immobility by hyperforin (Butterweck *et al.*, 2003).

As shown in Table 4, the *Hypericum* species contain various amounts of hypericin, pseudohypericin and individual flavonoids. The highest content of hypericins and flavonoids (5.76 mg/kg, 40.89 mg/kg respectively) are in *H. erectum* extract, and the lowest contents are in *H. seniavinii* extract. Therefore there is no direct correlation of the hypericins content with the antidepressant activity. Further studies are required to define their chemical profiles as well as the relationship between the main constituents and the antidepressant activities.

Table 4. Yields of the hydroalcoholic extracts of three *Hypericum* species and the contents of the main constituents in the extracts

Hypericum **species**	**Yield of extract (%)**	**Content in extract (mg/g)**	
		Hypericins HPLC	**Flavonoids UV**
H. hubeiense	14.2	3.30	33.76
H. seniavinii	21.8	1.30	26.41
H. erectum	15.0	5.76	40.89

In summary the results suggest that these three *Hypericum* extracts possess antidepressant activity, which may be mainly mediated by serotonergic system. The *H. hubeiense* and *H. erectum* zalso possess anxiolytic activity. The results indicate that the three Chinese Hypericum species, in particularly *H. hubeiense,* possess pharmacological activities similar to *H. perforatum* and have the potential to be used as new source of antidepressant product.

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Cytolytic and Toxic Effects of Ostreolysin, a Protein from the Oyster Mushroom (*Pleurotus ostreatus*)

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ABSTRACT

Ostreolysin is a 15 kDa cytolytic protein found in considerable amounts in oyster mushrooms (Pleurotus ostreatus). Ingestion of large quantities of these edible mushrooms can result in adverse reactions, and at least some of these effects can be attributed to ostreolysin. This protein is able to permeabilize erythrocytes and other cells at sub-micromolar concentrations, acting by a colloid-osmotic mechanism and inducing the formation of a membrane pore with a hydrodynamic radius of 2 nm. Its binding to and subsequent permeabilization of the membrane depend on interaction with cholesterol-enriched raft-like membrane domains. When injected intravenously into rodents, ostreolysin induces cardiorespiratory arrest and is lethal for mice with an LD_{50} of 1170 μg/kg. In rats, it induces a transient increase in arterial blood pressure, followed by a progressive drop of blood pressure, associated with noticeable bradicardia and myocardial ischemia. Continued drop of blood pressure is accompanied with ventricular extrasystoles, related to marked hyperkalemia. Moreover, sub-micromolar concentrations of ostreolysin induce a concentration-dependent increase in aortic ring tension.

Key words : Cardiorespiratory effect, hemolysis, hyperkalemia, lipid rafts, ostreolysin, *Pleurotus ostreatus*, toxicity, pore forming protein

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INTRODUCTION

Ostreolysin (Oly) is an acidic 15 kDa protein that is synthesized specifically in the primordia and young fruiting bodies of the oyster mushroom (*Pleurotus ostreatus*) (Berne *et al.*, 2002). The protein belongs to a larger group of highly homologous proteins called aegerolysins. Aegerolysin-like proteins have been isolated from several mushrooms, moulds, plants and bacteria (Berne *et al.*, 2005). They all have low isoelectric points, similar molecular weights, and are heat-labile. Furthermore, they are stable over a wide range of pH, mainly from 4 to 10 (Sakaguchi *et al.*, 1975; Berne *et al.*, 2005). Several aegerolysins are expressed during the formation of mushroom fruit bodies (Fernandez-Espinar & Labarère, 1997; Berne *et al.*, 2002; Vidic *et al.*, 2005) and during bacterial sporulation (Barloy *et al.*, 1998), which suggests their involvement in the developmental cycle of the organism that produces them. Oly can induce the formation of primordia and development of young fruit bodies of *P. ostreatus*, even when applied exogenously to the mushroom mycelium (Berne *et al.*, 2007).

Binding to natural and artificial lipid membranes followed by permeabilization is another common feature of many aegerolysins. Asp-hemolysin from the mould *Aspergillus fumigatus*, as well as mushroom-derived aegerolysins (aegerolysin from *Agrocybe aegerita*, Oly and pleurotolysin A (Ply A) from *Pleurotus ostreatus*, and eryngeolysin from *Pleurotus eryngii*) have been reported to lyse erythrocytes and various cell lines (Ebina *et al.*, 1985; Berne *et al.*, 2002; Sepčić *et al.*, 2003; Tomita *et al.*, 2004; Ngai & Ng, 2006).

The hemolytic and cytolytic effects of aegerolysin-like proteins appear to be associated with their toxicity. One of the most studied aegerolysins, Asp-hemolysin, can be considered as a virulence factor of *A. fumigatus* during infection of animals by this opportunistic pathogenic mould (Ebina *et al.*, 1982; Maličev *et al.*, 2007). The edible oyster mushroom, although being a massively cultivated species with important medicinal, biotechnological, and environmental applications (Kües & Liu, 2000; Cohen *et al.*, 2002) has been reported to induce sporadic local intoxications (Al-Deen *et al.*, 1987). These toxic effects appear to be connected with Oly, which causes cardiorespiratory arrest after intravenous administration to mice, with a half-lethal dose of 1170 μg/kg (Žužek *et al.*, 2006).

The lytic process caused by many pore-forming proteins starts by their recognition of a distinctive membrane component such as a membrane lipid. Aegerolysins show considerable variability in this respect. Asp-hemolysin, and recombinant PA0122 from *Pseudomonas aeruginosa* have been suggested to bind specifically to lysophosphatidylcholine (Kudo *et al.*, 2002; Rao *et al.*, 2008), PlyA to sphingomyelin (SM) (Tomita *et al.*, 2004) and Oly to sense specifically membrane domains enriched together in cholesterol

(Chol) and SM (Sepčić *et al.*, 2004). Lateral cell membrane domains, known as lipid rafts, are highly enriched in these two lipids (Pike, 2004). These transient membrane entities, existing in a liquid-ordered (l_o) state (McConnell & Vrljic, 2003), are more resistant to solubilization by detergents than lipids in liquid-disordered (l_d) domains (Lichtenberg *et al.*, 2005) and are therefore often termed detergent-resistant membranes (DRMs) (Simons & Ikonen, 1997). Lipid rafts are involved in several important biological functions, including exocytosis and endocytosis, signal transduction, pathogen entry, and attachment of ligands (Simons and Ikonen, 1997; London, 2002; Edidin, 2003), and stabilize on binding ligand molecules (Kenworthy *et al.*, 2000; Subczynski & Kusumi, 2003; Hancock, 2006).

Due to their important functions in the living cell and in cell-cell interactions, there is an increasing need for new techniques to study lipid rafts. Oly, which recognizes specifically the combination of the two main lipid raft components, SM and Chol, is in this regard a good candidate for a new marker of Chol-enriched raft-like membrane microdomains.

MEMBRANE ACTIVITY OF OSTREOLYSIN

The ability to bind to natural and artificial lipid membranes and form pores is a common feature of aegerolysin-like proteins. There have been several attempts to express recombinant aegerolysins in bacterial hosts, however most were devoid of lytic potential (Juarez-Perez & Delécluse, 2001; Kumagai *et al.*, 2002; Rao *et al.*, 2008; Rebolj & Sepčić, unpublished data). The sole exception is the recombinant form of PlyA, which acts as an active bi-component cytolysin in concert with the recombinant 59 kDa pleurotolysin B (PlyB) (Sakurai *et al.*, 2004).

Hemolytic and Cytolytic Activities

Oly lyses erythrocytes from a variety of species at sub-micromolar concentrations (Sepčić *et al.*, 2003). The time course of hemolysis is typical of pore-forming proteins that bind to the membrane in their monomeric form, oligomerize, insert and translocate their polypeptide segments across the lipid bilayers, resulting in the formation of a transmembrane pore consisting of several protein monomers (Gouaux, 1997). The hydrodynamic radius of an Oly-induced transmembrane pore, determined by the use of different osmotic protectants, is 2 nm (Sepčić *et al.*, 2003). Some aegerolysins have been shown to form rings and patches at the surface of erythrocyte membranes, detected by scanning electron microscopy (Ebina *et al.*, 1985; Tomita *et al.*, 2004). In the case of pleurotolysin, SDS-stable, ring-shaped 700 kDa complexes were observed when Ply A and Ply B were mixed in a 3:1 molar ratio (Tomita *et al.*, 2004). Incubation of Oly with lipid vesicles formed from total sheep erythrocyte lipids, followed by ultracentrifugation, showed that Oly is located exclusively in the sediment, also pointing to its

tight binding with lipid membranes. Furthermore, SDS-PAGE analysis of the diluted sediment showed the formation of membrane-bound Oly aggregates of around 34, 64, and 100 kDa, corresponding to dimers, tetramers, and hexamers (Sepčić *et al.*, 2003).

The pH-optima for Oly binding to the erythrocyte membrane, and for the consequent permeabilization, differ. Optimal binding is usually achieved between pH 6 and 7, while maximal hemolysis is observed between pH 7 and 8 (Berne *et al.*, 2005). Oly however retains its membrane activity over a wide range of pH, from 3.5 to 10.5 (Berne *et al.*, 2005). At physiological pH, the binding of Oly to a membrane correlates with an increase in its intrinsic tryptophan fluorescence and α-helical content (Sepčić *et al.,* 2003). Fluorescence titration experiments revealed that Oly changes its conformation more extensively in the lipid-bound than in the free form, suggesting that binding to the bilayers evokes further conformational transitions necessary for membrane insertion and pore formation (Berne *et al.*, 2005). Further, the involvement of intact Oly cysteine residues in the hemolytic process has been proved to be important (Berne *et al.*, 2002, 2005). Lysis can be prevented by micromolar concentrations of some divalent ions, like Hg^{2+} and Fe^{2+} (Berne *et al.*, 2002). Finally, Oly-induced hemolytic effects are abolished by the addition of micromolar concentrations of lysophospholipids (especially lysophosphatidylinositol and sphingosine-1-phosphate), fatty acids (Sepčić *et al.*, 2003), and lipid vesicles composed of an equimolar SM:Chol mixture (Sepčić *et al.*, 2004).

The differing sensitivity of erythrocytes from different species to aegerolysins is another interesting feature (Sakaguchi *et al.*, 1975; Bernheimer & Avigad, 1979; Tomita *et al.*, 2004; Ngai & Ng, 2006). Oly showed no difference in its ability to lyse sheep, bovine and human erythrocytes (Sepčić *et al.*, 2003) but was much less potent on those from rodents (Žužek *et al.*, 2006), dog, and amphibians (Rebolj & Sepčić, unpublished data) (Fig 1).

In addition to erythrocytes, Oly and several other aegerolysins have been found to be cytotoxic for cell lines at sub-micromolar concentrations (Sepčić *et al.*, 2003; Sepčić *et al.*, 2004; Maličev *et al.*, 2007; Rebolj *et al.*, 2007) (Table 1). Direct microscopic observation of some tumour cells exposed to Oly showed typical signs of colloid-osmotic lysis, like swelling, blebbing and degranulation of cells (Sepčić *et al.*, 2003). Similar events were observed during real-time monitoring of Oly-induced disruption of fluorescently stained chondrocytes and osteoblasts, using confocal microscopy (Maličev *et al.*, 2007). Lytic effects of Oly on different cells can induce hyperkalaemia and are therefore, at least partially, responsible for the observed toxicity of the protein after intravenous administration (Žužek *et al.*, 2006).

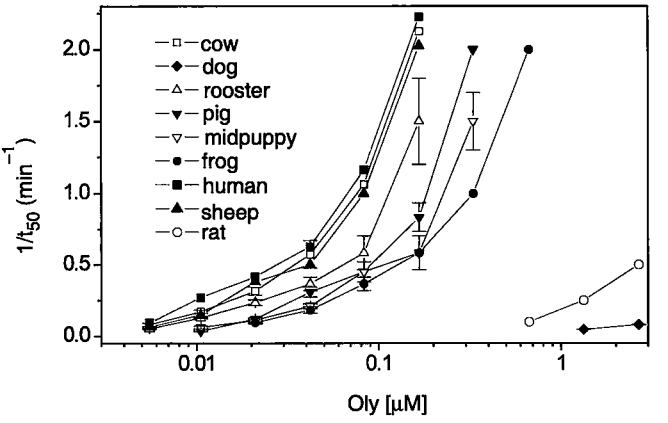

Fig 1. Hemolytic activity of ostreolysin on erythrocytes. Hemolysis was assayed by a turbidimetric method (Sepčić *et al.*, 2003). The reciprocal half-time of hemolysis, ($1/t_{50}$), is plotted against the concentration of ostreolysin, Oly (0.0055 to 3 μm). t_{50} is the time in which half of the erythrocytes were lysed. Each result is the mean±standard error of three independent experiments

Table 1. Cytotoxic properties of Oly on cell lines. ED_{50} is the concentration of ostreolysin producing 50% cytotoxicity

Cell line	ED_{50} (μM)	Time of exposure	Reference(s)
HT 1080 (fibrosarcoma, human)	0.67	2 h	Sepčić *et al.*, 2003
MCF 7 (mammalian tumour, human)	0.67	2 h	Sepčić *et al.*, 2003
Chinese hamster ovary cells	0.067	15 min	Sepčić *et al.*, 2004
Human chondrocytes	0.067	1 h	Maličev *et al.*, 2007
V-79-379A (lung fibroblasts of Chinese hamster)	0.087	1 h	Rebolj *et al.*, 2007
HUVEC (human umbilical vein endothelial cells)	0.15	1 h	Rebolj *et al.*, 2007

Mode of Ostreolysin Binding to Cell Membranes

In order to elucidate the structural characteristics of Oly-susceptible membranes, we performed an extensive preliminary study using artificial lipid membranes. Lipid monolayers or bilayers, the latter in the form of vesicles of different sizes, with a range of lipid compositions, were tested for their susceptibility to Oly. Binding was assessed either indirectly in a hemolysis-inhibition assay by incubating Oly with lipid vesicles and subsequent addition of erythrocytes (Sepčić *et al.*, 2003), or directly, using surface plasmon resonance on supported lipid monolayers (Sepčić *et al.*, 2004; Rebolj *et al.*, 2006; Chowdhury *et al.*, 2008). Membrane-permeabilizing activity was assessed mainly by measuring the leakage of the fluorescent dye calcein from lipid vesicles following the addition of

Oly (Sepčić *et al.*, 2003; Sepčić *et al.*, 2004; Rebolj *et al.*, 2006). We found that lipid vesicles composed of equimolar mixtures of egg phosphatidiylcholine with various other lipids were completely resistant to Oly binding and permeabilization (Sepčić *et al.*, 2003). The only vesicles showing susceptibility to Oly membrane activity were those composed of SM and Chol in a 1:1 molar ratio (Sepčić *et al.*, 2004). Due to the facts that (i) Oly cannot bind to pure SM or Chol, and (ii) the two lipids are known to be highly enriched in lipid rafts, Oly specific interaction with SM:Chol liposomes suggested its interaction with Chol-enriched raft-like membrane microdomains in living cells. This finding was confirmed by a series of experiments. First, Oly was found in isolated DRMs of both SM:Chol (1:1, mol:mol) vesicles and Chinese Hamster Ovary (CHO) cells (Sepčić *et al.*, 2004). Secondly, Oly bound to and permeabilized SM:Chol membranes in various molar proportions of the two lipids, but only above 30 mol% Chol (Sepčić *et al.*, 2004, Rebolj *et al.*, 2006), the concentration at which this sterol induces the formation of a liquid-ordered phase (de Almeida *et al.*, 2003). Further, binding of sub-lytic concentrations of Oly to various mammalian cells and cell lines (CHO cells, mouse somatotrophs, human chondrocytes and osteoblasts), followed by immunolocalization using fluorescence-labeled antibodies, showed selective binding and clustering of the protein on cell membranes, pointing to its association with distinctive membrane microdomains rich in Chol (Maličev *et al.*, 2007, Chowdhury *et al.*, 2008). Lipid rafts are sensitive to treatment with methyl-β-cyclodextrin (MβCD), which acts as a Chol scavenger and promotes disruption of the structural and functional integrity of low density membrane domains (Pike & Miller, 1998). In CHO cells pre-treated with 20 mM MβCD (and depleted by 43% of their Chol content), Oly membrane binding was dramatically decreased to only 2%. However, treatment with 10 mM MβCD, which resulted in depletion of membrane Chol by 35%, caused no significant reduction in the binding of Oly (Chowdhury *et al.*, 2008) (Fig 2). In accordance with the results obtained with SM:Chol vesicles in various molar ratios (Sepčić *et al.*, 2004), these data suggest that, to effectively bind and permeabilize lipid membranes, Oly requires a specific threshold membrane Chol concentration. The same trend was observed in experiments with CHO cell mutants having impaired Chol synthesis and consequently a lower fraction of raft-like membrane domains. Binding of Oly to these cells clearly correlated with the amount of their membrane Chol (Sepčić *et al.*, 2004).

However, not all our results suggest Oly as a classical lipid raft-binding protein. Our experiments using lipid mono- and bi-layers composed of SM and various sterols and Chol derivatives revealed that Oly membrane activity does not completely correlate with the ability of a particular steroid to induce the formation of l_o domains (Rebolj *et al.*,

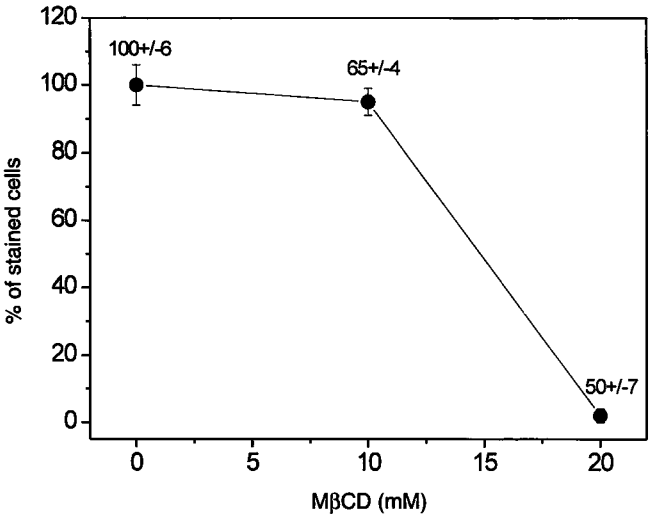

Fig 2. Effect of membrane cholesterol concentration on ostreolysin binding to Chinese Hamster Ovary cells. The graph represents percentage of cells able to bind ostreolysin (Oly) following their pre-treatment with methyl-β-cyclodextrin, MβCD, (0 to 20 mM). Symbols represent mean values of Oly bound±standard error from 16-20 micrographs in 2 independent experiments. Numbers adjacent to error bars indicate the percentage of cholesterol in cell membranes. Adapted from Chowdhury *et al.* 2008

2006). Further, Oly cannot exert its membrane activity on lipid membranes composed of the equimolar mixture of SM, Chol and unsaturated glycerophospholipids that are generally used to mimic the membranes of living cells (Sepčić *et al.*, 2004). Finally, immunostaining studies using CHO cells showed that Oly was not co-localized with typical raft-binding proteins like caveolin and cholera toxin B-subunit, suggesting that it binds to distinct Chol-enriched membrane domains (Chowdhury *et al.*, 2008). Altogether, these findings reinforce the emerging idea of the microheterogeneity of the lipid rafts (Roper *et al.*, 2000; de Almeida *et al.*, 2005; Hancock, 2006), comprising at least 2 Chol-enriched domain types – caveolin-containing (caveolae), and caveolin-lacking rafts (Pike, 2004). It appears that Oly, rather than recognizing the pure lipid component (*e.g.* Chol or SM), or a specific physical state of the membrane (*e.g.* a liquid-ordered state), recognizes and binds to specific patterns that are formed by Chol molecules at the surface of biological membranes. This conclusion is reinforced by the fact that the addition of Oly does not affect the electron paramagnetic resonance spectra of equimolar SM:Chol vesicles, which would point to the recruitment of the lipids after the ligand binding (Sepčić *et al.*, 2004).

WHOLE ANIMAL TOXICITY STUDIES OF OSTREOLYSIN AND *IN VITRO* STUDIES ON THE CELL AND ORGAN LEVEL

In vivo Effects of Ostreolysin

Despite its putative physiological role during the formation of mushroom fruit bodies (Berne *et al.*, 2002; Vidic *et al.*, 2005; Berne *et al.*, 2007), experiments on erythrocytes and artificial membranes revealed that Oly is also a pore-forming agent (Sepčić *et al.*, 2003). The hemolytic and cytolytic activity of many bioactive proteins from different sources appears to be associated with their toxicity. Many questions arise as to whether these properties are also responsible for Oly toxicity, as demonstrated for another aegerolysin-like protein, Asp-hemolysin (Ebina *et al.*, 1982; Maličev *et al.*, 2007). To investigate this, the toxic and lethal effects of Oly were further studied on experimental rodents *in vivo* and *in vitro*. The lethal and toxic effects, expressed as LD_{50} dose, have been determined in mice and rats, respectively. To determine LD_{50}, Oly was dissolved in physiological solution and injected as a bolus through the right tail vein of mice. Subsequent clinical signs comprised cyanosis, cessation of movement and hair bristle, and lethal outcome within 20 min for all mice at a dose of 1400 μg/kg or higher (Žužek *et al.*, 2006). The *i.v.* LD_{50} in mice was 1170 μg/kg (Žužek *et al.*, 2006).

In order to study its effects on the cardiovascular system and systemic response, *in vivo* experiments with Oly were performed on adult male Wistar rats. Oly was dissolved in physiological solution and applied as a bolus through the cannulated right jugular vein. Respiratory activity, electrocardiograms (ECG) and blood pressure were monitored. Since Oly is lytic to erythrocytes from various animal species and cell lines (Fig 1), some blood electrolytes were also monitored to find out whether the heart rhythm abnormalities caused by Oly are related to the altered ion concentration (in particular to potassium concentration related to its hemolytic action). In the sub-micromolar range, the toxin lysed rat erythrocytes *in vitro* in a dose-dependent manner. When injected into rats under general anesthesia, one mice LD_{50} of Oly causes death in all experimental animals, suggesting that rats are more sensitive than mice. Hemolysis, with a resulting rise in plasma potassium concentration, is probably an important factor in its toxic and lethal effects, as already shown for some other pore forming toxins (Šuput *et al.*, 2001; Andrade *et al.*, 2004).

In a dose of one mice LD_{50}, Oly produced a transient pressor response in rats, followed by a prolonged hypotensive response and a progressive, irreversible fall of blood pressure to its mid-circulatory value. In addition, transient apnea is observed after injection of Oly, followed by transient respiratory activity and respiratory arrest (Žužek, Sepčić & Frangež, unpublished results) (Fig 3). The transient pressor response induced by 1 LD_{50} of Oly lasted for approximately 1-3 min. The basal mean arterial

blood pressure (MAP) of anaesthetized rats increased from 87 ± 7.6 mm Hg (n=6) before the injection of Oly to 116 ± 9.7 mm Hg (n=6) afterwards. The pressor response was followed by a progressive and irreversible fall in arterial blood pressure (aBP) to the mid-circulatory blood pressure (6-8 mmHg) in all experiments (Fig 3). Oly produced a respiratory arrest in 10-20 s after the toxin injection, followed by a transient respiratory activity after 1-2 min. Two min after injection into anaesthetized rats, Oly significantly reduced the heart rate from 266 ± 14.6 to 121 ± 21.9 ($p \leq 0.05$, n=6). At the same time, changes in ECG appeared as sinus arrhythmia, with peaking and increased amplitude of the T-wave, and increased prolonged P-R interval. Elevation of the S-T segment, ventricular extrasystoles and widened QRS complexes were also observed regularly, suggesting myocardial ischemia (Fig 3) (Žužek *et al.*, 2006).

Oly binds specifically to raft-like Chol-rich membrane domains (Sepčić *et al.*, 2004; Rebolj *et al.*, 2006), followed by the formation of 4 nm transmembrane pores (Sepčić *et al.*, 2003). Severe hyperkalemia (10.28 ± 1.25 mmol/l, $p \leq 0.05$, n=6), caused by the lytic action of Oly on erythrocytes and other cells, may be responsible for bradycardia and irregular heart rate and rhythm in rats (Žužek *et al.*, 2006).

To evaluate the role of cholinergic stimulation of the heart, potentially triggered by the action of Oly on arterial blood pressure and on the ECG, its effects were studied in atropinized rats. Although the effects of Oly on heart rate were less pronounced than in the control group of rats, the muscarinic blocker, atropine, did not significantly abolish or alter the cardiorespiratory effects described for the non-atropinized group of animals injected with Oly. It was therefore concluded that parasympathetic stimulation of the heart plays only a minor role in Oly cardiotoxic action (Žužek *et al.*, 2006). It was also found that Oly produces the same changes in ECG, blood pressure, and biochemical blood parameters in artificially ventilated rats (n=4), which excludes hypoxia as the primary cause of cardiotoxic effects initiated by respiratory arrest (Žužek *et al.*, 2006).

Although hyperkalemia, suggesting *in vivo* lysis of erythrocytes and other cells, may be significant for its lethal action, the exact mechanism of the lethal effect of Oly remains unclear. Results from the *in vivo* experiments (arrhythmias, drop of blood pressure, elevation of S-T segment) suggest myocardial hypoxia as the primary effect. Hence, in order to identify the cellular and molecular mechanisms of the toxic action responsible for *in vivo* effects of Oly, its effects on vessel ring tension and endothelial cell viability have recently been studied (Rebolj *et al.*, 2007).

Effect of Oly on Isolated Coronary Rings

To identify its target organs and cells, *in vitro* experiments with Oly have been performed on thoracic aortic ring preparations, human umbilical vein

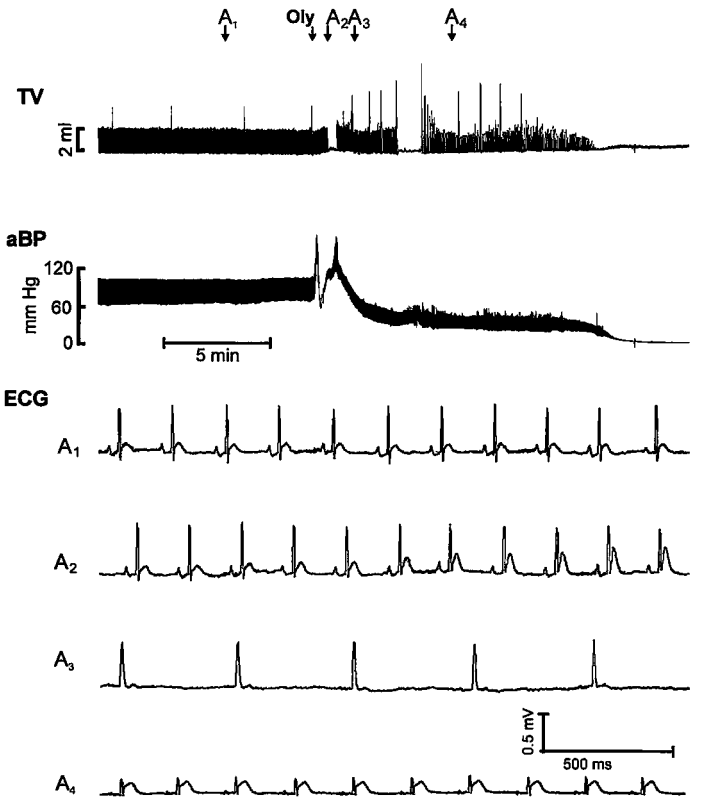

Fig 3. A representative experiment in which the effects of Oly on electrocardiogram (ECG), arterial blood pressure and breathing were recorded. Tidal volume (TV) and arterial blood pressure (aBP) represent the time course of both parameters recorded during the whole experiment. A_1 is the ECG recording before the toxin injection and A_2 to A_4 the ECG traces after the toxin injection as indicated on the top of the fig

endothelial cells (HUVEC), and lung fibroblasts of Chinese hamster (V-79-379A).

Oly induced concentration-dependent rat aorta ring contractions at final concentrations of 1 to 50 μg/mL. After the aorta rings were exposed to various concentrations of Oly, endothelium-mediated relaxation of rings pre-contracted with norepinephrine was diminished. This effect may be due to injury of the endothelial cells or to the breakdown of the mechanisms responsible for the acetylcholine (ACh) mediated endothelium relaxation response (Rebolj *et al.*, 2007). This result is in agreement with those related to the lytic effects of Oly on erythrocytes (Sepčić *et al.*, 2003) and umbilical vein endothelial cells (Rebolj *et al.*, 2007). The effective cytotoxic concentration (EC_{50}) of Oly evaluated after 48 h was 1.3 μg/mL for V-79, and 2.2 μg/mL for HUVEC cells in the growth medium (Rebolj *et al.*, 2007).

The direct damage of endothelial cells probably plays an important role in the cardiotoxicity of Oly (Žužek *et al.*, 2006). This view is additionally supported by the results obtained from the experiments with aortic rings having preserved endothelium, where a functionally defective ACh-mediated relaxation response was clearly demonstrated (Rebolj *et al.*, 2007). The final concentrations of Oly (15-30 μg/mL) in the bathing medium, which produced a significant increase in aorta ring tension *in vitro*, were similar to the maximal calculated concentrations of Oly in the blood during the *in vivo* experiments. It is suggestive that especially the coronary vessels contraction may be important as the underlying cause of death in experimental rodents (Rebolj *et al.*, 2007). The same effects on coronary vessels could result in a vasospasm leading to ischemia and hypoxic injury of cardiomyocytes. This view is also supported by the ECG data from *in vivo* experiments (Žužek *et al.*, 2006). Direct harmful effect of Oly on cardiomyocytes and cells of the conductive system of the heart also cannot be excluded.

CONCLUSIONS AND PERSPECTIVES

Since Oly may be a causative agent responsible for some documented adverse responses associated with eating oyster mushrooms, it is important to reveal the exact molecular mechanisms responsible for its cardiorespiratory and lethal action observed in rodents. Moreover, although its physiological role in the mushroom is still uncertain, Oly displays a variety of effects that could have valuable applications in various fields of human activity. First, its ability to trigger the fruiting of oyster mushrooms after its application to the mushroom mycelium (Berne *et al.*, 2007) could be used in the field of biotechnology. Secondly, because of its specific interaction with Chol-rich raft-like membrane domains (Sepčić *et al.*, 2004; Maličev *et al.*, 2007; Chowdhury *et al.*, 2008), Oly could find its place in the field of cell biology as a lipid raft marker. Fluorescence- or spin-labeled raft-binding mutants of cytolytic proteins, devoid of their lytic activity, are good candidates in this regard. One of these (the G_{M1} ganglioside-binding cholera-toxin B subunit (Bacia *et al.*, 2004) forms part of a commercially available raft-labeling kit. Finally, the cytolytic and hemolytic properties of Oly are comparable to those exerted by Asp-hemolysin, the putative virulence factor of the pathogenic mould *Aspergillus fumigatus* (Maličev *et al.*, 2007). Studies of the molecular mechanisms of Oly action could therefore shed some light on the action of Asp-hemolysin during the infection, and help in the development of vaccines or in the use of antibodies against infection by *A. fumigatus* spores, which is very frequent in hospitals associated with surgery.

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13

Antioxidant, Antisecretory and Gastroprotective Activities from *Leiothrix flavescens*

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ABSTRACT

Leiothrix flavescens, popularly know as "sempre viva" belongs to Eriocaulaceae family with important biological activity. Extracts of this specie contain mainly flavones and xanthones. The methanolic extract (MeOH) obtained the scapes of L. flavescens was evaluated for its ability to protect the gastric mucosa against gastric mucosa injuries agents in rodents such as HCl/EtOH, non-steroidal anti-inflammatory drug or stress. We also evaluated its activity against Helicobacter pylori and its antioxidant property. All pretreatment with MeOH extract from L. flavescens show significant gastroprotective action. No acute toxicity was observed in animals treated with MeOH extract at doses of 5 g/kg (p.o.). The MeOH extract from L. flavescens also increased intestinal motility and changed gastric juice parameters significantly by decreasing gastric acid secretion and promoting reduced acid output. The MeOH extract also shown effective action against H. pylori by inhibiting their growth and presented an interesting antioxidant action in vitro. The

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present data show the effectiveness of MeOH extract from L. flavescens as antacid/antiulcer and laxative.

Key words : Flavonoids, antiulcer, *Leiothrix flavescens,* xanthones, anti-*Helicobacter pylori*, antioxidant action

INTRODUCTION

Leiothrix Ruhland is an exclusively South American genus of about 37 species mainly restricted to Brazil, with only two species occurring elsewhere: *Leiothrix flavescens* (Bong.) Ruhland that occurs in Brazil, Venezuela, Guyana and Peru, and *Leiothrix celiae* Moldenke which is found only in Venezuela (Giulietti *et al.*, 1998). The other species are mostly endemic to small areas in Minas Gerais and in Bahia, states of central Brazil (Giulietti *et al.*, 1994). This genus and other species of the Eriocaulaceae family such as *Syngonanthus* and *Paepalanthus* are known as "sempre vivas". Chemical studies on species of *Leiothrix* are also scarce. Salatino *et al.* (1990) studied the contents of soluble phenolic compounds of the capitula of 21 species of *Leiothrix.* The presence of nepetin-7-O-β-D-glucopyranoside, nepetin-7-O-β-D-arabinopyranoside and luteolin *O*- and *C*-glucopyranoside were determined in *Leiothrix* species (Dokkedal & Salatino, 1992). Another study determined the presence of xanthones and flavonoids with antioxidant activity in capitula of *Leiothrix* species (Santos *et al.*, 2001).

Although there has been no pharmacological study about this species, the Eriocaulaceae family has presented significant effects on gastrointestinal tract. The antiulcerogenic activity of two species of *Syngonanthus* (Coelho *et al.*, 2006; Batista *et al.*, 2004) has also been determined. Di Stasi *et al.* (2004) reported the intestinal anti-inflammatory activity of paepalantin, an isocoumarin isolated from *Paepalanthus bromelioides* (Eriocaulaceae).Therefore, the present work was carried out in order to investigate the gastric effect of the methanolic extract (MeOH) from scapes of *L. flavescens* against acute experimental models of gastric ulcer in rodents as well as to perform its phytochemical investigation.

MATERIALS AND METHODS

Plant Material

Scapes of *Leiothrix flavescens* (Bong.) Ruhland were collected in May 1997, at Serra do Cipó, Minas Gerais state, Brazil and authenticated by Professor Dr. Paulo Takeo Sano from Instituto de Biociências (IB) da Universidade de São Paulo (USP), São Paulo. A voucher specimen (CFCR n° 4680) was deposited at the Herbarium of the IB-USP.

Animals

Male Swiss albino mice (25-35 g) from the Central Animal House of the Universidade Estadual Julio de Mesquita Filho (UNESP/Botucatu) were used. The animals were fed a certified Nuvilab CR-a® (Nuvital) diet with free access to tap water under standard conditions of 12 h dark-12 h light and temperature (22 ± 1%). Fasting was used prior to all assays because standard drugs and extract were always administered either orally (by gavage) or by intraduodenal route. The protocols followed the recommendations of the Canadian Council on Animal Care (Olfert *et al.*, 1993).

Drugs and Chemicals

Hydrochloric acid (Nuclear, Brazil), cimetidine, atropine (Sigma Chemical Co., St Louis, MO, USA), piroxicam (Hexal, Brazil) and lansoprazole (Medley, Brazil) were used in this study. Extract was dissolved in NaCl solution 0.9% (vehicle). All substances were prepared immediately before use and the reagents used were of analytical grade.

Acute Toxicity

The acute toxicity studies of *L. flavescens* were performed in mice. In this assay, increasing doses of MeOH extract were orally administered to groups of ten animals for each dose after a 6 h fast. Animals receiving the vehicle served as control. The signs and symptoms associated with the MeOH administration were observed at 0, 30, 60, 120, 180 and 240 min. after and then once a day for the next 14 days. At the end of the period the number of survivors was recorded. The acute toxicological effect was also estimated (Souza Brito, 1995).

Antiulcerogenic Effect

HCl/ethanol-induced ulcer: The antiulcerogenic activity obtained from *L. flavescens* was studied in HCl/ethanol-induced gastric ulcer (Mizui & Doteuchi, 1983). Mice were divided into groups of 7-8 animals, which fasted 24 h prior to receiving an oral dose of the vehicle, saline (10 mL/kg), lansoprazole (30 mg/kg) or *L. flavescens* (250, 500 or 1000 mg/kg). After 50 min. all groups were orally treated with 0.2 mL of a 0.3 M HCl/60% ethanol solution (HCl/ethanol) for gastric-ulcer induction. Animals were killed 1 h after the administration of HCl/ethanol, and the stomachs excised and inflated by saline injection (2 mL). The extent of the lesions was measured using ulcerative lesion index (ULI) and pH of gastric juice determined. This index was expressed as the sum of all lesions (Szelenyi & Thiemer, 1978).

Non-Steroidal Antiinflammatory Drug (NSAID)-Induced Gastric Ulcers

In this model (Puscas *et al.*, 1997), gastric ulcer was induced using piroxicam (30 mg/kg, s.c.) administered to mice. The MeOH extract from *L. flavescens* (250, 500 or 1000 mg/kg), cimetidine (100 mg/kg) or saline were administered orally 30 min before the induction of gastric ulcer. The animals were killed 4 h after treatment with the ulcerogenic agent and gastric damage was determined as described above.

Hypothermic Restraint Stress Ulcer

After fasting, the mice received an oral administration of MeOH extract from *L. flavescens* (250, 500 or 1000 mg/kg), cimetidine (100 mg/kg) or saline (10 mL/kg). 1 h after treatment, mice were immobilized in a restraint cage at 4°C for 4 h to induce gastric ulcer. The animals were killed and gastric lesions were determined as described above (Levine, 1971).

Determination of Gastric Secretion

All groups of mice fasted for 24 h, with free access to water. Immediately after pylorus ligature, MeOH extract from *L. flavescens* (250, 500 or 1000 mg/kg), cimetidine (100 mg/kg) as positive control, or the vehicle was administered intraduodenally. The animals were killed 4 h later and the abdomen was opened and another ligature placed around the esophagus close to the diaphragm. The stomachs were removed and the gastric content collected to determine the pH values and total amount of gastric-juice acid. Distilled water was added and the resultant solution centrifuged at 2500 G for 10 min. Total acid in the gastric secretion was determined in the supernatant volume by titration to pH 7.0 with 0.01 N NaOH (Shay *et al.*, 1945).

Anti-Helicobacter pylori Activity

The MeOH of *L. flavescens* was tested to detect anti-*Helicobacter pylori* activity (Hachem *et al.*, 1996). The stain of *Helicobacter pylori* (ATCC 43504) was isolated from patients with duodenal ulcer disease. Frozen *H. pylori* isolate was thawed and grown on 5% sheep blood agar plates for 3 to 4 days at 37°C in 10% CO_2 and 98% humidity. Each plate was swabbed with a sterile cotton-tipped applicator and the cells were suspended in sterile saline to obtain turbidity equivalent to a 2.0 McFarland standard. Muller-Hinton broth containing 10% horse serum was added to all wells of a 96-well microtiter plate (Corning-USA). Each well was incubated with *H. pylori* at a final concentration of 1 × 105 CFU/mL. The plates were incubated for 5 days in a microaerobic atmosphere at 37°C. Following incubation, the plates were examined visually and spectrophotometrically and the lowest concentration showing complete inhibition of growth was recorded as the MIC (minimum inhibitory concentrations). *Staphylococcus aureus* ATCC

25923 and *E.coli* ATCC 25922 were used as control organisms for clarithromycin and ampicillin, respectively. The results were considered valid only when the MIC values for the control organisms were within the ranges established by the National Committee for Clinical Laboratory Standards (NCCLS).

Determination of Activity on Intestinal Motility

Mice submitted to a 6 h fast were treated orally with the vehicle (saline, p.o.), atropine (1 mg/kg, p.o.), MeOH (250, 500 or 1000 mg/kg, p.o.). 30 min after the treatments, all animals received 10% activated charcoal (0.1 mL/ 10 g, p.o.) and were killed 30 min later. The distance traveled by activated charcoal up to the last portion of small intestine was determined (Stickney & Northup, 1959). The results were expressed as percentage of the total length of the small intestine traveled by this marker.

Antioxidant Activity

The antioxidant activity was evaluated through the methodology of lipidic peroxidation with membrane of brain from Wistar rats (Stocks, 1974), with a serial dilution of the methanolic extract of *L. flavescens* (1, 14; 2, 27; 4, 55; 9, 09; 18, 18 e 36, 36 μg/mL) with the purpose to obtain IC_{50} (inhibitory concentration of 50% of the oxidation) through the production of malonidialdehide (MDA) (Fee & Teitelbaum, 1972).

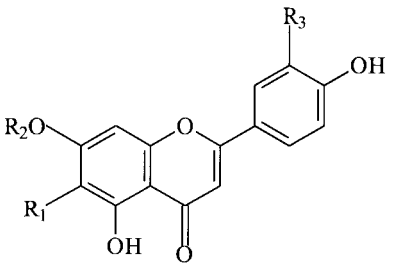
Statistical Analysis

Results were expressed as the mean ± S.E.M. Statistical significance was determined by one-way analysis of variance followed by Dunnett's test, with the level of significance set at $p<0.05$.

Extraction and Isolation

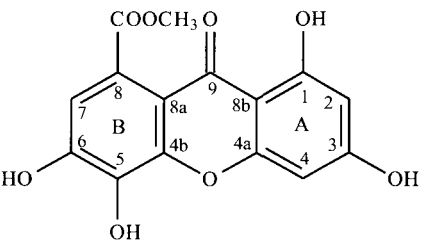
The scapes of *L. flavescens* (500 g) were separated from the capitula, powdered and successively extracted with chloroform, methanol and 80% methanol (1 week for each solvent). The solvents were evaporated in a vacuum yielding black syrups. A portion (2.0 g) of the ethanol extract of *L. flavescens* was submitted to CC on sephadex LH-20 (100 × 5 cm), with MeOH as eluant. Fractions (8 mL) were collected and checked by TLC on silica gel in Si gel plates, *n*-BuOH-AcOH-H_2O (20:5:8, v/v/v)]. Fractions 90-100 (40 mg) were purified by repeated CC on polyvinylpolypyrrolidone (Sigma) eluted with MeOH yielding compounds **1** (7.0 mg) and **2** (9.0 mg). Fractions 65-73 (69.8 mg) were further purified by HPLC using MeOH:H_2O (1:1, v/v) as eluant to afford **3** (4.2 mg, R_T = 8 min), and **4** (10.8 mg, R_T = 11 min). Fractions 47-51 (12.0 mg) using MeOH:H_2O (2:3, v/v) as eluant gave compound **5** (6.5 mg, R_T = 18 min). Fractions 40-46 using MeOH:H_2O (2:3, v/v) as eluant afforded compound **6** (8.5 mg, R_T = 9 min) and **7** (5.8 mg, R_T = 16 min.). Fractions 82-83 contained pure **8** (13.3 mg). Substances (Fig 1) were identified by NMR (nuclear magnetic resonance, as ^{13}C-NMR, ^{1}H-NMR), spectra and by

comparison with previous data reported in the literature (Santos *et al.*, 2001; Agrawal, 1989; Harborne, 1996).



1, 2, 4-8

Compounds	R_1	R_2	R_3	Name	Reference(s)
1	H	H	OH	Luteolin	(Agrawal,
2	H	H	H	Apigenin	1989; Harborne,
4	OH	glc	OH	3',4',5,6-tetrahydroxy-7-methoxy-flavone	1996)
5	glc	CH_3	OH	3',4',5-triihydroxy-7-metoxy-6-C-glucopyranosylflavone	
6	CH_3	glc	OCH_3	4',5-dihydroxy-3',6-dimethoxy-7-O-β-D glucopyranosylflavone	
7	glc	H	H	3',4',5, 7-tetrahydroxy-6-C-glucopyranosylflavone	
8	OH	H	OH	3',4',5,6,7,8-hexahydroxyflavone	



3- 8-carboxy-methyl-1,3,5,6-tetrahydroxyxanthone (Santos *et al.*, 2001).

Fig 1. Flavonoids and xanthones isolated from the scapes of *Leiothrix flavescens*

Experimental Section

NMR spectra in DMSO-d_6 were obtained using a Varian INOVA 500 spectrometer operating at 500 MHz for ^1H and 150 MHz for ^{13}C. HPLC separations were achieved on a Chance HPLC System equipped with a R401 refractive index detector and with a phenomenex Luna reversed phase C-18 columns (250 × 10 mm × 10 mm) and Rheodyne injector. TLC were performed on silica gel SiF254 (Merck) eluted with $CHCl_3$:MeOH:H_2O (80:18:2, v/v/v) and *n*-BuOH:HAc:H_2O (65:35:25, v/v/v), the plates were visualized using UV light (254 and 365 nm).

HPLC-UV-PDA Analysis

The chromatography profile of the methanolic extract of *L. flavescens* was established with a liquid chromatograph equipped with a PU-2089 quaternary solvent pump, a MD-2010 photodiode array detector (Jasco, Tokyo, Japan) and a Rheodyne 7725 sample injector with a 20 µl sample loop (Rheodyne, Cotati, CA, USA). The analytical column was a Phenomenex Luna RP18 (250 × 4.6 mm I.D. × 5 µm, equipped with a Phenomenex security guard 4.0 × 2.0 mm, Torrance, CA, USA). The mobile phase composition used was: water (eluent A) and acetonitrile (eluent B), both with 0.05% of TFA. The gradient program was as follows: 15-22% B (25 min.), 22-40% B (15 min.), 40-78% B (5 min.), 78-100% B (5 min.) and 100% B isocratic (5 min.). Total run time was 55 min. The flow rate of the mobile phase was 1.0 mL/min. EZChrom Elite Data System software was used for both the operation of detector and for data processing. The methanolic extract (2 mg) was dissolved in 2 mL methanol, filtered through a 0.45 µm membrane PTFE filter (Millex), resuspended in 3 mL of water and 20 µl submitted to the analysis by HPLC. The HPLC profile of the methanolic extract prepared from scape of *L. flavescens* was presented in Fig 2.

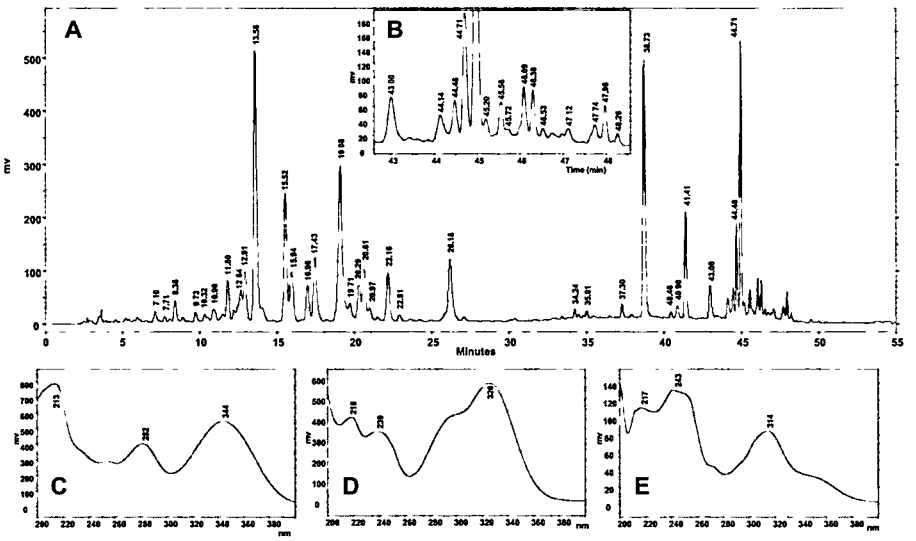


Fig 2. (A) HPLC fingerprint profile of crude methanolic extract from scape of *L. flavescens*. Retention times of major peaks are indicated in chromatogram. B) The regions of xanthones are shown as insets. Representative UV for the classes of compounds found in the methanolic extract is shown in the lower part of the chromatogram. C) Flavones derivatives, D) Cafeoyl quinic derivatives and E) xanthones derivatives

RESULTS AND DISCUSSION

Biological experiments may be designed with the objective of determining the dose of a chemical required to produce any specific effect. The measured effect need not be death of the animal but may be any type of biologic effect that can be quantified. So as part of the pharmacological evaluation of MeOH extract of *L. flavescens,* we investigated the acute toxicity in mice. A single oral administration of MeOH extract (5,000 mg/kg) did not produce any sign or symptom of toxicity in the treated animals. After 14 days of extract administration, no animal died and no significant changes in daily body or organ weight were observed. There were no significant alterations in water or food consumption. In autopsy, no significant change or lesion was observed in the viscera of each animal. Loomis & Hayes, (1996) described the classification of some chemicals agents with categories of toxicity and chemicals may be categorization by practically nontoxic at dose of 5 g/kg. Thus, this result indicates that the MeOH extract from *E. ligulatum* has no acute toxicological effect when administered orally. These results guarantee the continuance from the pharmacological studies from this specie. For this reason, a 5-fold lower dose (1000 mg/kg) was used as the maximum doses in all experiment to determine the general profile of antiulcer effect of extract from *L. flavescens* scapes.

We evaluated the preventive effect of three oral doses of 250, 500 or 1000 mg/kg of the MeOH extract obtained from *L. flavescens* administered to mice using different standard experimental models in order to verify their action on the gastrointestinal tract. This approach allows a general profile to be established for the gastroprotective activity from the vegetal extract for a future investigation of its action mechanism.

In the HCl/EtOH-induced gastric ulcers, the lesions were characterized by multiple-hemorrhage red bands of different sizes along the long axis of the glandular stomach. As shown in Fig 3, all doses of the MeOH extract showed significant antiulcer activity. The above results observed with MeOH extract (250, 500 or 1000 mg/kg) demonstrated significant inhibition of ulcerative lesion by 51% (24 ± 4.1 mm), 76% (12 ± 4.1 mm) and 87% (6.2 ± 1.9 mm), respectively, compared to the control value (49 ± 7.7 mm).

The ability of the gastric mucosa to resist to injury caused by endogenous secretions (acid, pepsin and bile) and by exogenous irritants (*e.g.* alcohol) can be attributed to a number of factors that have been collectively referred to as mucosal defense (Kinoshita *et al.*, 1995). The formation of gastric mucosal lesions by necrotizing agents such as HCl and EtOH has been reported to involve the depression of these gastric defensive mechanisms and also promote stasis in gastric blood flow that contributes to the development of the hemorrhagic and necrotic aspects of tissue injury (Morimoto *et al.*, 1991).

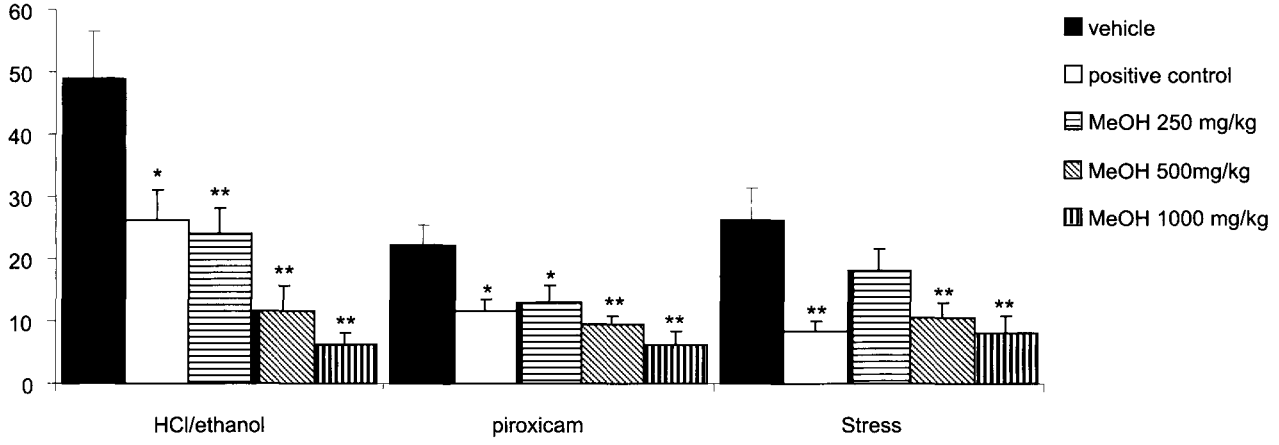


Fig 3. Effects of oral treatments with MeOH extract from *L. flavescens* on ulcerative lesion index (ULI) in gastric lesions induced by HCl/ethanol, NSAID (piroxicam) and hypothermic-restraint-stress in mice

Data are presented as mean ± S.E.M. ANOVA: $F_{(4,32)} = 82.8$ ($p<0.05$) for pH-HCl/ethanol; $F_{(4,32)} = 8.88$ ($p<0.05$) for ULI-HCl/ethanol; $F_{(4,44)} = 4.85$ for pH-NSAIDS ($p<0.05$); $F_{(4,44)} = 6.27$ for ULI-NSAIDS ($p<0.05$); $F_{(4,25)} = 0.74$ ($p>0.05$) for pH-stress model and $F_{(4,25)} = 6.45$ ($p<0.05$) for ULI-stress model. Dunnett's test: * $p<0.05$, **$p<0.01$.

Gastric lesions induced by ethanol are not inhibited by antisecretory agents such as cimetidine, but are inhibited by agents that enhance mucosal defensive factors such as prostaglandins (Garner, 1992). These results shown that the MeOH extract of *L. flavescens* probably has an antiulcerogenic effect related to the cytoprotective action since this extract protected against the necrotizing action of ethanol.

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin®, piroxicam and indomethacin remain among the most commonly used pharmacological agents (Garner, 1992). However, these classes of substances cause gastrointestinal ulceration. This effect is correlated to the ability of these agents to suppress the prostaglandin synthesis (Wallace, 2001).

Fig 3, shows the antiulcerogenic effect of MeOH extract in the NSAID-induced lesion model. Significant reductions in the damage to gastric mucosa ($p<0.05$) of 41% (250 mg/kg), 57% (500 mg/kg) and 72% (1000 mg/kg), respectively, were observed with the use of the MeOH extract when compared to the control value. The increased synthesis of mucous and/or prostaglandins can be the probable cytoprotective mechanism involved with this activity (Guth *et al.*, 1984).

Stress is the major causes of gastric disturbances in modern society. Gastric stress ulceration is probably mediated by histamine release with enhanced acid secretion and reduced mucous production. Moreover, stress-induced ulcer can be prevented partially or entirely by vagotomy. Vagal over-activity has been suggested to be the principal factor in stress-induced ulceration (Singh & Majumdar, 1999).

In the gastric ulcer induced by hypothermic-restraint stress (Fig 3), the MeOH extract also showed significant gastroprotection ($p<0.05$); and the gastric-lesion inhibition for the extract were 60% (500 mg/kg) and 70% (1000 mg/kg) respectively, when compared with the vehicle.

Ares *et al.* (1995) identified gastroprotective properties in the flavone class and phytochemical analyses of *L. flavescens* shown that this species produces mainly flavones that certain contributed to gastroprotective action of this plant (Silva *et al.*, 2007).

In the next step, we investigated the biochemical alterations promoted by *L. flavescens* extract in gastric-juice parameters. The MeOH extract was administered intraduodenally after submitting the mice to pylorus ligature (Table 1). Ligation of the pylorus for 4 h produced accumulation of gastric juice and lesion in gastric mucosa, whereas the intraduodenal route showed systemic action for all of the treatments. As shown in Table 1, the MeOH extract significantly decreased the gastric-acid secretion ($p<0.05$) and promoted reduced acid output ($p<0.01$). Batista *et al.* (2004) shown that antisecretory activity from *Syngonanthus*

arthrotrichus were observed only by flavonoids rich fraction. This results obtained with MeOH extract of *L. flavescens* as antisecretory agents is important as prophylaxis of ulcer gastric and gastric carcinogenesis. But gastric carcinogenesis is a complex, multistep and multifactorial event, in which the role of *Helicobacter pylori* infection has been established. We also evaluated the anti-*Helicobacter pylori* action of MeOH extract of *L. flavescens*. In the course of the study the MeOH extract was found to present an important antibacterial activity against the standard strain (ATCC 43504). The results showed that MIC of MeOH against *H.pylori* was 125 μg/mL. The literature reported that MIC<500 μg/mL is considered interesting for vegetal extracts (Gadhi *et al.*, 2001; Hachem *et al.*, 1996). We conclude that the MeOH has an excellent antimicrobial action against one of the most important factors that cause gastric ulceration.

Table 1. Effects of MeOH extract from *L. flavescens* administered intraduodenally (i.d.) on the biochemical parameters of gastric juice obtained from pylorus-ligature mice

Treatment group (p.o.)	Dose (mg/kg)	N	pH (unit)	Gastric juice volume (mL/4h)	Acid output (mEq/mL/4h)
Control	-	9	2.78 ± 0.40	1.55 ± 0.13	25.8 ± 2.29
Cimetidine	100	5	3.75 ± 0.75	1.12 ± 0.11	15.7 ± 3.71 *
L. flavescens	250	6	4.00 ± 0.45	1.08 ± 0.18	18.3 ± 2.33
	500	6	3.67 ± 0.76	0.94 ± 0.17 *	3.25 ± 0.48 **
	1000	6	3.50 ± 0.62	0.72 ± 0.11 **	5.00 ± 0.91 **

The results are the mean±S.E.M. N: number of mice used. ANOVA, $F_{(4,27)} = 0.80$ ($p>0.05$) for pH; $F_{(4,27)} = 5.17$ for gastric juice volume ($p>0.05$); $F_{(4,27)} = 18.4$ ($p<0.05$) for $[H^+]$. Dunnett's Test: * $p<0.05$; ** $p<0.01$

Farinati *et al.* (2008) shown that among the pathways relevant to gastric carcinogenesis and correlated with *H. pylori* infection, the production of reactive oxygen species may be quite important. The eradication of *H. pylori* to prevent the production of reactive oxygen species and accumulation of oxidative DNA damage are strategic point to prevent gastric ulcer and gastric carcinogenesis. The reactive oxygen species (ROS) generate by metabolism of arachidonic acid, platelets, macrophages and smooth muscle cells contribute to gastric mucosal damage (Repetto & Llesuy, 2002). MeOH extract from *L. flavescens* has an antioxidant activity *in vitro* with IC_{50} of 21.55 ± 0.02 μg/mL. This value is considered a good antioxidant activity when it comes from a crude extract and thus justifying the antiulcerogenic activity observed in other models.

We also observed that MeOH extract from *L. flavescens* was able to increase the propulsion of charcoal meal in a dose dependent manner (Table 2). The velocity of intestinal travel is one of the factors that affected the intensity of luminal absorption and regulated the biodisponsibility of the

drug administered orally. Thus gut transit test with activated charcoal was carried out to determine the effect of MeOH from *L. flavescens* on intestinal motility. This result indicated that MeOH extract from *L. flavescens* also has a laxative property. There are few laxative products related to intestinal peristalsis that have been in use for decades. Many of these products in use today are of plant origin (Fintelmann, 1991). Although laxative effects have been described for some antiulcer drugs (Motiva *et al.*, 1996; Bobrove, 1990), such effects of MeOH extract is being currently investigated by our group.

Table 2. Effects of MeOH extract from *L. flavescens* on intestinal transport, induced by charcoal, in mice (n=7)

Treatment group (p.o.)	**Dose (mg/kg)**	**Charcoal transport (arcsine)**
Control	-	62.8 ± 12.5
Atropine	5	40.2 ± 16.4 #
L. flavescens	250	77.0 ± 5.75
	500	79.8 ± 5.30 *
	1000	83.5 ± 7.58 **

The results are the mean ± S.E.M. ANOVA, Dunnett's Test: # $p<0.01$, * $p<0.05$, ** $p<0.01$. $F_{(4, 30)} = 20.3$

The phytochemical analyses carried out in this work led to the isolation of xanthones and flavones in the MeOH extract of scapes from *L. flavescens* (Figs 1, 2). The literature reveals the gastroprotective action of various species containing xanthones and flavones (Gonzalez & Di Stasi, 2002; La Casa *et al.*, 2000; Sartori *et al.*, 1999; Ares *et al.*, 1995).

The flavones isolated from *L. flavescens* are mostly derived from luteoline which presents the catechol nucleus at ring B. Such compounds have been correlated with the antiulcerogenic activity of many plant extracts since they possess antioxidant action (La Casa *et al.*, 2000). Moreover, the isolated xanthone **3** also displays *ortho*-hydroxyl groups at position 5, 6 of the A ring, thus providing another system with a catechol nucleus.

Besides the presence of xanthones and flavonoids in the MeOH extract of *L. flavescens*, this extract also has potential as a new alternative in the management of gastric ulcers by the presence of luteolin that has a proven effect against *Helicobacter pylori* (Chung *et al.*, 2001). In light of the fact that *H. pylori* is considered the most important risk factor in development of duodenal and gastric ulcers, this study with MeOH extract from *L. flavescens* as antiulcerogenic agent may contribute an excellent pathway for treating the universal problem of this gastric pathology.

In conclusion, our study evidenced the gastroprotective factor of the MeOH extract obtained from *L. flavescens*, with antioxidant, antisecretory and gastroprotective activities. The phytochemical investigation of the

MeOH extract led to the isolation of flavones and xanthones with catechol nucleus in their structures, which can be correlated with antioxidant activity.

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14

Evaluation of the Immunity Potency and Security of Inactivated Oil-Adjuvant Vaccine against Chlamydiosis in Dairy Cattle

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ABSTRACT

The inactivated oil-adjuvant vaccine against chlamydiosis in dairy cattle was successfully developed using Chlamydia psittaci SX5 field strain with strong and stable virulence in China. The minimal effective dosages for adult dairy cow and calf are 3 mL and 1.5 mL, respectively, and the clinically used dosages are 4~5 mL (adult cattle) and 2 mL (calves), respectively. The average protection rate reaches 94.4% (ranging from 88.9% to 100%) in the vaccines potency tests. The immunity persistent periods of the vaccine is ten months (adult cattle) and six months (calf). The vaccines conserved for 12 months at 4°C has effects same as the new one. The safe tests for cattle of various ages and small areas of field trials (1216 cattle were vaccinated) confirmed safety of the vaccine is reliable and there were not obviously abnormal changes in appetite, milk production and the site of vaccination in the vaccinated cattle herds.

Key words : Chlamydiosis in dairy cattle, inactivated vaccine, protection rate, safety

INTRODUCTION

Chlamydiae are widely distributed throughout the world, causing various forms of disease in animals and humans. Several species, particularly *Chlamydophila (Cp.) psittaci* and *Cp. abortus*, are known to be transmissible

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from animals to humans, causing significant zoonotic infections (Gnutov *et al.*, 1980; Stěpánek *et al.*, 1983; Sachse *et al.*, 2002). *Chlamydiae* is strictly intracellular microorganism, which have unique biphasic lifestyle, namely infective extracellular phage and parasitic intracellular phage, which renders the respective diseases difficult to control. This is compounded by the specialist growth conditions for the organisms and the lack of a genetic based system for the transformation of chlamydiae, both of which have hampered research on these pathogens. To escape the host immune response these bacteria are capable of transforming into persistent stages of development characterised by a distinct antigenic profile.

Bovine chlamydial abortion is one of all serious diseases induced by Chlamydia, which may result in large loss in dairy industry (Daniel *et al.*, 1993; Kaltenboeck *et al.*, 2005). The disease is named as epizootic bovine abortion caused by *Cp abortus*, and characterized by abortion, premature birth, still birth or weak birth with wide prevalence (Martel *et al.*, 1983; Martinov, 1984).

The disease often aggrieves virgin cows, heifers or cows from non-epidemic areas and results in 25% to 75% of affected pregnant cows to abort and infertility (Bowen *et al.*, 1978). The causative agents may induce uteritis and infertility in cows with bad curative effect despite drugs (Vězník *et al.*, 1996), conjunctivitis (Otter *et al.*, 2003), mastitis and polyarthritis (Martinov *et al.*, 1981), etc. In a regional survey in Germany, *Cp. psittaci/Cp. abortus* were detected at a rate of up to 100% in affected herds. Economic losses as a consequence of a drop in milk production and milk quality, as well as abortions and reduced fertility rates were estimated to be Euro 40,000 per year at an average farm of 60 dairy cows and 20 heifers. Investigations carried out in Italy have shown that the chlamydial organisms involved in these outbreaks belong to the so-called "non-invasive" strains classified as *Cp. pecorum*. The same agent was also detected in cases of encephalomyelitis in water buffaloes used for milk production (Qiu, 2003).

In 1980's, investigations of bovine chlamydiosis carried out in some provinces in China showed infection rate of chlamydiosis in dairy cows was higher than in yellow cattle or buffalo (Qiu, 2005). In current years, the dairy farming has been developing rapidly and at present has reached above ten million cattle in China. However, the production level of dairy cattle was very low owing to prevalence of infectious diseases (including chlamydiosis) (Qiu *et al.*, 2006) and absence of specific bio-preparations for monitor and control of dairy cattle diseases. So we carried out studies on inactivated vaccine against chlamydiosis in dairy cattle.

MATERIALS AND METHODS

The Vaccine Strain

The vaccine strain is *Chlamydia psittaci* SX5 which was isolated from a dairy cow affected with chlamydial abortion on a dairy farm standing at the north of Xi-an city of Shaanxi province locating in the North-western of China, and identified and preserved in our laboratory and its virulence value reached $10^{-12}ELD_{50}$/0.4 mL (Qiu *et al.*, 2006).

The Virulent Strain Used in the Vaccine Potency Test

The virulent strain used in the vaccine potency test is *Chlamydia psittaci* SX5 with virulence value of $10^{-12}ELD_{50}$/0.4 mL. In the potency tests, the calves were challenged at a dosage of 1 mL and adult dairy cattle at dosage of 2 mL by neck vein route (The concentration of the virulent strain SX5 used in the vaccine potency test was 10% of suspension of the strain SX5 chicken embryo yolk sac cultures). And the challenges were carried out at the third week post vaccinations in the potency tests (Li *et al.*, 1995).

The Tested Vaccine

The ten batches of inactivated oil-adjuvanted vaccines against chlamydiosis in dairy cattle were prepared for potency test and security test of target animals and the vaccines were composed of inactivated SX5 antigens and Montanide ISA 206 oil adjuvant made in France with 0.2% formaldehyde as inactivator. In these trials, calvies and adult cattle were intramuscularly vaccinated at a doses of 2 mL and 4~5 mL, respectively.

The Vaccine Tests

The contents of vaccine evaluation examination included potency test, safety test, immune persistence period test for dairy cattle and conservation time of the vaccine in the laboratory as well as field tests of vaccinations of target animals.

Animals used in the tests: All cattle were purchased from several cattle herds which had been confirmed to be negative for Chlamydia infection by serologic method.

RESULTS

The Minimal Effective Dosages for Target Animal

The minimal effective dosages of the vaccine for adult cattle and calf were seen in the Tables 1 and 2, respectively.

Table 1. The minimal effective dosages for adult dairy cow

Groups	Vaccine batch no.	Cattle	Date	Dose (mL)	Challenge (mL)	Protection rate
1	030312	5	03-03-30	2.0	2.0	2/5
2	030317	5	03-03-30	2.0	2.0	2/5
3	030327	5	03-03-30	2.0	2.0	2/5
4	030312	5	03-03-30	3.0	2.0	4/5
5	030317	5	03-03-30	3.0	2.0	5/5
6	030327	5	03-03-30	3.0	2.0	5/5
Control		5	03-03-30	0	2.0	0/5

Table 2. The minimal effective dosages for dairy calvies

Groups	Vaccine batch no.	Cattle	Date	Dose (mL)	Challenge (mL)	Protection rate
1	030312	5	03-03-30	1.0	1.0	1/5
2	030317	5	03-03-30	1.0	1.0	2/5
3	030327	5	03-03-30	1.0	1.0	2/5
4	030312	5	03-03-30	1.5	1.0	5/5
5	030317	5	03-03-30	1.5	1.0	4/5
6	030327	5	03-03-30	1.5	1.0	5/5
Control		5	03-03-30	0	1.0	0/5

According to the results of minimal effective dosages tests, the minimal effective dosages of the vaccine for adult cattle and calf were 3.0 mL and 1.5 mL, respectively. Clinically, the dosages used for calves and adult cattle were affirmed as 2 mL and 4-5 mL, respectively.

The Vaccine Potency Tests

Six batches of the vaccines were used for two potency tests, and in the first potency test, the vaccine protection rate was 8/9, and in the second potency test, the vaccine protection rate was 9/9, with an average protection rate of 94.4%. The detail was seen in the Table 3.

Table 3. Potency tests of the vaccine for dairy cattle

Groups	Vaccine batch no.	Cattle	Dose (mL)	Challenge (mL)	Smear	Isolation	Protection rate(%)
1	030312	3	5.0	2.0	3/3 -	3/3 -	3/3 (100.0)
2	030327	3	5.0	2.0	3/3 -	3/3 -	3/3 (100.0)
3	030403	3	5.0	2.0	3/3 -	2/3 -	2/3 (66.7)
Control 1		3	0	2.0	3/3+	3/3+	0/3 (0)
4	040113	3	5.0	2.0	3/3 -	3/3 -	3/3 (100.0)
5	040210	3	5.0	2.0	3/3 -	3/3 -	3/3 (100.0)
6	040317	3	5.0	2.0	3/3 -	3/3 -	3/3 (100.0)
Control 2		3	0	2.0	3/3+	3/3+	0/3 (0)

The Safety Tests

The security tests of the vaccine would deal with weaned calves (30 to 40 days old), pregnant cows and adult cattle. The details were seen in the Tables 4, 5, 6, respectively.

Table 4. Safety tests of the vaccine for dairy calvies

Groups	Vaccine batch no.	Calves	Dose (mL)	Site of vaccination	Appetite	Evaluation
1	030312	5	5.0	No swelling	Normal	Safe
2	030327	5	5.0	No swelling	Normal	Safe
3	030403	5	5.0	No swelling	Normal	Safe
4	040113	5	5.0	No swelling	Normal	Safe
5	040210	5	5.0	No swelling	Normal	Safe

Table 5. Safety tests of the vaccine for pregnant dairy cows

Groups	Vaccine batch no.	Pregnant cows	Dose (mL)	Site of vaccination	Parturition	Evaluation
1	030312	5	10.0	absorded in 3 days	Normal	Safe
2	030327	5	10.0	absorded in 2-3 days	Normal	Safe
3	030403	5	10.0	absorded in 2-3 days	Normal	Safe
4	040113	5	10.0	absorded in 2-3 days	Normal	Safe
5	040210	5	10.0	absorded in 2-3 days	Normal	Safe

Table 6. Safety tests of the vaccine for adult dairy cattle

Groups	Vaccine batch no.	Adult cows	Dose (mL)	Site of vaccination	Clinically	Evaluation
1	041210	5	10.0	absorded in 2-3 days	Normal	Safe
2	050103	5	10.0	absorded in 3 days	Normal	Safe
3	050114	5	10.0	absorded in 2-3 days	Normal	Safe
4	050120	5	10.0	absorded in 2-3 days	Normal	Safe
5	050220	5	10.0	absorded in 3 days	Normal	Safe

The test results of safety of the vaccine for target animals well indicated that after the vaccinations, no tested cattle showed serious manifestations with basically normal appetite and milk crops clinically. And all of the vaccinated pregnant cattle did not aborted within the whole test duration. A few of the tested animals had a high temperature (to 39°C or so) but returned normal in the second day post the vaccinations.

The Immune Persistence Period Test of the Vaccine

Nine adult cattle and nine calvies were inoculated with the vaccines and then the sera were continuously sampled from the tested animals for ten months (adult cattle) or six months (calves) for measuring the antibody levels to chlamydiosis. The antibody titer changes were seen in the Fig 1 and 2 in detail.

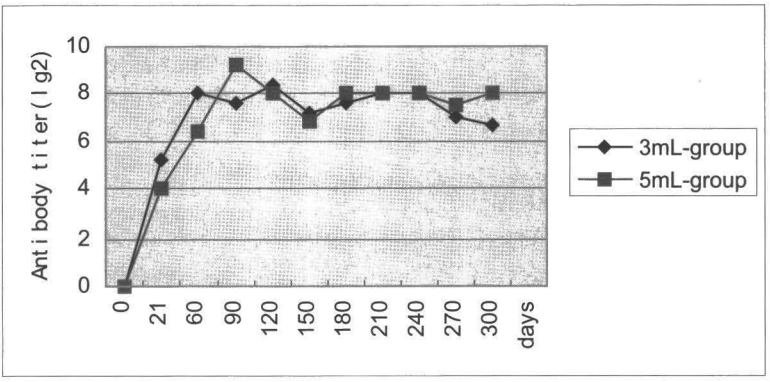

Fig 1. The antibody curves in adult dairy cattle inoculated with various doses of the vaccine within 10 months duration

As showing in the Fig 1, the antibody titers of the both group animals reached 1:16~1:32 on the day 21 post vaccination; the antibody titers of the both group animals were kept in an increasing status and reached 1: 512 on the day-90 post vaccination; the antibody titers of the both group animals were steady in 1: 256 or so within 4 to 8 months after vaccinations; in 9 to 10 months post vaccinations, the antibody level of the 3 mL-group animals began to fall but the antibody level of the 5 mL-group animals was kept in 1: 128~1: 256.

The adult cattle of the 5 mL-group were challenged with virulent strain SX5 10 months after the vaccinations and all inoculated animals were protected and the test results were seen in the Table 7 in detail.

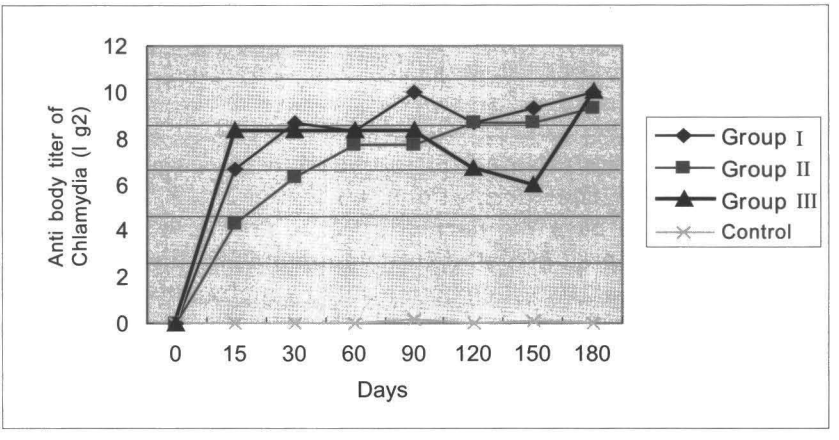

Fig 2. The antibody curves in calvies inoculated with the vaccine within 6 months duration

Table 7. The challenge test in the immunity persistent period of the vaccine for adult cattle

Groups	Vaccine batch no.	Cattle	Dose (mL)	Challenge (mL)	Smear	Isolation	Protection rate(%)
1	030312	3	5.0	2.0	3/3 -	3/3-	3/3 (100.0)
2	030327	3	5.0	2.0	3/3 -	3/3 -	3/3 (100.0)
3	030403	3	5.0	2.0	3/3 -	3/3 -	3/3 (100.0)
Control		3	0	2.0	3/3+	3/3+	0/3 (0)

15 days after the vaccinations, the antibodies (1:64) could be detected from most of the inoculated calves. And 6 months after the vaccination, the antibody titers were kept at higher levels in inoculated calves of the 3 groups and the mean antibody titers were 10 Lg2, 9.3 Lg2 and 10 Lg2, respectively. While all of the calves were challenged, the animals of the vaccinated groups all were protected (3/3, 3/3, 3/3), but no animals of the control group were protected (0/3). The test results were seen in the Table 8 in detail. The 3/3 calves of the control group showed high body temperature (39.8°C to 40.1°C and the 1/3 calves diarrhoea occurred after challenging.

Table 8. The challenge test in the immunity persistent period of the vaccine for calvies

Groups	Calvies	Inoculated dose(mL)	Antibody titer 180 days post vaccination	Challenge dose(mL)	Protection rate%
I	3	2.0	10Lg2	1.0	3/3 (100.0)
II	3	2.0	9.3Lg2	1.0	3/3 (100.0)
III	3	2.0	10Lg2	1.0	3/3 (100.0)
Control		3	0	1.0	0/3(0)

The conservation time of vaccine

The three batches of the vaccine which had been hold for 12 months at 4°C were used to inoculate 9 dairy cattle, each team 3 animals. Challenging on the day 21 post the vaccinations, all of the vaccinated animals were protected but the 3 control animals fell ill showing high body temperature, bad appetite or abortion. The test results were seen in the Table 9.

Table 9. Storage period tests of the vaccine

Groups	Vaccine batch no.	Cattle	Vaccination date	Dose (mL)	Challenge date	Dose (mL)	Protection rate%
1	030312	3	04-04-05	5.0	04-04-26	2.0	3/3 (100.0)
2	030327	3	04-04-05	5.0	04-04-26	2.0	3/3 (100.0)
3	030403	3	04-04-05	5.0	04-04-26	2.0	3/3 (100.0)
Control		3	04-04-05	0	04-04-26	2.0	0/3(0)

According to the test results, the quality of the vaccines kept for 12 months at 4°C did not go down. So the conservation time of the vaccine was a year.

The Vaccine Field Test

In order to further know effect and safety of the vaccine for dairy cattle in field, the three batches of the vaccine were used in field tests and 1216 dairy cattle were vaccinated at some of the dairy farms in Gansu, Ningxia, Inner Mongolia, Shaanxi, Henan, Guangdong, Zhejiang and Sichuan provinces, China, which proved that the vaccines were effective for preventing and controlling bovine chlamydiosis and very safe. For example, in 2003 year, on a dairy farm with above one thousand cattle, six hundred of which became pregnant in Ningxia province, chlamydiosis took place,which induced above 100 pregnant cows to abort within a month with suffering heavy economic loss. Afterwards, when all animals of the herd were vaccinated with the vaccines, abortions of dairy cows were stopped rapidly and the production of the farm recovered normality also.

DISSCUSSION

Bovine chlamydial abortion is one of chlamydiosises leading to very severe economic loss in dairy farming in China. Now the natural transmission manner of the disease has been unclear. The affected animals and latently infected hosts carrying the pathogen and showing no clinical manifestations were very important infection origins (Reinhold *et al.*, 2008). It might be possible that the disease was transmitted between cattle and sheep each other (Griffiths *et al.*, 1995). While pregnant cows with chlamydiosis aborted or laboured, a lot of Chlamydia would be discharged out with the amniotic fluids (Wittenbrink *et al.*, 1994) and polluted environment around and infected other susceptible animals via ingestive tracts (Wittenbrink *et al.*, 1993) and respiratory tracts (Ehret *et al.*, 1975). If a breeding bull was infected by *Chlamydia psittaci*, cow herds copulated with the bull might be infected by Chlamydia- contaminated sperm (Storz *et al.*, 1968; Amin 2003; Kauffold *et al.*, 2007). Investigations revealed that if not controlling, bovine chlamydiosis would be rapidly spread and induce very severe economic loss in cattle farming (Stěpánek *et al.*, 1983). Because of extensive occurrences and many infection routes of *Chlamydia* spp, the complex strategy characterized by vaccination and strong management would be needed for prevention and control of chlamydiosis in cattle (Rodolakis *et al.*, 1987; Rodolakis *et al.*, 1998; DeGraves *et al.*, 2004; Biesenkamp-Uhe *et al.*, 2007).

The very pivotal aspect for developing a vaccine is selection of immunogenecity-broad, virulence-steady vaccine strain. In the study, *C. psittaci* SX5 was confirmed to be virulence-strong and stable by continuous passages in chick embryos, clone and sequence of the MOMP gene and

potency test of target animal and highly identical in homology of the MOMP genes of prevalence strains from other regions (Qiu *et al.*, 2006). So the SX5 is a more ideal strain for vaccine.

According to minimal vaccination dosage test, the dosages used clinically for calvies and adult cattle were 2 mL and 4~5 mL, respectively. The dosages for adult dairy cows would be chosen on the basis of the milk production and individual bodily form size, if the milk production was higher and the individual bodily form size was bigger, the vaccination dose would be 5 mL, otherwise, 4 mL.

In the security tests, because two-months-pregnant cows were easily infected with *Chlamydia* sp the safety of the vaccine to two-months-pregnant cows was examined which showed that the vaccine was much safe for both mother and fetus. So the efficacy of vaccinations for preventing chlamydial infection in dairy cows would be reasonable in a month before or after artificial inseminations.

On the basis of prevalence characteristics of bovine chlamydiosis and duration of the vaccine protection, the use procedure of the vaccine: cows will be vaccinated in a month before or after artificial inseminations; calves will be vaccinated in a month after weaning.

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Annona crassiflora Wood Constituents: Antimalarial Efficacy, Larvicidal and Antimicrobial Activity

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ABSTRACT

*Wood of Annona crassiflora was phytochemically evaluated and three annonaceous acetogenins, annofolin (**1**) ACM5 (**2**) and ACM6 (**3**), the alkaloids aterospermidine (**4**) and liriodenine (**5**), the steroids β-sitosterol (**6**) and stigmasterol (**7**), and fatty acids were isolated. Their structures were elucidated by a combination of chemical and spectral methods including MS and NMR experiments. Antimalarial activity, antimicrobial activity in vitro against pathogenic bacteria and brine shrimp lethality of wood extracts of Annona crassiflora were evaluated. The crude extract and fractions were very toxic to brine shrimp (DL_{50}<30 μg/mL). Doses of the extract and fractions of 500 mg/kg and 250 mg/kg caused animal death upon injection in P.berghei-infected mice. Doses of 12.5 mg/kg and 1.25 mg/kg produced 67% suppression of parasitaemia. The alkaloidic fraction showed good results when compared with chloramphenicol against pathogenic bacteria.*

Key words : *Annona crassiflora*, Annonaceae, antibacterial activity, antimalarial efficacy, larvicidal activity, *Plasmodium berghei*

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INTRODUCTION

It is estimated that Brazil has about 60-250000 plant species that still are lesser known and under explored. Probably, 40% of them possesses interesting biological activities, constituting source of new useful molecular standards for the development of new plant-derived drugs. Our team has been investigating Annonaceae family for over ten years (Santos *et al.*, 1995; Pimenta *et al.*, 1996; Pimenta *et al.*, 2001; Santos *et al.*, 2006) to find plants with potential activity as antimicrobial, against endemic illnesses such as malaria, and chronic-degenerative diseases as the cancer. Annonaceae family has been source of several structural types of isoquinoline alkaloids and Annonaceous acetogenins. Annonaceous acetogenins are long chain fatty acid derivatives isolated exclusively from plants belonging to the Annonaceae family. These natural products exhibit a broad range of biological properties such as cytotoxic, immunosuppressive, pesticidal, antiparasitic, and antimicrobial activities. Furthermore, its potential to inhibit multiple drug resistant cells have attracted increasing interest in these compounds (Bermejo *et al.*, 2005). Our previous phytochemical study with the seeds of *Annona crassiflora* has yielded three new and four known annonaceous acetogenins that showed good cytotoxic activity against some human tumoral cells lines (Santos *et al.*, 1995; Pimenta *et al.*, 1996; Pimenta, 1995). Insecticidal activity against *Spodoptera frugiperda* was also detected from leaves and seeds extracts of *Annona crassiflora* (Pimenta *et al.*, 2000). The Annonaceous acetogenins had also been determined earlier to possess antiparasitic activities, such as antimalarial *in vivo* (Rupprecht *et al.*, 1990; Fang *et al.*, 1993). Isoquinoline alkaloids have shown great activity against resistant strains of *P. falciparum*. (Fisher *et al.*, 2004). *Xylopia aromatica* bark extract (Annonaceae) was active against *Plasmodium falciparum* chloroquine resistant strain *in vitro* but inactive against *P. berghei* ANKA strain *in vivo* (Garavito *et al.*, 2006). Considering that malaria is the most prevalent disease among the insect-borne individuals and kills around two million people every year, it is very important to look for antimalarial active new drugs. The success of the antimalarial drugs, quinine and artemisinin, from plant's sources, associated to the ethnopharmacological information about the *Xylopia aromatica* activity *in vitro* against *P. falciparum* (Garavito *et al.*, 2006) and also the presence of the acetogenins and isoquinoline alkaloids in this species drove our attention to the evaluation of the antimalarial activity of the wood's crude extracts of *A. crassiflora* and some fractions from them.

The species *Annona crassiflora*, popularly known as "araticum, cabeça-de-negro", or "marolo", is a native tree from Brazilian "cerrado". It ocurrs in the states of Minas Gerais, Bahia, Ceará, Goiás, Mato Grosso and São Paulo. Its fruit is edible and used as juice, ice-cream, jelly and liqueur. Its pulp is laxative and the seeds are used against diarrhoea and once pulverized they are toxic and reputed as anti-parasitic. It has been used as efficient

remedy for lice elimination. There are reports of leaves being used as medication for sweat, antirheumatism by oral route. It may also be used externally, as a mouth rinse, in treatment of stomach inflammations, and in neuralgias and headaches (Lorenzi *et al.*, 2002). Prior to our investigation some alkaloids have been isolated from the leaves (Hocquemiller *et al.*, 1982), and cytotoxic annonaceous acetogenins from the seeds (Santos *et al.*, 1994; Santos *et al.*, 1999; Pimenta, 1995) of this plant.

Annona crassiflora is disappearing due to the fire used by local farmers for preparing the field for crops. Neither chemical nor biological studies of this plant's wood are reported in the literature.

The present paper reports the fractionation of *A. crassiflora* wood extract bioguided by *Artemia salina* lethality test which lead to the isolation of three annonaceous acetogenins, named annofolin, ACM5 and ACM6 (**1**, **2**, **3**), the alkaloids aterospermidine (**4**) and liriodenine (**5**), the steroids β-sitosterol (**6**) and stigmasterol (**7**), and fatty acids. The antimalarial efficacy and the general toxicity of the extracts and some fractions were evaluated *in vivo* in the *Plasmodium berghei*-infected mice following the conventional four-day-test (Peters *et al.*, 1986). Antimicrobial activity was determined *in vitro* against pathogenic bacteria *S. aureus*, *B. subtilis*, *E. coli*, *M. luteus* and *P. aeruginosa* (Takahashi *et al.*, 2006).

MATERIALS AND METHODS

Melting points determinations were determined on a Mettler FP5 apparatus and are uncorrected. Optical rotations were taken on a PERKIN ELMER 341 polarimeter. IR spectra were measured on a Perkin Elmer spectrometer version 3.02.1 and Spectrum One-FT-IR Spectrometer Universal ATR Sampling Accessory. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra (all in $CDCl_3$) were obtained in BRUKER DPX-400 spectrometer. ESIMS was recorded in a MICRO TM Q-TOF MICROMASS spectrometer. HPLC was carried out with WATERS HPLC instrument with a UV-VIS 486 detector set at 220 nm, using a Shim-pack PREP-ODS C-18 column (4.6 mm i.d. × 250 mm). Control of the equipment, data acquisition, processing, and management of chromatography information were performed by the WATERS 746 integrator. Chloroquine diphosphate was purchased from Sigma-Aldrich, USA. N,N-dimethylacetamide and PEG 300 were provided by Vetec. The solvents were from Quimex, Vetec, Carlo Erba and J.T. Baker.

Plant Materials

The pieces of wood used in the present work were collected from the protected area inside of Zoo-Botanic Foundation of Belo Horizonte, Belo Horizonte, Minas Gerais state, Brazil. Voucher specimens (No. 22988) were deposited at the Instituto de Ciências Biológicas Herbarium (BHCB), UFMG, Belo Horizonte, MG, Brazil.

Extraction and Isolation

The wood of *A. crassiflora* was dried at 40°C, weighted (1166.00 g), grounded and extracted at room temperature with ethanol which was removed under vacuum to give the crude extracts (ACWF01; 150.9 g). Part of this extract (130.60 g) was dissolved in ethanol/water (7:3) and successively extracted with hexane and chloroform. After solvent removal, the hexane (ACWF02), chloroform (ACWF03) and hydroalcoholic (ACWF04) fractions were obtained. The fraction ACWF03 was extracted with acid aqueous solution pH 4 followed by chloroform resulting in organic layer (ACWF03 O) and an acid aqueous layer (ACWF03 A). The acid aqueous phase was alkalinized and extract with chloroform, which after solvent removal yielded an alkaloidic fraction (ACW A). The ACWF03 O was partitioned between hexane and MeOH-H_2O (9:1), furnishing the hydroalcoholic (ACWF05) and hexane (ACWF06) fractions. The ACWF05 O fraction was submitted to biological guided fractionation on silica gel, followed by gel filtration on Sephadex LH-20 and preparative reverse phase HPLC with acetonitrile-water gradients, furnishing the acetogenins **1** (2.0 mg), **2** (2.0 mg), and **3** (1.5 mg). The alkaloidic fraction (ACW A) was fractionated on silica gel column affording the alkaloids **4** (3.0 mg), and **5** (2.5 mg). The ACWF02 fraction after separation by silica gel column chromatography eluted with hexane, dichlorometane, acetylacetate and methanol gradients afforded the compounds **6** and **7** (7.0 mg) as a mixture.

Biological Evaluation

Brine Shrimp Test

Artemia salina encysted eggs (10 mg) were incubated in 100 mL of seawater under artificial lighting at 28°C, pH 7-8. After incubation for 24 h, nauplii were collected with a Pasteur pipette and kept for an additional 24 h under the same conditions to reach the metanauplii stage. The samples (triplicate) to be assayed were disolved in dimethylsulfoxide (DMSO) (2 mg/400 μL or 2 mg/1000 μL) and serially diluted (10, 20, 30 and 50 μL/5mL) in seawater. About 10-20 nauplii were added to each set of tubes containing the samples. Controls containing 50 μL of DMSO in seawater were included in each experiment. As a positive control, lapachol dissolved in DMSO was used. Twenty four h later, the number of survivors was counted, recorded and the lethal concentration 50% (LC_{50}) and 95% confidence intervals were calculated by Probit analysis (Finney, 1976).

Antibacterial Assay

The following bacterial strains were used: *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Micrococcus luteus* ATCC 9341, *Pseudomonas aeruginosa* ATCC 27835 and *Staphylococcus aureus* ATCC 25923. They were cultured on Miller-Hinton agar at 37°C for 48 h. Antibacterial activity was assayed by agar diffusion method (Bauer *et al.*, 1966). Dimethylformamide

(DMF) was used for extracts solubilization. Sterile 6 mm diameter filter paper discs were impregnated with 1 mL of each extract (100 μg/mL). Inhibition zones surrounding the discs were measured after 24 and 48 h of bacterial growth. Chloramphenicol (30 μg/disc) was used as positive control and discs containing only DMF were used as negative control. Inhibition zone diameters around each disc (diameter of inhibition zone minus diameter of the disc) were measured and recorded at the end of incubation time. The experiment was carried on in triplicate and an average zone was calculated from all measurements. Only inhibition zones equal or bigger than 7 mm (observed after 24 h) were considered of interest.

Antimalarial Activity in P. berghei-Infected Mice.

Animal experiments were carried out according to the Principles of Laboratory Animal Care and legislation in force in Brazil and the protocol was approved by the animal ethical committee of the University. Female outbred Swiss albino mice weighing 20 to 24 g were supplied by the Animal Facility of Universidade Federal de Ouro Preto. They were kept in a normal diurnal cycle and had free access to food and water throughout the experiments.

The classical 4-day suppressive test adapted from Peters *et al.* (1986) for determination of antimalarial activity against chloroquine sensitive *Plasmodium berghei* NK65 strain in Swiss albino mice was used for monitoring *in vivo* activity of the extracts and fractions. An infective inoculum was prepared from a previously infected donor mouse with rising parasitemia (20%). On day 0 the mice were infected intravenously with a million of infected erythrocytes of *P. berghei* in 0.2 mL of phosphate-buffered saline. They were randomly divided in groups of 5 mice and treated by the intraperitoneal route once daily (1.25; 12.5, 250 and 500 mg kg^{-1}) with different extracts or fractions of *A. crassiflora* for four consecutive days (days 0 to 3). The extracts and fractions were dissolved in N,N-dimethylacetamide and polyethyleneglycol (PEG 300) at 1:2 proportion and further diluted 15-fold in saline to obtain the desired concentration in 0,3 mL for injection. Control groups received 0.2 mL of phosphate-buffered saline (untreated) or chloroquine diphosphate (60 mg/kg/day) as positive control. Thin blood smears were made from tail blood on days 4, 7, and 16 after infection. The parasitemia levels were assessed on Giemsa-stained thin blood smears. At least 3000 cells were checked to calculate parasitemia percent. Drug activity was determined on the basis of average parasitemia per group of mice. The percent reduction of parasitemia in treated groups as compared to untreated groups was calculated as follows: % parasitemia in control group (Pcg) - % parasitemia in test group/Pcg × 100.

Statistical Evaluation

All RBC counts and parasitemia levels are expressed as mean values ± standard deviations. The parasitemia data were analysed by using the one-

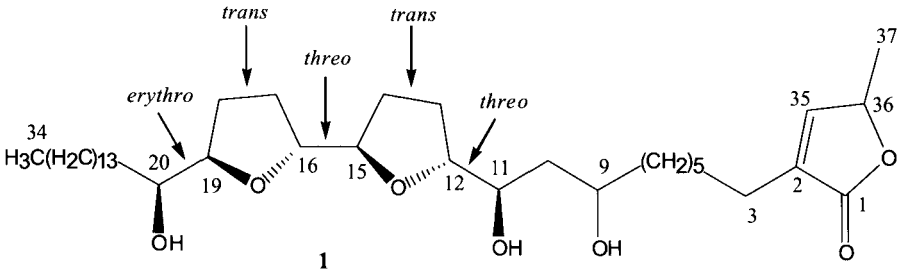
way analysis of variance (ANOVA) test using Prisma® 4.0 software. Mean survival times were compared by using Student's *t* test, considering a probability of 5% to be significant.

RESULTS AND DISCUSSION

Isolation & Characterization

The molecular formula of **1** was determined as $C_{37}H_{66}O_7$ from the $[M+H]^+$ peak at *m/z* 623.53 in the electrospray ionization mass spectrum (ESIMS). A positive Kedde test (Alali *et al.*, 1999) and IR absorptions at 3400, 2920, 2850, 1745, 1650 cm^{-1} suggested **1** to be an annonaceous acetogenin bearing an α,β-unsaturated γ-lactone moiety. The presence of a typical α, β-unsaturated-γ-lactone, without an hydroxyl group at C-4 (Alali *et al.*, 1999; Kim *et al.*, 2001) was suggested by the signals at δ (H) 6.98 (1 H, td, $J_{H\text{-}35/H\text{-}36} = J_{H\text{-}35/H\text{-}3} = 1.40$ Hz, H-35); 4.99 (1 H, qdd, $J_{H\text{-}36/H\text{-}37} = 6{,}80$ Hz; $J_{H\text{-}35/H\text{-}36} = J_{H\text{-}36/H\text{-}3} = 1.40$ Hz, H-36); 1.41 (1 H, d, $J_{H\text{-}36/H\text{-}37} = 6.80$ Hz, H-37) e 2.26 (2 H, tt, $J_{H\text{-}3/H\text{-}4} = 7.6$ Hz; $J_{H\text{-}3/H\text{-}35} = J_{H\text{-}3/H\text{-}36} = 1.4$ Hz, H-3) in the ^{1}H-NMRspectrum (Table 1), which showed correlations with carbons in the HMQC and HMBC spectra, permitting the assignment of carbons signals δ (C) 173.94 (C-1), 148.91 (C-35), 134.40 (C-2), 77.42 (C-36) and at 19.20 (C-37). The presence of an adjacent *bis*-THF rings system with two flanking OH groups was suggested by the four carbon signals between δ 83.30 and 82.14, which were correlated to the proton signals at δ 3.91 (5H) in the HMQC spectrum. The one-bond ^{1}H-^{13}C correlations detected in the HMQC spectrum, along with those observed in COSY, permitted the assignment of the signals at δ 74.00 and 71.80 to oxygenated methine carbons and at δ 3.42 and δ 3.91 to the hydrogen adjacent to the THF-rings. COSY spectrum showed correlation between these signals at δ 3.91 (5H) and the multiplet at δ 3.42 (1H). The presence of a third hydroxyl group in the hydrocarbon chain was suggested by the signals at δ 3.60 (1H) and δ 71.40 (in ^{1}H NMR and ^{13}C NMR spectra, respectively). The placement of THF rings and the additional hydroxyl group (δ 3.60 and δ 71.40) was determined by a close examination of the ESIMS fragmentation of **1** after induced collision of the ion at *m/z* 623.53. The hydroxyl group was located at C9 on basis of fragments at *m/z* 439, 411 e 181 in ESIMS of **1** (Figs 1,2). Only the relative stereochemistry at C-11/C-12, C-15/C-16 and C-19/C-20 was determined by comparing the ^{1}H- and ^{13}C-NMR data to those of model compounds of known relative stereochemistry (Cavé *et al.*, 1997; Jossang *et al.*, 1990; Sahai *et al.*, 1994). This comparison showed the relative configurations between the carbons C-11 to C-20 being as *threo-trans-threo-trans-erythro* considering the similarity of the ^{1}H- and ^{13}C-NMR data of **1** with those of the bullatacin-type of acetogenins (Zeng *et al.*, 1996; Sahai *et al.*, 1994). The planar structure of this adjacent *bis*-THF acetogenin annofolin (**1**) with a hydroxyl group in C-9, to the best of our knowledge, was described by our

group for the first time from *Annona cornifolia* seeds (Lima, 2007; Pimenta *et al.*, 2008) and now for the first time in *A. crassiflora*.



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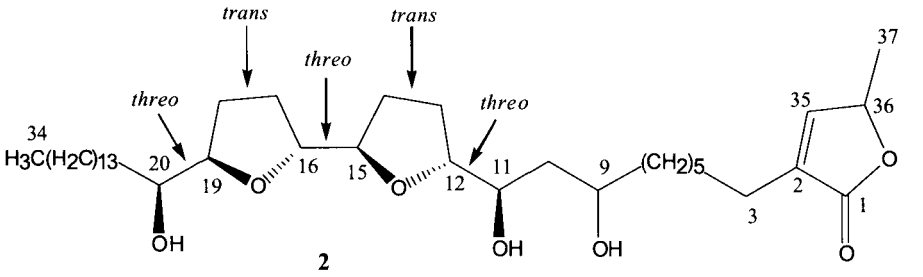

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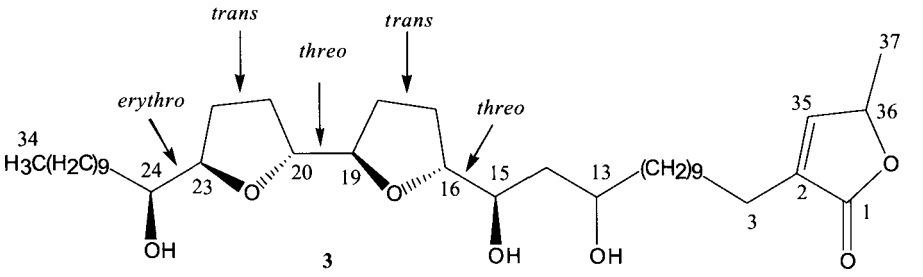

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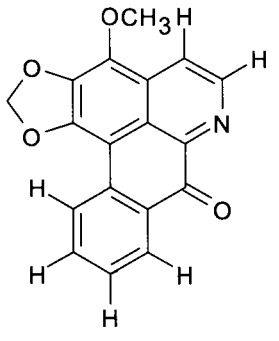

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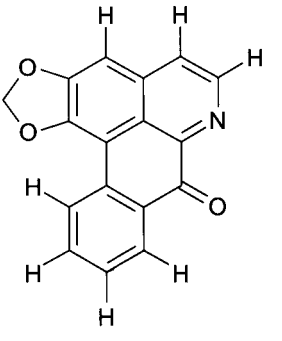

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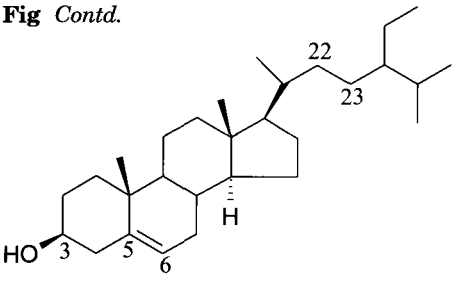

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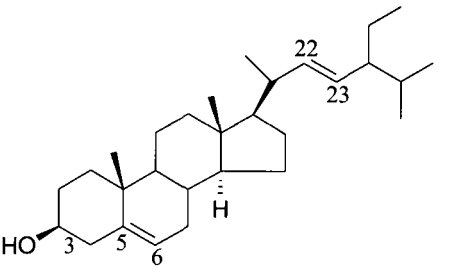

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Fig 1. Structures of the compounds isolated from *Annona crassiflora* wood

The planar structure of the ACM5 (**2**) is similar to annofolin (**1**) differing only in the relative stereochemistry around the THF rings that was determined as *threo-trans-threo-trans-threo* by ^{1}H NMR data (Table 1).

Table 1. ^{1}H[a] and ^{13}C NMR spectral data of anofolin (**1**), and ^{1}H[a] NMR data of ACM5 (**2**) and ACM6 (**3**)

Carbon	Annofolin		ACM5	ACM6
	^{1}H (*J*)	^{13}C	^{1}H (*J*)	^{1}H (*J*)
1	-	173.9	-	-
2	-	134.4	-	-
3	2.26 tt (7.60, 1.40)	25.0	2.26 tt (7.66, 1.52)	2.26 tt (7.66, 1.00)
4	1.54 m	27.0	1.58 m	1.56 m
5-7	1.26-1.30 m	22.5-29.5	1.26 m	1.26 m
8	1.40 m	32.0	1.40 m	1.26 m
9	3.60 m	71.4	3.60 m	1.26 m
10	1.40 m	32.0	1.40 m	1.26 m
11	3.42 m	74.0	3.41 m	1.26 m
12	3.91 m	83.2	3.86-3.91 m	1.39 m
13	1.54-2.00	27.0	1.54-2.00	3.61 m
14	1.54-2.00	28.8	1.54-2.00	1.39 m
15	3.91 m	82.0	3.86-3.91 m	3.47 m
16	3.91 m	82.0	3.86-3.91 m	3.93 m
17	1.54-2.00	28.8	1.54-2.00	1.56-1.97 m
18	1.54-2.00	28.3	1.54-2.00	1.56-1.97 m
19	3.91 m	82.7	3.86-3.91 m	3.93 m
20	3.91 m	71.8	3.41 m	3.93 m
21	1.40 m	32.0	1.40	1.56-1.97 m
22	1.26 m	22.5-29.5	1.26 m	1.56-1.97 m
23	1.26-1.30 m	22.5-29.5	1.26 m	.93 m
24	1.26-1.30 m	22.5-29.5	1.26 m	3.93 m
25-33	1.26-1.30 m	22.5-29.5	1.26 m	.26-1.39 m
34	0.89 t (6.60)	14.0	0.89 t (6.40)	0.88 t (6.80)
35	6.98 d (1.40)	148.9	6.98 d (1.52)	6.99 d (1.00)
36	4.99 qd (6.80, 1.40)	77.4	4.99 qd (6.80, 1.52)	4.99 qd (6.40, 1.00)
37	1.41 d (6.80)	19.2	1.41 d (6.80)	1.41 d (6.40)

[a] $CDCl_3$, 400 MHz. (*J* in Hz)

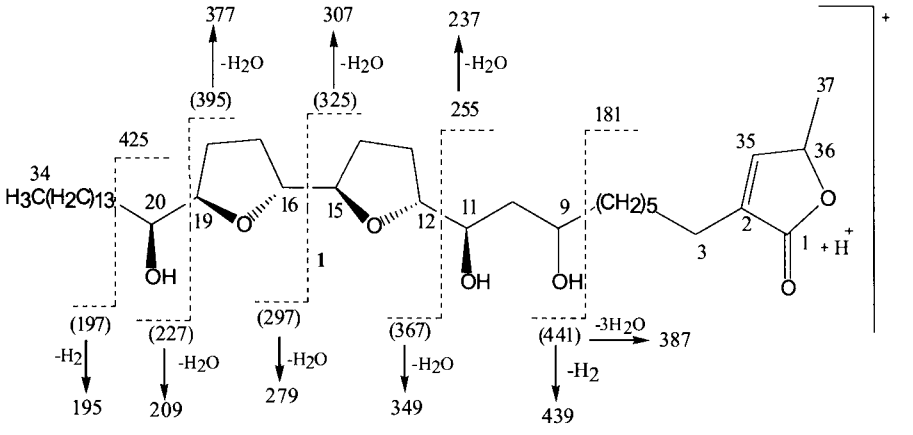

Fig 2. Diagnostic ESIMS fragment ions (*m/z*) for annofolin (**1**). Peaks in parentheses were not observed

The planar structure of ACM6 (**3**) was suggested based on the ^{1}H-NMR (Table 1), COSY and ESI mass spectra. The signals at δ 6.99 (1H, d, H-35); 4.99 (1H, qd, H-36); 1.41 (3H, d, H-37) and 2.26 (2H, tt, H-3), indicated the presence of a α, β-unsaturated-γ-lactone , without an hydroxyl group at C-4 (Alali *et al.*, 1999). The signals at δ 3.47 (1H, m, H-15); 3.61 (1H, m, H-13) e 3.93 (5H, m, H-16, 19, 20, 23 e 24) are characteristics of de hydrogens of oxygenated carbons, with the signal at δ 3.61 assigned to a hydroxyl group in the hydrocarbon chain (Hisham *et al.*, 1991) and the others assigned to a bis-THF system with two flanking hydroxyl groups. The relative stereochemistry from C-15 to C-24 was suggested as *threo-trans-threo-trans-erythro* considering the similarity of the ^{1}H- and ^{13}C-NMR data of **3** with **1** and those of the bullatacin-type of acetogenins (Zeng *et al.*, 1996). The ESIMS data revealed the presence of one acetogenin with molecular formulae of $C_{37}H_{66}O_7$. Based on analysis of fragment ions obtained after induced collision of the ion *m/z* 623.40 $[M + H]^+$ the the system *bis*-THF α, α′-dihydroxylated was located between C-15 and C24, and the third hydroxyl group at C-13 (Fig 3).

The structures of the alkaloids aterospermidine (**4**) and liriodenine (**5**) and the steroids β-sitosterol (**6**) and stigmasterol (**7**), were confirmed by one and bidimensional ^{1}H- and ^{13}C-NMR data and MS measurements, compared to those of the literature (Guinadeau *et al.*, 1975; Hossain *et al.*, 1995; Castro *et al.*, 1996; Goulart *et al.*, 1993). This is the first report of the alkaloid presence in the wood of *Annona crassiflora,* and the first report of aterospermidine in this species.

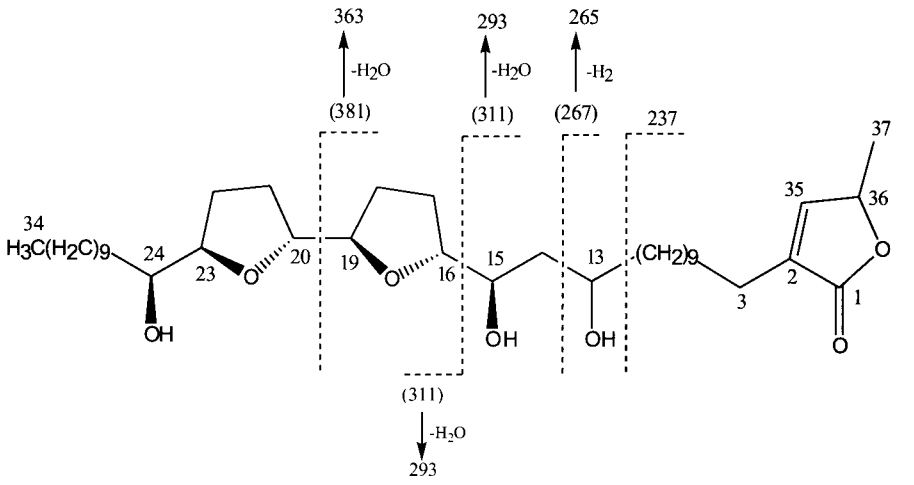

Fig 3. Diagnostic ESIMS fragment ions (*m/z*) for annofolin (**3**). Peaks in parentheses were not observed

Biological assays

The crude extract (ACWF01) and the fractions ACWF02, ACWF03, ACWF03 O, ACWA, ACWF05, ACWF06 showed good bioactivity in brine shrimp lethality test with the DL_{50} range from 1.29 to 30 µg/mL (Table 3). This is in accordance with the presence of annonaceous acetogenis as well as aporfine alkaloids.

Plasmodium berghei NK-65 strain, sensitive to chloroquine (Peters *et al.*, 1986), was used for antimalarial evaluation *in vivo*. This strain is known to induce high mortality in mice, providing a good model to estimate survival and antimalarial efficacy in reducing parasitemia, and is sensitive to all currently used antimalarial drugs. It was kindly supplied frozen in nitrogen by Prof. E.M. Braga, Malaria Laboratory, Parasitology Department, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil.

The antimalarial activity was determined using the four-day suppressive test in *P. berghei* infected mice. Doses of the extract and fractions of 500 mg/kg, 250 mg/kg, 12.5 mg/kg and 1.25 mg/kg were tested. The crude extract caused 100% of death in a dose of 500 mg/kg, and 60 % of death with 250 mg/kg in mice, by intraperitoneal route in the first 24 h after administration.

Table 2 summarizes the effects of extracts and fractions of *A. crassiflora* at concentrations of 1.25 and 12.5 mg/kg/day *in vivo*. The results showed that alkaloidic fraction (ACWA) has a significant antimalarial activity compared with untreated control ($p<0.05$) and a mean survival time

greater than the control indicating the smaller toxicity of this fraction. However, the other fractions especially hexane fraction (ACWF02) exhibits higher toxicity even at the lower doses used in this protocol. In general, the percentage of parasitemia reduction increases with the dose and particularly with the time, suggesting that the pharmacokinetic profile of the substances responsible for the activity possesses long half-lives in mice. No fraction or extract was able to suppress 100% parasitemia (Table 2). However all the extracts and fractions produced some activity reducing the parasitaemia in 13 to 79% range. As expected the *P. berghei* NK65 strain was chloroquine-sensitive parasite and 60 mg/kg/day had a suppressive activity of 100%. The solvents DMA/PEG/saline had no significant effect ($p>0.05$) on parasitemia and on the mean survival time (data not shown) compared to controls.

In the antibacterial activity test by disc diffusion, the alkaloidic fraction, ACW A, showed good results against pathogenic bacteria *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella typhimurium* and *Escherichia coli* (Table 3). This is the first report of phytochemical contituents of *Annona crassiflora* wood with evaluation in different biological activity tests. The results indicated this plant as a potential source of lead compounds for the development of antitumoral, pesticide, antimicrobial and antimalarial drugs.

Table 2. Mean Parasitemia (%), mean survival time and parasitemia reduction (%) in *P. berghei* NK65 infected mice treated with extracts and fractions of *Annona crassiflora*

Codes	Fractions	Dose mg/Kg/ day	MST# Days ± SD	Mean Parasitemia(%)#		Parasi-temia Reduction (%) (days 4/7)
				D4	D7	
Control	Not treated	-	16.2 ± 3.3	14.4 ± 3.5	24.6 ± 3.3	-
Chloroquine	-	60.0	>30	0	0	100/100
ACWF01	Crude extract	1.25	14.3 ± 4.0§	12.6 ± 1.7§	15.9 ± 0.7*§	13/35
ACWF01	Crude extract	12.5	14.8 ± 4.4§	8.9 ± 1.7*§	11.4 ± 0.7*§	38/54
ACWF02	Hexanic	1.25	13.3 ± 2.1§	11.6 ± 2.0§	10.4 ± 0.5*§	19/58
ACWF02	Hexanic	12.5	10.2 ± 7.8§	11.1 ± 2.6§	5.0 ± 0.2[a]	23/79
ACWF05	Hydroalcoholic	1.25	13.3 ± 4.8§	10.6 ± 2.2*§	13.5 ± 0.6*§	26/45
ACWF05	Hydroalcoholic	12.5	16.8 ± 4.1§	12.0 ± 1.8§	12.4 ± 0.6*§	16/49
ACWA	Alkaloidic	1.25	20.8 ± 4.8§	7.2 ± 1.4*§	7.0 ± 1.3*§	50/71
F1-G24	Fraction from ACWF03 O	1.25	14.3 ± 4.0§	9.2 ± 2.1*§	16.8 ± 1.3*§	36/32
F1-G24	Fraction from ACWF03 O	12.5	11.5 ± 4.7§	8.5 ± 2.1*§	8.2 ± 2.7*§	41/67

#n=5; MST: mean survival time; *different from control ($p< 0.05$); § different from chloroquine group ($p< 0.05$) using student t test, [a]two mice.

Table 3. Brine shrimp lethality and antimicrobial data for the extract and selected fractions from *A. crassiflora*

Compound	BST[b] (μg/mL)	Diameter[a] (mm) of inhibition zone of bacterial growth							
		S. aureus		*S. typhimurium*		*S. pyogenes*		*E. coli*	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
ACWF01	27.7	ND[c]	ND	ND	ND	ND	ND	ND	ND
ACWF02	3.8	7.0	6.0	9.0	8.0	ND	ND	9.0	9.0
ACWF03	2.9	ND	ND	11.0	10.0	11.0	11.0	ND	ND
ACWF05	4.6	ND	ND	10.0	10.0	11.0	11.0	ND	ND
ACWF06	1.6	8.0	7.0	11.0	11.0	15.0	14.0	ND	ND
ACWF03 O	1.8	ND	ND	8.0	8.0	9.0	9.0	ND	ND
ACW A	-	12.0	12.0	14.0	14.0	14.0	13.0	10.0	10.0
Chloramphenicol	-	20.0	22.0	22.0	22.0	20.0	19.0	ND	ND

[a] Diameter of inhibition zone minus diameter of the disc after 24 and 48 h observed for the tested extracts, fractions (100 μg/mL) and chloramphenicol (30 μg/disc).

[b] Brine Shrimp Lethality test. [c] Not detected.

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Pharmacokinetics of Active Constituents of *Rhodiola rosea* SHR-5 Extract

A. PANOSSIAN[2,*], A. HOVHANNISYAN[1], H. ABRAHAMYAN[1], E. GABRIELYAN[1] AND G. WIKMAN[2]

ABSTRACT

The active constituents of Rhodola rosea extract SHR-5 of the popular plant adaptogen Rhodiola rosea are tyrosol, rhodioloside and rosavin. Validated capillary electrophoretic methods for the determination of these components in the blood was developed and used for a pharmacokinetic study in rats and human (n=16) volunteers. Rhodioloside was found to be quickly and completely absorbed into the blood of rats (bioavailability-75-90%), distributed within organs and tissues, and rapidly metabolised to tyrosol following oral administration of SHR-5 at doses of 20 and 50 mg / kg. Many of the measured pharmacokinetic parameters of rhodioloside were significantly different when the pure compound was administered rather than the extract. The basal level of tyrosol in blood plasma of rats increased following administration of SHR-5 as a result both of absorption of free tyrosol present in the extract and of biotransformation of rhodioloside into tyrosol, which occurred within the first 2 h. The pharmacokinetics and the rate of biotransformation of rhodioloside were essentially the same following single or multiple regimes of administration of SHR-5. Rosavin has low bioavailability (20-26%) and was quickly eliminated from the blood of rats that have been administered SHR-5. The results of the study in humans showed that 16 healthy volunteers, after oral administration of two tablets of Rosenroot, reached a maximum concentration of rhodioloside and rosavin in blood plasma after 2 h, where the absorption rate constant was equal in

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both components. Although, in contrast to rhodioloside, rosavin was not detected in blood plasma at 0.5 h and 1 h after oral administration of two tablets of Rosenroot. The maximum concentration and elimination half-life of rhodioloside was 2-3 x higher than in rosavin. The $AUC_{0-\infty}$ C_{max} *and* AUC_{0-t} *values of rhodioloside were 3.5, 2.2 and 3.1–fold higher than in rosavin. Thus, the elimination of rhodioloside from the blood was 1.8 fold longer than the elimination time of rosavin.*

Key words : Capillary electrophoresis, pharmacokinetic, *Rhodiola rosea* L., rhodioloside, rosavin, tyrosol, SHR-5

INTRODUCTION

Rhodiola rosea is one of the most popular plant adaptogens in Russia. Since 1969 *Rhodiola rosea* has been recommended by the Pharmacological Committee of the Ministry of Health of the USSR for the medicinal use as a stimulant of fatigue in patients experiencing aesthetic states or in healthy people with a tendency to asthenisation during their work, which requires high mental exertion or to recover their work capacity during and after long periods of intensive physical work. In addition, *Rhodiola rosea* is recommended in cases of borderline nervous-mental diseases, neuroses, neurosis-like disorders, and psychopathies. In psychiatric practice, *Rhodiola rosea* extract is indicated for the correction of neurological side effects of psychopharmacological therapy and for the intensification and stabilisation of remissions of aesthetic and apathico-abulic type schizophrenia patients (Saratikov *et al.*, 1965; Krasik *et al.*, 1970; Saratikov, 1973; Mikhailova, 1983; Brichenko *et al.*, 1986; National Pharmacopoeia of the USSR, 1987; Saratikov & Krasnov 1987).

A number of double-blind, placebo-controlled randomised clinical trials of the standardised extract (SHR-5) of *R. rosea* have shown that use of the drug can reduce general fatigue (as determined by tests measuring complex perceptive and cognitive cerebral functions) under certain stress conditions by increasing total mental performance (Darbinyan *et al.*, 2000), and can significantly improve general well-being, physical fitness, mental fatigue, and coordination in healthy subjects when taken repeatedly for a short period of time (7-10 days) (Spasov *et al.*, 2000 a,b). A similar effect was observed after single dose administration of standardised SHR-5 extract on the capacity for mental work against a background of fatigue and stress on a highly uniform population comprising of 161 cadets (Shevtsov *et al.*, 2003). The anti-fatigue effect of *Rhodiola* SHR-5 extract, as well as improved attention in fatigue and stressful conditions, were earlier observed in patients with chronic stress induced fatigue (Olsson *et al.*, 2008). Moreover, it has been beneficial in mild and moderate depression (Darbinyan *et al.*, 2007).

The active principles of *Rhodiola* extracts are tyrosol (*syn.* salidrosol) [2-(4-hydroxyphenyl)ethanol], and the phenolic glycosides, rhodioloside (*syn.* rhodosin, salidroside) [2-(4-hydroxyphenyl)ethyl-1-β-D-glucopyranoside] and rosavin (*syn.* cinnamyl alcohol β-vicianoside) [3-phenyl-2-propenyl-*O*-(α-L-arabinopyranosyl-(1-6)-β-D-glucopyranoside] (Saratikov *et al.*, 1968; Saratikov, 1973; Barnaulov *et al.*, 1986; Sokolov *et al.*, 1990; Linh *et al.*, 2000; Panossian *et al.*, 2007; Panossian *et al.*, 2008). In order to determine the contribution of individual components with respect to the overall curative effect of an herbal extract, it is necessary to study the pharmacokinetics of the active compounds in the particular preparation, since the presence of even inactive ingredients can influence the bioavailability, absorption, distribution, metabolism and excretion of the active compounds. The aim of the present study was to the investigate the pharmacokinetics of rhodioloside and rosavin in rats (Panossian *et al.*, 2002) and healthy human volunteers after single and multiple oral administration of *Rhodiola rosea* SHR-5 extract–used as an active ingredient of "Rosenroot ®" tablets used in Sc andinavia as an adaptogen to increase impaired performance in fatigue and weakness.

MATERIALS AND METHODS

Chemicals

HPLC-grade methanol (Rotisolv®; # 7342.1) and acetone (# 7328.2) were purchased from Carl Roth (Barcelona, Spain); HPCE-grade water (# 5062-85780), 50 mm borate buffer (pH 9.3; # 5062-8573) and 0.1M sodium hydroxide (# 5062-8575) were from Hewlett-Packard (Palo Alto, CA, USA); ethyl acetate (# 15485-7; lot KI 01058DI) and β-glucuronidase (# G-7770; lot 22K8942) were from Sigma-Aldrich (St Louis, MO, USA); and heparin lock flush solution (containing 500 USP heparin units/mL) was from Abbott Laboratories (Chicago, IL, USA). Unless stated otherwise, the water employed was deionised and distilled.

Study drug

Rhodiola rosea L. roots soft extract SHR-5 (batch No EX 20465, Swedish Herbal Institute, Sweden), quantified for rhodioloside content, (10 ± 2 mg/g), rosavin (20 ± 3 mg/g) and tyrosol (1.5 ± 0.3 mg/g). Rosenroot tablets (400 mg, batch Nr L211) contain 144 mg SHR-5, 3.63 mg rhodioloside and 4.2 mg rosavin.

Standard Solutions

Reference standards stock solution. T A standard solution of the reference compounds tyrosol (Swedish Herbal Institute, Goteborg, Sweden; series 22.10.2001), rhodioloside (VILAR, Moscow, Russia; series S10402) and rosavin (VILAR; series S10402) was prepared by accurately weighing 5.0 mg of each

compound into a 5 mL volumetric flask, dissolving and adjusting to volume with methanol

Study animals

Three hundred time-mated male Wistar rats were obtained from the Institute of Fine Organic Chemistry of the National Academy of Science, Yerevan, Armenia. All animals were clinically examined upon arrival and any animal showing signs of abnormality or disease was replaced before the start of the study. No animals were replaced after the study had commenced. Animals were kept for 10-15 days prior to the start of the study under a 12/12 h light/dark cycle and had free access to standard rat chow. During the study, a standardised diet for rats (Combi/Yerevan, Combi-Corm plant; for composition, Zapadnyuk *et al.,* 1983) was provided, but feeding was discontinued before administration of a test substance. Only tap water was offered *ad lib.*

Throughout the observation period, animals were kept separately in cages (55 × 35 × 25 cm), each consisting of a polystyrene case and a lattice-framed steel lid, and supplied with wood-sawdust bedding. A 12/12 h light/dark cycle was maintained in the animal room throughout the study period, and the temperature and humidity were 22 ± 4°C and 40 ± 5%, respectively, with 1-2 changes of air/h. Each day, or after completion of work, the floor of the animal room was swept and mopped with an atmospheric biocide-cleanser. Excretions were collected every second day on trays lined with absorbent paper which were suspended beneath each cage.

Dosage and Administration

Dosage, Sampling, Drug Safety Control.

The concentrations of rhodioloside, rosavin and tyrosol in the SHR-5 solutions were analysed by HPCE before their administration to the animals. The administered concentrations were 57.6, 97.5 and 6.5 μg/mL, respectively. In addition, two doses of SHR-5 were used in this study. The 20 mg/kg dose shows maximum antiarrhythmic and antistressor effect in rats (Saratikov, 1973; Sokolov *et al.*, 1990) and its rhodioloside content was equivalent to a human therapeutic dose (10 drops of Rhodiola extract), which is close to one tablet of "Rosenroot ®". The 50 mg/kg dose contained rhodioloside equivalent to a human mean daily dose (25 drops of Rhodiola extract) (Saratikov, 1973), which corresponds to two tablets of "Rosenroot ®". Three sets of experiments aimed at determining the pharmacokinetics of tyrosol, rhodioloside and rosavin in the blood of animals were performed, whilst two further groups of animals were used to study the biotransformation of the active components of SHR-5 (Table 1).

Table 1. Experimental design of the study of pharmacokinetics and biotransformations following administration of special extract SHR-5 of *Rhodiola rosea* L. to rats

Group No	Drugs	Total number of animals	Dosage (mg/kg)	Administration	Administrated route	Administrated volume (mL/kg)	Administrated dose of tyrosol (mg\mL/kg)	Administrated dose of rhodioloside (mg/mL/kg)	Administrated dose of rosavin (mg\mL/kg)	Blood and urine sampling time after infusion of drug, h
1	SHR-5	54	20	Single	I.V.	2	0.026	0.230	0.390	0; 0.06; 0.33; 0.5; 0.75; 1.0;1.5; 2.0; 3.0
2	SHR-5	54	20	Single	Oral	10	0.026	0.230	0.390	0; 0.5; 1.0;1.25; 1.5; 2.0; 4.0; 5.0; 6.0
3	SHR-5	54	50	Single	Oral	10	0.065	0.576	0.975	0; 0.5; 1.0; 1.25; 1.5; 2.0; 4.0; 6.0; 8.0
4	SHR-5	48	20/daily	Multiple (5 days)	Oral	10	0.026	0.230	0.390	0; 0.5; 1.0; 1.25; 1.5; 2.0; 4.0; 6.0;
5	SHR-5	36	50/daily	Multiple (5 days)	Oral	10	0.065	0.576	0.975	0; 1.0; 1.5; 2.0; 4.0; 5.0;
6	Rhodiolo-side	42	0.570	Single	Oral	10	-	0.576	-	0; 0.5; 1.0; 1.5; 2.0; 3.0; 5.0
7	Water	42	-	Single	Oral	10	-	-	-	0; 0.5; 1.0; 1.5; 2.0; 3.0; 5.0

Pharmacokinetic Studying Rats

In the first set of pharmacokinetic experiments, SHR-5 was administrated intravenously *via* the tail vein at a dose of 20 mg/kg to 48 animals in group 1 (mean weight 112 ± 10 g; range 100-120 g). Blood samples (4-6 mL each) were collected in heparinised centrifuge tubes immediately before administration of drug and at various times between 0.06 and 3 h after injection (Table 1). Following collection, blood samples were centrifuged at 300 × g for 15 min to obtain blood plasma which was stored at –20°C until required for analysis.

In the second set of experiments, SHR-5 was administrated orally to two groups of 48 animals that had been deprived of food for 12 h: free access to food was provided 2.5 h after the administration of the drug. Animals in group 2 (mean weight 167 ± 10 g; range 150-180 g) received SHR-5 solution at a dose of 20 mg/kg, whilst those in group 3 (mean weight 120 ± 8 g; range 110-130 g) received a dose of 50 mg/kg. Blood samples (4-6 mL each) were collected in heparinised centrifuge tubes before administration and at various times between 0.5 and 6 h after administration (Table 1).

In the third set of experiments, oral administration of SHR-5 was repeated for 5 days at daily doses of 20 mg/kg (group 4: mean weight 157

± 8 g; range 150-180 g) and 50 mg/kg (group 5: mean weight 145 ± 10 g; range 130-160 g). Animals were deprived of food for 12 h prior to the administration (at 9.00 pm) of a single daily dose of SHR-5 solution: 2.5 h after drug administration, animals were allowed free access to food until 9.00 am. Blood samples (4-6 mL each) were collected in heparinised centrifuge tubes before administration and at various times between 1.0 and 5 h after the fifth administration (Table 1).

Study of Biotransformation of Rhodioloside

In order to study the biotransformation of active compounds, a group of 36 animals (group 6; mean weight 125 ± 9 g; range 110-140 g) was fasted for 16 h and rhodioloside was administrated orally at a dose of 0.570 mg/kg. After administration of the drug, only water was supplied during the whole experiment. Blood samples (4-6 mL each) were collected in heparinised centrifuge tubes before administration and at various times between 0.5 and 5 h after administration (Table 1).

Human Study Design

The study was performed as a blind randomized study, in compliance with the revised declaration of Helsinki (Hong Kong, 1989) and The Ministry of Health in Armenia.

Volunteers

The clinical part of the study was carried out at the Centre of Traumatology, Orthopedics, Burns and Radiology at the Ministry of Health of Armenia and written informed consent was obtained from each subject before the study was initiated. In total, 16 normal and healthy volunteers were scheduled to participate (two groups of 8-male and 8-female). All volunteers had been donors at the Centre of Hematology for more than four years and a written and signed agreement between the head of investigation and the volunteer were included in the study. Prior to the study, each subject underwent a brief physical examination and had blood drawn for serum chemistry and hematology analysis, according to the inclusion/ exclusion criteria.

During the study each volunteer received a number and a special code, in compliance with their sex.

Study Schedule

The study included the following stages: selection of volunteers, receiving written signed agreements (information for volunteers), medical examination and blood biochemistry and hematology analysis, disposition in groups, drug administration and blood sampling, determination of drug in blood samples, and calculation of pharmacokinetic parameters. The study was provided according to the scheme notified in the investigation protocol.

Selection of Volunteers

The volunteer inclusion criteria focussed on healthy males and females aged 28-55, without allergenic anamnesis or illnesses, such as chronic diseases of the kidney, liver, cardiovascular and nervous system. In addition, the volunteers have to have signed an agreement written by the head of the investigation. Finally, they needed to be able to collaborate adequately during the entire length of the study. The volunteer exclusion criteria concerned volunteers suffering from chronic illnesses, such as cardiovascular diseases, diabetes (etc.), and arterial hypotension (etc.). In addition, volunteers addicted to, for example, medicines, narcotics and tobacco were also excluded. Finally, they were not allowed to participate in any other trials. In addition, volunteers were removed from the investigation if they experienced an individual reaction, adverse reactions, serious adverse events or if their health and physical condition deteriorated.

The Dates of Volunteers Enrolled in the Study

Bearing in mind the expected intra-individual variability, 16 healthy volunteers (Caucasians), which had not received any drugs in the past month prior to this study were enrolled. The volunteers, aged 28-55, were both female (mean age 41.37 ± 11.6) and male (mean age 45.50 ± 6.9).

Dosage, Blood Sampling and Safety Control

Dosage and Blood Sampling

The subjects were fasting during one night and then received two tablets of Rosenroot with 200 mL water two h before breakfast at 9 a.m. The standard breakfast time was at 9.30 a.m. – 10.00 a.m., while the standard dinner time was 15.30–16.00. The period measured was 10 h. In addition, the subjects agreed to refrain from the use of other prescription or nonprescription drugs (including vitamins), alcohol and coffee during the entire study period. At the end of the study, each subject underwent a brief physical examination and had blood drawn for serum chemistry and hematology analysis. The volunteers were all Armenian and live in the capital Yerevan.

A 20-gauge heparinised catheter was inserted into a vein of the forearm to collect blood samples in a dilute heparin solution (10 to 15 U/mL). Two millilitres of blood was drawn and discarded prior to collection of each blood sample (6-8 mL) into heparinised tubes. These tubes were centrifuged at 300 g for 15 min to prepare plasma and stored at –20°C until further analysis. For each volunteer, a set of blood samples (6-8 mL each) were drawn before (0) administration and at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0 and 10.0 h thereafter.

Safety Control

Drug safety was rated from physical examination, measurement of blood pressure, heart rate, and laboratory examination, such as hematology (leukocytes, erythrocytes, and hemoglobin), biochemistry and documentation of adverse events (AE), which were asked and recorded by the clinical staff every h after drug intake. The classification of the AE's was in accordance with the EC-guideline 111/3445/91-EN and termed as mild, moderate and severe. In addition, the causes were classified as definite, probable, possible, unlikely, not related and not possible to judge.

HPCE Analyses

In order to analyse rhodioloside and rosavin in blood, 1.5 mL of acetone was added to 1.5 mL of the plasma sample, the mixture was vortex-mixed, a further 5 mL of acetone added, the whole vortex-mixed again and the proteins precipitated at 4°C by centrifugation at 2000 × g for 15 min. The supernatant was removed, evaporated to dryness at 40°C using a vacuum rotary evaporator and the residue dissolved in 100 mL of methanol, transferred into HPCE vials and subjected to analysis. For the analysis of tyrosol in blood, 1.0 mL of methanol was added to 1.0 mL of the plasma sample, the mixture was vortex-mixed, a further 3.0 mL of methanol added, the whole vortex-mixed and the proteins precipitated at 4°C by centrifugation at 2000 × g for 15 min. The supernatant was removed and treated exactly as described above.

For the analyse of tyrosol in urine, *ca.* 50 μl of β-glucuronidase solution was added to 1.0 mL of urine sample, the whole mixed vigorously for 30 s and then incubated at 55°C for 3 h. After incubation, the mixture was purified *via* solid phase extraction by applying the sample to Supelclean LC-18 SPE tubes (Supelco, Belefonte, PA, USA; 3 mL; lot SP1662A), which had been pre-washed with 1 mL of methanol and 1 mL of water, and eluting with 3 mL each of water : methanol mixtures of compositions 95:5, 85:15 and 50:50. The second and third eluents were evaporated to dryness at 50°C using a vacuum rotary evaporator: the residue was dissolved in 1.0 mL of acetate buffer (pH 5.2), 5.0 mL of ethyl acetate added, the whole vortex-mixed for 60 s, and the two layers separated. The organic phase was evaporated to dryness at 50°C using a vacuum rotary evaporator, the residue was dissolved in 100 ml of methanol and subjected immediately to HPCE analysis.

Analyses were carried out using a Hewlett Packard HPCE system comprising a model HP3DCE apparatus interfaced to a HP Kayak XA workstation and a HP Laser Jet 4000 printer, and equipped with a HP fused silica capillary (# G 1600-61232; total length 56 cm; effective length 50 cm; i.d. 50 mm; optical path length 150 mm) maintained at 10°C. The column was preconditioned by flushing for 1.0 min. with 0.1 M sodium hydroxide, rinsing with water, and then flushing with mobile phase [50 mM

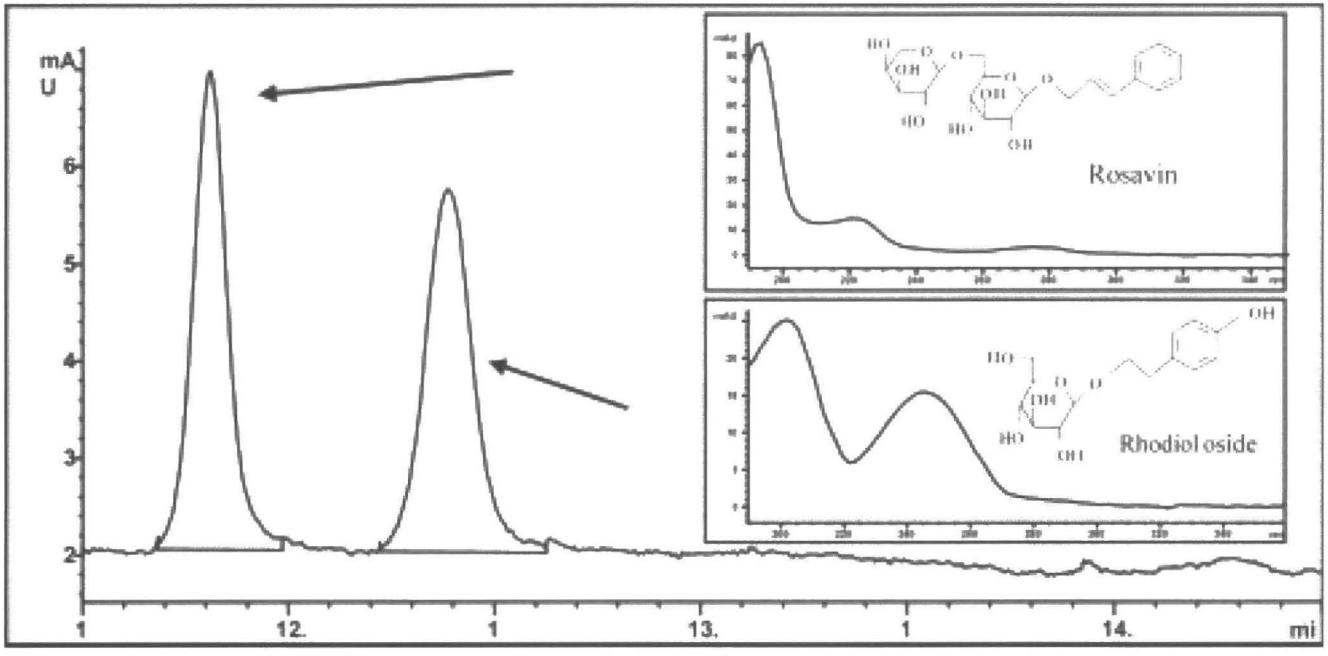

Fig 1. Electropherogram of standard solutions of salidroside and rosavin (25 mg/l) monitored at 201 nm [the solvent system was 50 mM borate buffer (pH 9.3): methanol (85:15, v/v): the migration times for salidroside and rosavin were 11.86 ± 0.06 and 12.39 ± 0.06 min, respectively: the relative migration time salidroside/rosavin was 0.9573 (CV = 0.09%)]

borate buffer (pH 9.3) : methanol in the ratio 85:15 (v/v) for rhodioloside and rosavin, and 60:40 (v/v) for tyrosol] for 2 min. Injection was at 50 mbar for 4 s, the running voltage was 25 kV, detection was at 201 nm (reference 300 nm), 251 nm (reference 450 nm) and 275 nm (reference 400 nm), and the run time was 15 min. The capillary was washed for 5 min. after very fifth run and the mobile phase replaced.

Fig 1 shows a capillary electropherogram of rhodioloside and rosavin (migration times 11.86 ± 0.06 and 12.39 ± 0.06 min, respectively), whilst Fig 2 depicts the electropherogram for tyrosol (migration time 12.79 ± 0.10 min). Calibration curves for all analytes were linear in the range 2.50 – 125.00 mg/l with correlation coefficients of 0.9986, 0.9962 and 0.9984, respectively, for tyrosol, rhodioloside and rosavin. The limit of detection for all analytes was 1.0 mg/l (at a signal/ noise ratio of 3) and the limit of quantification was *ca.* 2.5 mg/l. The accuracy was 98.59% for rhodioloside, 97.20% for rosavin and 97.34% for tyrosol. The recoveries were 90.38 and 93.82%, respectively, for rhodioloside and rosavin from blood samples, and 86.5 and 99.5%, respectively, for tyrosol from blood and urine samples. The repeatability for all analytes was *ca.* 94%, and the relative standard deviation (RSD) value was established at <5% for all validation parameters.

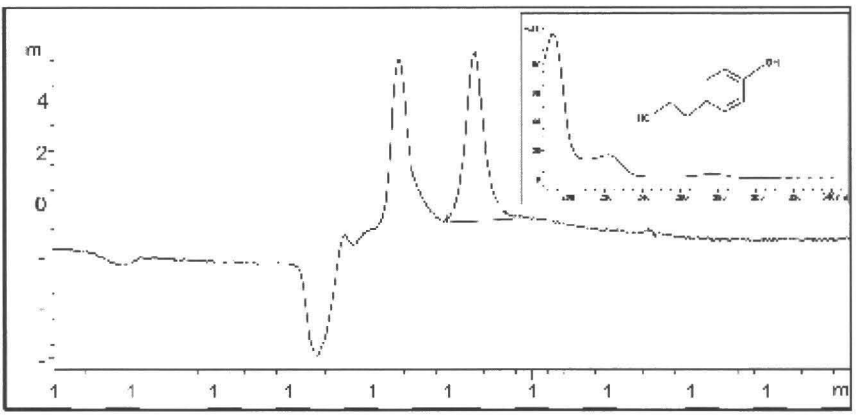

Fig 2. Electropherogram and UV spectrum of a standard solution of tyrosol (25 mg/l) monitored at 201 nM [the solvent system was 50 mm borate buffer (pH 9.3): methanol (60:40, v/v): the migration time for tyrosol was 12.615 min.]

Calculation of Concentration of Analytes in Blood and Urine Samples

The mean concentrations of rhodioloside and rosavin in blood plasma ($C_{MEAN\ PLASMA}$) were calculated from the mean values measured by HPCE ($C_{MEAN\ HPCE}$) using the equation $C_{MEAN\ PLASMA} = C_{MEAN\ HPCE} / (K_1 X K_2)$, where K_1 is the concentration factor of the sample during pre-treatment (= 15) and K_2

is the appropriate coefficient of recovery (0.904 and 0.938 for rhodioloside and rosavin, respectively). The mean endogenous concentration of tyrosol ($C_{MEAN\ ENDOGENOUS}$) in blood plasma and urine was determined from $C_{MEAN\ ENDOGENOUS} = C_{MEAN\ HPCE}/(K_1 X K_2)$, where K_1 is the concentration factor of the sample during pre-treatment (= 10) and K_2 is the appropriate coefficient of recovery (0.869 and 0.995 for blood plasma and urine, respectively). The mean exogenous concentration of tyrosol in blood plasma after administration of drug ($C_{MEAN\ PLASMA}$) was calculated from $C_{MEAN\ PLASMA} = C_{MEAN\ TOTAL} - C_{MEAN\ ENDOGENOUS}$, where $C_{MEAN\ TOTAL}$ is the mean total concentration (endogenous + exogenous) of tyrosol found by HPCE in blood plasma after administration of a drug, and $C_{MEAN\ ENDOGENOUS}$, is the mean endogenous concentration of tyrosol in control samples of blood plasma.

The amount of rhodioloside excreted in the urine as tyrosol and expressed as a percentage of the amount of SHR-5 administered (D_E), was determined from:

$$D_E = \frac{(R_2 - R_1) \times t}{(k \times D_S + D_{FT}) \times BW} \times V_t \times 100$$

where R_1 is the rate (mg/mL/h) of urinary excretion of endogenous tyrosol before administration of SHR-5, R_2 is the rate (mg/mL/h) of urinary excretion of tyrosol after administration of SHR-5, t is the time (h) after administration of SHR-5, k is the molecular weight ratio of tyrosol : rhodioloside (= 0.46), D_S is the dose (mg/kg) of rhodioloside administrated, D_{FT} is the dose (mg/kg) of free tyrosol administrated, BW is the total body weight (kg) of the two animals in the chamber, and V_t is the total volume (mL) of urine collected over time t.

The Measured Pharmacokinetic Parameters

The pharmacokinetic parameters were calculated using the TOPFIT software (version 1.1; Godecke, Freiburg, Germany; Schering, Berlin, Germany, Thomae, Biberach-an-den-Riss, Germany): parameters for multiple administrations were calculated using the method of Dost (1968). The measured parameters were: (i) C_{MAX} (ng/mL) - the maximum concentration taken directly from the concentration course; (ii) C_0 ng/mL - initial drug concentration in blood plasma after the first dose; (iii) F (%) - bioavailability; (iv) K_A (/h) - the absorption rate constant; (v) K_{EL} (/h) - the elimination rate constant calculated from a log/linear regression of the concentration/time data (terminal slope); (vi) $t_{1/2}$ (h) - the elimination half-life ($0.693/k_{EL}$); (vii) $AUC_{0-\infty}$ (μg.h/mL) - area under the curve after extrapolation from time x to infinity, where x is the last time point with a concentration above the lower limit of quantification ($= AUC_{0-t} + C_X/K_{EL}$); (viii) Cl_t (mL/min) - clearance ($= F \times dose/AUC_{0-\infty}$); (ix) MRT (h) - mean residence time (= AUMC /AUC where AUMC is the area under the first statistical moment curve - $\int_{0-\infty} t.C.dt$ (ng/mL)h^2); (x) K_{12} (/h) - distribution

rate constant for the transfer of drug from central to peripheral compartment; (xi) K_{21} (/h) - distribution rate constant for the transfer of drug from peripheral to central compartment; (xii) Vc (l) - apparent volume of distribution of central or plasma compartment (= D/C_0); (xiii) V_{DSS} (l) - apparent volume of distribution at steady state (= F × dose/C_0 or $K_{12} + K_{21}/K_{21}$ × Vc for a one or two-compartment model, respectively); (xiv) C^{SS}_{MAX} (ng/mL) - maximum drug concentration in blood plasma in multiple dosing at steady state; (xv) C^{SS}_{AV} (ng/mL) - mean steady state blood plasma concentration; (xvi) C^{SS}_{MIN} (ng/mL) - minimum drug concentration in blood plasma in multiple dosing at steady state; and (xvii) τ (h) - dosing interval.

RESULTS

Pharmacokinetics Study in Rats

Rhodioloside

Following intravenous injection of SHR-5 at a dose of 20 mg/kg, the concentration of rhodioloside in blood plasma decreased rapidly from 920 to 367 ng/mL after 30 min., and then diminished slowly reaching a value of 190 ng/mL after 1 h (Fig 3). When SHR-5 was given orally at a dose of 20 or 50 mg/kg, the maximum concentration of rhodioloside in plasma was attained 1 h after administration (Fig 3). For both doses, the concentration of rhodioloside fell sharply during the interval from 1-2 h after administration, and then declined slowly. After 1 h following intravenous injection of SHR-5, or 5-6 h after oral administration of the drug, rhodioloside was no longer detectable in blood plasma since the level of the analyte fell below the limit of detection (100 ng/mL) of the analytical method.

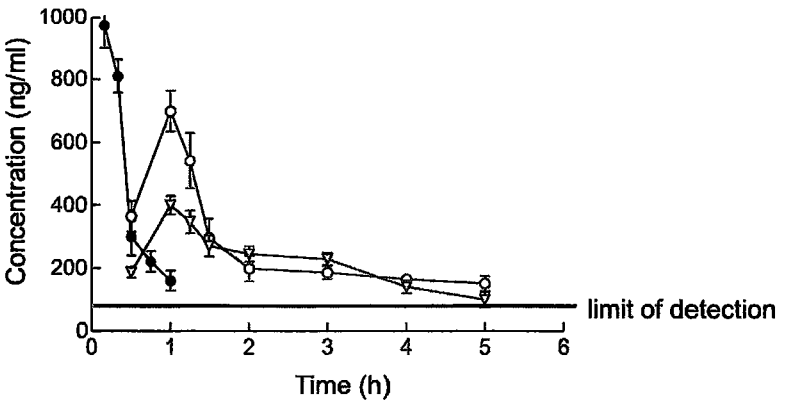

Fig 3. Profiles of plasma concentration of salidroside *versus* time following a single intravenous (20 mg/kg, -• -) and oral administration of SHR-5 solution at doses of 20 mg/kg (Δ) or 50 mg/kg (o). The values are means of replicate (n = 6) measurements with their respective standard deviations

Fig 4 shows that after the fifth oral administration of SHR-5 at a dose of 50 mg/kg, the maximum concentration of rhodioloside in plasma was reached 1-1.5 h after drug administration. During the period 1-2 h after administration, the concentration of the analyte decreased sharply, following which the decline was much slower. Around 5 h after multiple administration of SHR-5, the blood level of rhodioloside fell below the limit of detection.

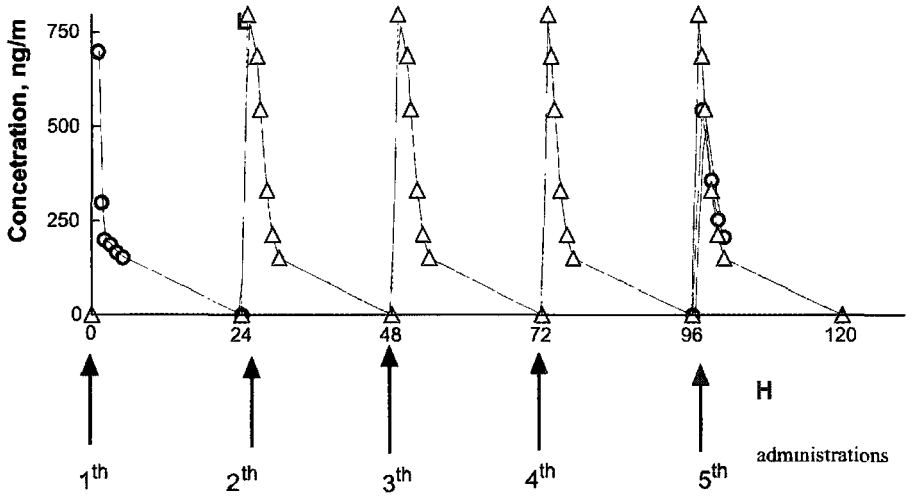

Fig 4. Profiles of plasma concentration of rhodioloside *versus* time following multiple oral administration of SHR-5 solution (50 mg/kg/day) showing experimental values (O) and calculated values (Δ) based on single dosing; each are means of replicate (n = 6) measurements with their respective standard deviations

The pharmacokinetic parameters for rhodioloside, determined for all routes of administration and doses used in the study, are shown in Table 2. Clearly, rhodioloside was rapidly and completely absorbed into the blood (K_A - 0.7-2.0 /h; F - 78-93%) after single and multiple oral administrations of SHR-5 to rats at doses of 20 and 50 mg/kg. Following oral administration, rhodioloside was quickly distributed within tissues but returned somewhat slowly to the circulatory blood system (K_{12}/K_{21} > 2). For both dose levels studied, no significant differences were found in the values of the pharmacokinetic parameters of rhodioloside following single and multiple oral administrations of the drug. The rates of absorption, distribution and elimination after administration of SHR-5 at a dose of 20 mg/kg/daily were, however, lower than those following administration of the drug at a dose of 50 mg/kg/daily, but the bioavailability for both doses was the same (Table 2). Analysis of the pharmacokinetic curve of rhodioloside plotted with semi-logarithmic coordinates showed that in all cases the pharmacokinetics could be described by a two compartment

pharmacokinetic model. The differences between the concentrations of rhodioloside in plasma measured experimentally and those calculated using pharmacokinetic parameters for single dose administration, were not significant.

Table 2. The comparison of pharmacokinetic parameters of rhodioloside after SHR-5 administration

Parameters	Route of administration				
	I.V. single	Oral single		Oral multiple after 5th administration	
Doses, mg/kg	20	20	50	20	50
Ka, h^{-1}	-	2.06 ± 0.06	0.95 ± 0.07	0.73 ± 0.13	1.70 ± 0.18
C max, ng/mL	974 ± 27	400 ± 8	700 ± 26	472 ± 9	545 ± 8
Vdss, l	0.087 ± 0.020	0.023 ± 0.001	0.025 ± 0.006	0.040 ± 0.002	0.059 ± 0.012
$AUC_{0-\infty}$ [(ng /mL) ·h]	809 ± 26	1121 ± 27	1703 ± 33	1013 ± 23	2114 ± 61
$K_{1/2}$, h^{-1}	0.738 ± 0.316	4.781 ± 0.770	1.446 ± 0.267	0.582 ± 0.200	1.301 ± 0.033
$K_{2/1}$, h^{-1}	1.493 ± 0.265	0.720 ± 0.020	0.392 ± 0.088	0.185 ± 0.024	0.661 ± 0.16
Cl_t, mL/min	1.681 ± 0.084	0.560 ± 0.060	0.531 ± 0.011	0.350 ± 0.008	0.536 ± 0.02
$t_{1/2}$, h	0.57 ± 0.04	3.57 ± 0.18	4.45 ± 0.64	5.59 ± 0.52	3.48 ± 0.35

Rosavin

Following intravenous injection of SHR-5 at a dose of 20 mg/kg, the concentration of rosavin in blood plasma fell rapidly from 775 to 274 ng/mL after 30 min, and then diminished slowly (Fig 5). The maximum plasma concentration of rosavin following single oral administrations of SHR-5 at doses of 20 and 50 mg/kg was attained 1 h after drug administration, following which the blood level of the analyte sharply decreased within 1-1.5 h and then declined slowly to a level (<100 ng/mL) which was no longer detectable (Fig 5). It is noteworthy that the HPCE method developed for the determination of rosavin in blood plasma only allowed detection of the analyte between 1-1.5 h after oral administration of SHR-5 at a dose equivalent to a single therapeutic human dose of the drug: in all other blood samples the concentration of rosavin was below the limit of detection of the method. This may be explained by the very low bioavailability of rosavin (F = 20-26%; Table 3) and its rapid disappearance ($t_{1/2}$ = 0.5-0.6 h). Similar results were found following multiple administration of SHR-5 at a dose of 50 mg/kg one time daily where, again, it was possible to determine the analyte in blood only between 1-1.5 h following the fifth administration. The results demonstrate that the concentration of rosavin determined 1 h after the fifth administration of the drug (620.2 ± 131.0 ng/mL) is somewhat variable and near to the calculated value of C^{SS}_{MAX} (574 ng/L).

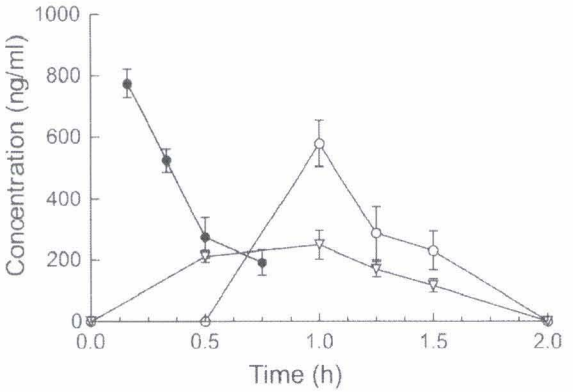

Fig 5. Profiles of plasma concentration of rosavin *versus* time following a single intravenous (20 mg/kg, -• -) and oral administration of SHR-5 solution at doses of 20 mg/kg (▽) or 50 (○) mg/kg and The values are means of replicate (n=6) measurements with their respective standard deviations

Table 3. The comparison of pharmacokinetics parameters of rosavin after SHR-5 intravenous and oral administration

Parameters	Route of administration		
	I.V. single	Oral single	Oral single
Doses, mg/kg	20	20	50
Ka, h^{-1}	-	0.489 ± 0.017	1.935 ± 0.438
C max, ng/mL	525.3 ± 15.1	249.6 ± 19.4	579.1 ± 30.2
Vdss, l	0.054 ± 0.007	0.058 ± 0.008	0.057 ± 0.001
$AUC_{0-\infty}$[(ng /mL) ·h]	485 ± 56	171 ± 10	492 ± 73
Cl_t, mL/min	1.30 ± 0.11	1.37 ± 0.08	1.76 ± 0.12
$t_{1/2}$, h	0.532 ± 0.065	0.469 ± 0.026	0.631 ± 0.044
F, %	100	20.3 ± 1.9	26.0 ± 6.5

Tyrosol

It is a well-known fact that tyrosol is consumed through food and remains at a stable level in the blood and urine during fasting conditions (Visioli, 2000: Miro-Casas *et al.*, 2001). Preliminary experiments showed that the basal level of free tyrosol in the blood plasma of rats subjected to a wash out period was 499.8 ± 15 ng/mL. After intravenous administration of SHR-5 at a dose of 20 mg/kg, the concentration of exogenous tyrosol in the plasma increased, reaching a maximum value after 1 h, but within 3 h the level decreased sharply and returned to the endogenous basal level (Fig 6). Following oral administration of SHR-5 at doses of 20 and 50 mg/kg, the concentration of exogenous tyrosol attained its maximum value within 1.5 and 2.0 h, respectively, and then decreased exponentially (Fig 6).

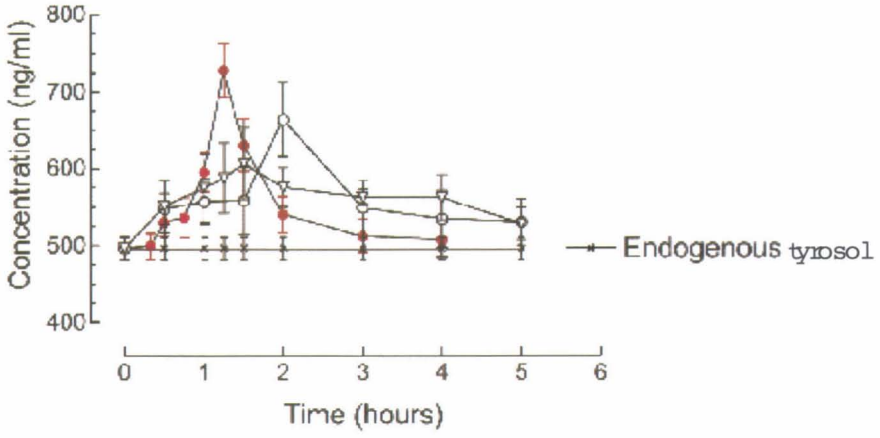

Fig 6. The mean plasma concentration vs time profiles for tyrosol after a single intravenous (20 mg/kg, -• -) and oral administration of SHR-5 solution of two doses: 20 mg/kg (-▽-) and 50 mg/kg-o-). The values are mean ± SD, n=6

In order to establish whether tyrosol could be generated from rhodioloside *in vivo*, the pharmacokinetic of tyrosol and rhodioloside were studied following oral administration of rhodioloside at a dose of 0.570 mg/kg (corresponding to that amount of rhodioloside present in a 50 mg/kg dose of SHR-5). The concentration/time courses of tyrosol and of rhodioloside in the blood plasma of rats fed with rhodioloside are shown in Fig 7. It was observed that the concentration of tyrosol reached its maximum value within 1 h, fell sharply within the period 1-1.5 h after administration, and then followed an exponential decrease. Within the first 2 h following administration of rhodioloside, the concentration of tyrosol was significantly higher than that of rhodioloside, but after 2 h their concentrations were very similar. These results can only be explained by assuming that tyrosol measured *in vivo* was derived (enzymatically or as a result of hydrolysis in acidic conditions in stomach) from the rhodioloside administered to the animal, scheme 1.

The comparison of pharmacokinetic curves and parameters (Fig 7 & Table 4) of rhodioloside after administration of SHR-5 or rhodioloside showed that its concentration in blood plasma was significantly higher, particularly in the first 1.5 h after administration. The results also showed that the maximum concentration time of rhodioloside in blood plasma appeared later when rhodioloside was administered alone, as a pure substance, in contrast to SHR-5 administration. Some other pharmacokinetic parameters were also significantly different after rhodioloside administration (as a pure substance), where the values of C_{max}, $AUC_{0-\infty}$, $t_{1/2}$ were less than 1.2 x - 2.0 x., in comparison to rhodioloside administration in SHR-5. The half-life time of rhodioloside was shorter and the total amount in the blood was less

2-(4-Hydroxyphenyl)ethanol-1-b-D-glucopyranoside (rhodioloside)

2-(4-Hydroxyphenyl)ethanol (tyrosol)

Scheme 1

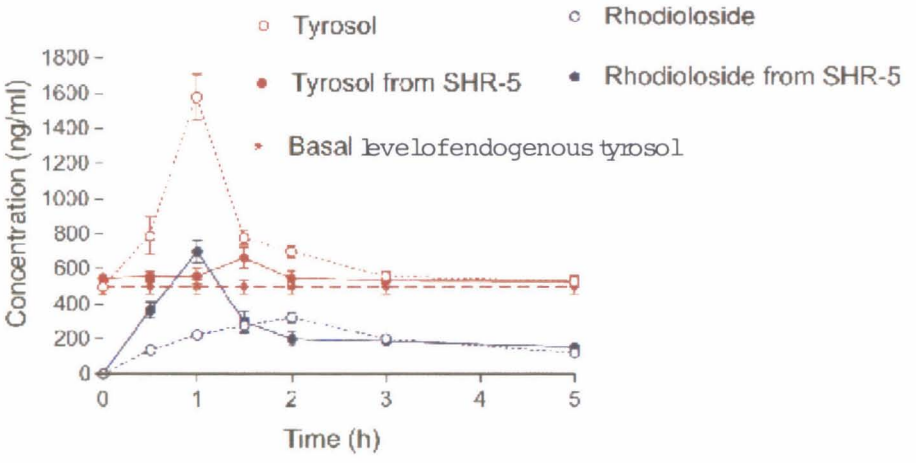

Fig 7. The mean plasma concentration vs time profiles for tyrosol (-o-) and rhodioloside (-o-) in the dose of 0.570 mg/kg after single oral administration of rhodioloside, and tyrosol (-•-) and rhodioloside (-•-) after single oral administration of SHR-5 solution in doses 50 mg/kg, n=6. The values are mean ± SD

when rhodioloside was administered alone. At the same time, its distribution rate in tissues was almost the same since the values of the volumes of distribution were similar when both pure rhodioloside and SHR-5 were administered. Thus, the differences observed might be due to the presence of other chemically similar compounds in SHR-5, which might have been metabolised by the same enzyme as rhodioloside. If this suggestion is correct, it means that the tyrosol content in blood plasma is higher after administration of pure rhodioloside than after administration of SHR-5.

The comparison of pharmacokinetic curves of tyrosol after administration of SHR-5 and rhodioloside showed that after administration of rhodioloside its concentration in blood plasma was significantly higher, particularly in the first 1.5 h after administration (Fig 9). In the interval between 2-3 h after administration, the concentration of tyrosol decreased approximately at the same rate, while at 3 h it reaches the basal level in both cases. Moreover, the maximum concentration of tyrosol was detected only 1 h after intravenous administration of SHR-5. This fact may also

indicate that the increase in tyrosol level detected in the blood originated not only from free tyrosol of SHR-5, but also from biotransformation of rhodioloside and/or other precursors present in SHR-5.

Table 4. The comparison of main pharmacokinetic parameters of rhodioloside in rats after oral administration of rhodioloside and SHR-5 (rhodioloside dose: 0.570 mg/kg). The difference is significant at $p<0.05$. The values are mean ± SD, (n=6)

Parameters	Rhodioloside after rhodioloside administration	Rhodioloside after SHR-5 administration	Significance of the dereference, p value
t_{max}, h	1,44 ± 0,18	1,01 ± 0,03	*0.0013*
C_{max}, ng/mL	334 ± 7	700 ± 26	*<0.0001*
V_{dss}, l	0.127 ± 0.011	0.155 ± 0.007	*0.0660*
$AUC_{0-\infty}$, [(ng /mL) •h]	1370 ± 46	1702 ± 40	*0.0003*
Cl_t, mL/min	0.846 ± 0.026	0.682 ± 0.016	*0.0004*
$t_{1/2}$, h	2.58 ± 0.39	4.54 ± 0.40	*0.0055*

Pharmacokinetics of Rhodioloside and Rosavin in Human

The study showed that the maximum concentration of rhodioloside, in blood plasma, was reached 2 h after oral administration of two tablets of Rosenroot. Although, in the interval between 1 h - 8 h the concentration decreased exponentially, while after 8 h the level of rhodioloside was not detection (concentrations in blood plasma was less then the limit of detection) (Fig 8). In contrast to rhodioloside, rosavin was not detected at 0.5 and 1 h in blood plasma after oral administration. The maximum concentration of rosavin in blood plasma was reached after 2 h and decreased exponentially in the interval between 1-8 h after administration the concentration (Fig 8).

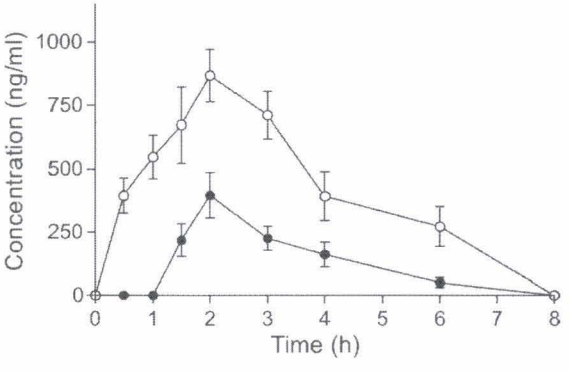

Fig 8. The mean plasma concentration vs time profiles for mean concentration of rhodioloside (-o-) and rosavin (-•-) in blood plasma of volunteers after a single oral dose of two tablets of Rosenroot. The values are mean ± SD, n=16

The study showed that the absorption rate constant and $t_{max,}$ of rhodioloside were similar to that of rosavin (Table 5), while the values of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$, of rhodioloside were significantly greater (2.12x, 3.14x and 3.7x, respectively), in comparison to rosavin. Therefore, the elimination of rhodioloside from the blood took longer (1.8x) than rosavin.

Table 5. The comparison of pharmacokinetic parameters of rhodioloside and rosavin in human volunteers evaluated through individual parameters. The mean ± SD, n=16. The difference is significant at $p<0.05$

Parameters	Rhodioloside A	Rosavin B	Ratio A : B	Significance of the dereference, p value
Dose, μg	9340	7740	**1.2**	
Ka, h^{-1}	1.06 ± 0.19	1.04 ± 0.13	**1.0**	>0.05
C_{max} ng/mL	948 ± 112	446 ± 90	**2.12**	<0.001
$t_{max,}$ h	2.0	2.0	**1.0**	>0.05
$AUC_{0-t,}$ ng ·h/mL	2569 ± 344	819 ± 142	**3.14**	<0.001
$AUC_{0-\infty}$, μg ·h/L	4477 ± 645	1192 ± 169	**3.7**	<0.001
$t_{1/2}$, h	2.5 ± 0.7	1.4 ± 0.3	**1.8**	<0.001
$AUC_{0-\infty}$/ Dose	0.48	0.26	**1.8**	

Table 6. Comparison of the main pharmacokinetic parameters of tyrosol, following oral administration of salidroside and SHR-5 (both in a dose containing 0.570 mg/kg salidroside) to rats, as calculated from the mean concentrations of exogenous tyrosol in the blood

Parameters	Tyrosol (after administration of salidroside)	Tyrosol (after administration of SHR-5)
K_A (/h)	1.794	0.958
t_{MAX} (h)	1.000	2.000
C_{MAX} (ng/mL)	1081.154	159.3400
$AUC_{0-\infty}$ (ng.h/mL)	1185.491	334.117
$t_{1/2}$ (h)	2.074	2,005
MRT (h)	2.993	2.982

DISCUSSION

The pharmacokinetic parameters of rhodioloside determined following administration of the pure compound and as a component of the special extract (SHR-5) of *R. rosea*, are compared in Table 4. Most of the measured parameters differed significantly according to the manner in which rhodioloside was administered: thus the maximal concentration of rhodioloside in blood plasma was attained later (t_{MAX}) when the pure compound was given, and the values of C_{MAX}, $AUC_{0-¥}$, $t_{1/2}$ and MRT were

some 1.2-2.0 times lower than those determined when the compound was administrated as SHR-5. In contrast, the rate of distribution of rhodioloside within tissues was almost the same since the values of V_{DSS} determined following administration of pure rhodioloside and SHR-5 were similar. The differences outlined above could be accounted for by the presence in SHR-5 of compounds which are chemically similar to rhodioloside and which compete with rhodioloside for the same metabolic enzymes. If this suggestion is correct, it implies that the content of tyrosol in blood plasma should be higher after administration of pure rhodioloside than after administration of SHR-5.

Comparison of the pharmacokinetics of tyrosol following administration of pure rhodioloside and of SHR-5 (Fig 7) shows that the initial concentration of tyrosol in blood plasma was significantly higher (particularly in the first 2 h) after administration of the pure compound, but then decreased at approximately the same rate for both forms of administration. At the same time the values for C_{MAX} and $AUC_{0-\infty}$ of tyrosol are significantly higher after administration of rhodioloside, but the values of the elimination parameters, such as $t_{1/2}$ and MRT, are almost the same (Table 6). It may thus be concluded that the biotransformation of rhodioloside was more rapid following oral administration of the pure compound than after administration of SHR-5. Moreover, the maximum concentration of tyrosol was detected after only 1 h following intravenous administration of SHR-5 indicating that tyrosol detected in blood originated not only from the free tyrosol in SHR-5 but also from other precursors present in SHR-5.

The above results indicate that the biotransformation of rhodioloside occurs within the first 2 h following administration of the compound either in pure form or as SHR-5 but, when administered as a component of the extract, its metabolism takes place more slowly because of the presence of other compounds such as rosavine, rosarine, rosine etc, which compete for the metabolising enzymes and hence decrease the rate of formation of tyrosol from rhodioloside.

It can be seen from Fig 7 that the maximum concentration of tyrosol in blood plasma was observed 1 h after multiple administration of SHR-5 at doses of 20 and 50 mg/kg: after 1-1.5 h the concentration fell sharply and then decreased exponentially. Whilst within the first 2 h following the fifth administration of SHR-5 the concentration of tyrosol in blood was significantly higher than the concentration of rhodioloside, the levels of the two analytes in blood plasma became almost the same at longer time periods.

These results indicate that the rate of biotransformation of rhodioloside is practically the same following single or multiple regimes of administration of the drug, and that component of SHR-5 cannot induce the activation of metabolising enzymes. These results also help to explain why

there are no statistically significant differences in the pharmacological effects of rhodioloside and tyrosol, such as in the increase of proteolytic activity of muscles or in alterations of levels of ATP, ADP, AMP etc, after exhausted physical loading (Saratikov, 1973).

The low bioavailability of rosavin (20 - 26%) means that the native compound cannot be detected in blood plasma after multiple administration of SHR-5 at a dose of 20 mg/kg. This suggests that rosavin is rapidly metabolised, perhaps during the first passage through the liver. The low bioavailability of rosavin may also explain why some effects of this compound are observed only when it is administrated in very high doses (50 mg/kg in mice) and not with lower doses (3 and 10 mg/kg in mice) corresponding to single therapeutic and daily human doses of SHR-5 (Sokolov *et al.*, 1990).

The pharmacokinetics of rosavin in humans is quite different to that in rats. For example, both t_{max} and elimination rate are longer in humans. A similar tendency is also seen in the pharmacokinetics of rhodioloside, where it is better available in the blood of patients than rosavin. For example, the ratio of $AUC_{0-\infty}$ /dose is nearly twice as high for rhodioloside, in comparison to rosavin (Table 5). As a matter of fact, the ratio of elimination half times has the exact same value of 1.8 (Table 5). This clearly indicates that there is a higher, and thus longer-lasting, concentration of rhodiolside in the blood, in comparison to rosavin. These data favours rhodioloside as the major biologically active constituent of Rhodiola SHR-5 extract. It is noteworthy to mention the beneficial effect of *Rhodiola rosea* on mental performance in humans, which was observed one h after oral administration and lasted for more than three h. During this time period, it was observed that the concentration of rhodioloside in human blood was about 587 ± 102 ng/mL (after 1 h) and 483 ± 102 ng/mL (after 4 h) (Fig 8).

ACKNOWLEDGEMENTS

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Metabolism and Pharmacokinetic Studies of Coumarins

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ABSTRACT

Coumarin is a naturally occurring compound found in a wide variety of plants, microorganisms and in some animal species, with multiple uses. The metabolism, toxicity and results of tests for carcinogenicity of coumarins present in foodstuffs and cosmetic products have been reviewed with respect to safety for humans. Coumarin is a natural product, which exhibits marked species differences in both metabolism and toxicity. Coumarin is metabolized mainly by two important pathways: 7-hydroxylation and metabolism of the lactone ring which involves ring opening and the cleavage carbon atom 2 to yield CO_2 (also known as 3,4-epoxidation). While 7-hydroxylation is the major pathway of coumarin in most subjects, humans can also metabolize coumarin by the 3,4-epoxidation and possibly other pathways to various other metabolites. In contrast, the major route of coumarin metabolism in the rat and mouse is by 3,4-epoxidation pathway resulting in the formation of toxic metabolites. These Phase I metabolites are further conjugated either with glucuronate, sulfate, acetate and amino acids (Phase II reactions). Coumarin metabolism is catalyzed by various cytochrome P450 (CYP) isoforms in humans and other species, thus showing marked species differences. There is an extensive literature available on the disposition and metabolism of coumarin in a range of animal species and humans. Both in vitro and in vivo metabolic studies have been performed. Studies have shown that coumarins are metabolized in the body by hepatic as well as extra-hepatic organs. In one of our in vitro research study, two coumarin

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derivatives, 7-ethoxy coumarin (7-EC) and 7-hydroxy coumarin (7-HC) showed significant degradation in rat and mice hepatic as well as extra-hepatic (intestine, kidneys, lungs and brain) subcellular fractions, thus showing the relative contribution of hepatic and extra-hepatic metabolism to total clearance of both drugs, being remarkably high, ranging from 62% to 77% and 22% to 38% respectively of the total metabolic clearance in rats. The majority of tests for mutagenic and genotoxic potential suggest that coumarin is not a genotoxic agent. The target organs for toxicity and carcinogenicity in the rat and mouse are primarily the liver and lung. Moreover, the dose-response relationships for coumarin-induced toxicity and carcinogenicity are non-linear, with tumour formation only being observed at high doses which are associated with hepatic and pulmonary toxicity. The maximum daily human exposure to coumarin from dietary sources for a 60 kg consumer has been estimated to be 0.02 mg/kg/day. From fragrance use in cosmetic products, coumarin exposure has been estimated to be 0.04 mg/kg/day. The total daily human exposure from dietary sources together with fragrance use in cosmetic products is thus 0.06 mg/kg/day. No adverse effects of coumarin have been reported in susceptible species in response to doses, which are more than 100 times the maximum human daily intake. The mechanism of coumarin-induced tumour formation in rodents is associated with metabolism-mediated, toxicity and it is concluded that exposure to coumarin from food and/or cosmetic products poses no health risk to humans.

Key words : Coumarin, metabolism, pharmacokinetics, genotoxicity, carcinogenicity, species differences

INTRODUCTION

Coumarin (1,2-benzopyrone; coumarinic anhydride) is a white crystalline solid (molecular weight 146.15, melting point 68-70°C, boiling point 297-299°C), freely soluble in ethanol, chloroform, diethyl ether and oils, but slightly soluble in water (Cohen, 1979). The majority of coumarin used commercially is synthesized from salicylaldehyde, although high-grade coumarin is still isolated from tonka beans (DeGarmo & Raizman, 1967; Hawley, 1971).

As described below, coumarin exhibits marked species differences in both metabolism and toxicity. The objective of this review which incorporates our own research findings on Drug Metabolism and Pharmacokinetics (DMPK) aspects of two coumarin derivatives is to evaluate data on coumarin metabolism. Further, a general discussion has been made on toxicities with respect to the hazards caused by this compound in humans, arising from its presence in food and fragrance products.

Uses of Coumarin

Coumarin is used as a fixative and enhancing agent in perfumes and it is also used as one of the ingredients in toilet soap, detergents, toothpaste, tobacco products and some alcoholic beverages (Cohen, 1979; Opdyke, 1974). Large quantities of coumarin are also used in rubber and plastic materials and in paints and sprays to mask unpleasant odours (Fentem & Fry, 1993).

Coumarin has also been used in clinical medicine because of its ability to activate macrophages that underlies its use for the treatment of high protein oedema and its immunomodulatory properties used in the treatment of brucellosis (Egan *et al.*, 1990). The use of coumarin as a food flavour was banned in USA in 1954 based on reports of hepatotoxicity in rats, prior to the existence of any carcinogenicity and mutagenicity data and was recommended for withdrawal from use in the UK in 1965 (Cohen, 1979; Opdyke, 1974).

Occurrence and Human Exposure

Coumarin is a naturally occurring compound, found in a wide variety of plants, microorganisms and in some animal species (Feuer, 1974; Soine, 1964). It was first isolated from tonka beans, and is found at high levels in some essential oils, particularly cinnamon bark oil (7000 ppm), cassia leaf oil (up to 87,300 ppm), peppermint oil (20 ppm) and lavender oil. Coumarin is also found in fruits (*e.g.* bilberry, cloudberry), green tea and other foods, such as chicory (TNO, 1996). Many coumarin derivatives have also been found in plants, being present in free state and as glucosides.

Humans are exposed to coumarin from food, beverages, caramel confectionery, chewing gum and alcoholic beverages. They are also exposed to coumarin from fragrance use in cosmetic products (Cohen, 1979; Opdyke, 1974). Such products include antiperspirant deodorants, bath products, body lotions, face creams, fragrance creams, hair sprays, shampoos, shower gels and toilet soaps. Overall, a realistic total daily human exposure to coumarin from the diet and from fragrance use in cosmetic products would be 0.06 mg/kg/day.

Pharmacokinetics of Coumarin

Extensive literature exists on the disposition and metabolism of coumarin in a range of animal species and humans. Both *in vitro* and *in vivo* metabolic studies have been performed in animal species.

Absorption, Distribution and Excretion

Following oral administration, it was found that coumarin was rapidly absorbed from the gastrointestinal tract and distributed throughout the body (Cohen, 1979; Fentem & Fry, 1993; Pelkonen *et al.*, 1997). The compound appears to be extensively metabolized in all species with little

unchanged coumarin being excreted. No evidence of significant tissue accumulation of coumarin and/or coumarin metabolites was obtained after oral administration to rats and rabbits or intraperitoneal (ip) administration to rats (Kaighen & Williams, 1961; van Sumere & Teuchy, 1971). Important quantitative differences exist between species in the routes of elimination of coumarin metabolites. The majority of studies have demonstrated a relatively large amount of biliary excretion in the rat with an appreciable proportion of the dose being excreted in the faeces. After a 50 mg/kg oral or ip dose of coumarin to rats some 50% of the dose was excreted in the bile as unknown metabolites within 24 h (Williams *et al.*, 1965).

The urine appears to be the major route of coumarin metabolite excretion in species such as the Syrian hamster, rabbit and baboon. The rapid excretion of coumarin, primarily as 7-hydroxycoumarin (7-HC), in the urine of human subjects given coumarin suggests that there is little or no biliary excretion of coumarin metabolites in humans (Shilling *et al.*, 1969).

The pharmacokinetics of coumarin has been studied in a number of species including the rat (Hardt & Ritschel, 1983; Piller, 1977; Ritschel & Hussain, 1988), dog (Ritschel & Grummich, 1981), gerbil (Ritschel & Hardt, 1983), rhesus monkey (Ritschel *et al.*, 1988) and in man. The elimination half-life ($t_{1/2}$) of coumarin was found to be similar in all species examined, being around 1-2 h in humans and between 1 and 4 h in other species.

In the rat, following an ip dose between 2.5 and 60 mg/kg, coumarin was rapidly eliminated from whole blood with an elimination half-life varying from 1 to 3 h (Hardt & Ritschel, 1983). In a later study conducted in rat, it was found that coumarin was rapidly eliminated from the systemic circulation ($t_{1/2}$ less than 1 h) after an intravenous (iv) dose of 1 mg/kg and 20% of an oral dose of coumarin (1 mg/kg) was absorbed intact due to extensive first-pass metabolism (Ritschel & Hussain, 1988). In the gerbil, coumarin at an ip dose of 40 mg/kg, was rapidly absorbed into the blood, and eliminated with a half-life of approximately 1 h (Ritschel & Hardt, 1983), whereas in the dog, the elimination of coumarin following an iv dose of 0.25 or 0.5 mg/kg was slower, the $t_{1/2}$ being 2.7 and 3.7 h, respectively (Ritschel & Grummich, 1981). In contrast to the rat, approximately 45% of an oral dose of coumarin of 1 mg/kg was absorbed intact in the dog. The pharmacokinetics of coumarin in the rhesus monkey were found to be similar to that in the dog (Ritschel *et al.*, 1988), with oral bioavailability approximately 45% after an oral dose of 1 mg/kg and elimination half-life approximately 2 h.

Pharmacokinetic studies in humans have demonstrated that coumarin is completely absorbed from the gastrointestinal tract after oral administration and extensively metabolized by the liver in the first-pass, with only between 2 and 6% reaching the systemic circulation intact

(Ritschel *et al.*, 1977, 1979; Ritschel & Hoffman, 1981). The elimination of coumarin from the systemic circulation is rapid, the half-lives following iv doses of 0.125, 0.2 and 0.25 mg/kg being 1.82, 1.46 and 1.49 h, respectively (Ritschel *et al.*, 1976).

Coumarin Metabolism Pathways

The metabolism of coumarin has been studied *in vivo* and *in vitro* in a wide range of species including humans. *In vitro* models employed include tissue slices, hepatocytes, subcellular fractions, and purified and c-DNA-expressed enzymes. Coumarin may be metabolized by hydroxylation at all six possible positions (*i.e.* carbon atoms 3, 4, 5, 6, 7 and 8) to yield 3-, 4-, 5-, 6-, 7- and 8-hydroxycoumarins (3-, 4-, 5-, 6-, 7- and 8-HCs) and by opening of the lactone ring to yield various products including o-hydroxyphenylacetaldehyde (o-HPA), o-hydroxyphenylethanol (o-HPE), o-hydroxyphenylacetic acid (o-HPAA) and o-hydroxyphenyllactic acid (o-HPLA). Additional metabolites of coumarin include 6,7-dihydroxycoumarin (6,7-diHC), o-coumaric acid (o-CA), o-hydroxyphenylpropionic acid (o-HPPA) and dihydrocoumarin (DHC). The known pathways of coumarin metabolism are summarized in Fig 1.

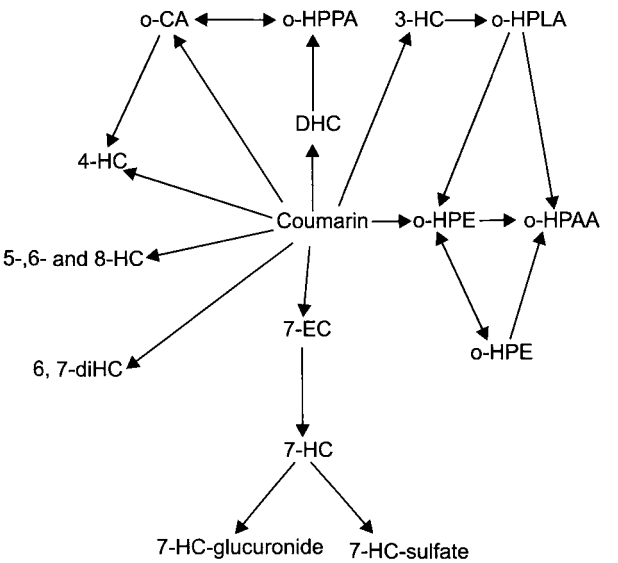

Fig 1. Some pathways of coumarin metabolism (Born *et al.*, 1997c; Cohen, 1979; Fentem *et al.*, 1991a; Lake *et al.*, 1992a, b; Norman & Wood, 1984). Abbreviations used are o-coumaric acid (o-CA), dihydrocoumarin (DHC), 6,7-dihydroxycoumarin (6,7-diHC), hydroxycoumarin (HC), o-hydroxyphenylacetaldehyde (o-HPA), o-hydroxyphenylethanol (o-HPE), o-hydroxyphenylaceticacid (o-HPAA), o-hydroxyphenyllactic acid (o-HPLA), o-hydroxyphenylpropionic acid (o-HPPA)

The two important pathways of coumarin metabolism are 7-hydroxylation and metabolism of the lactone ring which involves ring

opening and the cleavage of carbon atom 2 to yield carbon dioxide. The latter pathway is often referred to as the "3-hydroxylation" pathway of coumarin metabolism (Cohen, 1979; Fentem & Fry, 1993; Kaighen & Williams, 1961; Lake *et al.*, 1989a; Pelkonen *et al.*, 1997).

In vivo Metabolism Studies

Early studies into the *in vivo* metabolism of coumarin were conducted by Booth *et al.* (1959) and by Mead *et al.* (1958a, b) using unlabelled material. Booth *et al.* (1959) reported that coumarin was metabolized to o-HPAA in both the rat and rabbit, whereas only the rabbit appeared to excrete appreciable quantities of 3-HC and 7-HC. Mead *et al.* (1958a) observed that coumarin was converted to 3-HC, 7-HC and 8-HC in the rat and rabbit, with 3-HC, 5-HC, 7-HC and 8-HC being formed in the mouse, guinea pig and ferret. Additional studies with 3-HC, 4-HC, 5-HC, 6-HC, 7-HC and 8-HC in the rabbit demonstrated that all six hydroxycoumarins were excreted in the urine as glucuronide conjugates and that all except 4-HC were also excreted as sulfate conjugates (Mead *et al.*, 1958b). Generally, studies with 7-HC in several other species (Cohen, 1979) have demonstrated that if formed, this coumarin metabolite is extensively conjugated with either D-glucuronic acid and/or sulfate.

Coumarin was also extensively metabolized in the rat with only small amounts of unchanged coumarin being detected in the excreta. Various metabolites including o-HPAA were detected in the faeces, with urinary 3-HC, 7-HC, o-HPLA and o-HPAA accounting for 1.8, 0.4, 0.8 and 20% of the dose, respectively (Kaighen & Williams, 1961). The metabolism of [2-^{14}C] coumarin in the rat administered by the ip route was studied by van Sumere and Teuchy (1971). In this study a large percentage of the label was excreted in the expired air (*i.e.* 0as $^{14}CO_2$), because carbon atom 2 is removed when the lactone ring is opened to form o-HPA and subsequent metabolites (see Fig 2). Analysis of the urine revealed a number of coumarin metabolites including 5-HC, 7-HC, 8-HC, o-CA, o-HPAA and o-HPAA (van Sumere & Teuchy, 1971). Clear species differences in the extent of coumarin metabolism to o-HPAA have been reported. For example, o-HPAA appears to be a major urinary metabolite in the marmoset, but only a minor metabolite in the baboon (Lake *et al.*, 1989b; Waller & Chasseaud, 1981). Apart from glucuronide and sulfate conjugates of hydroxycoumarins, some other phase II coumarin metabolites have been identified. For example, o-CA may be conjugated with glycine (Feuer, 1974).

There is some evidence that coumarin may be metabolized by the gastrointestinal microflora (Cohen, 1979). For example, Scheline (1968) reported that rat caecal microflora could convert coumarin to DHC and o-HPPA.

Because of the relative ease of measurement of 7-HC, many *in vivo* studies have examined this pathway of coumarin metabolism and marked species differences have been reported. Overall, several species including the rat, most mouse strains, Syrian hamster, guinea pig, ferret, dog, marmoset and squirrel monkey appear to be poor 7-hydroxylators of coumarin excreting 5% or less of the administered dose as urinary 7-HC. Certain mouse strains, such as DBA/2 and 129/Rr strains, which have relatively high hepatic coumarin 7-hydroxylase activity, excrete up to 26% of an ip administered dose of coumarin as 7-HC (Lush & Andrews, 1978). Species such as the rabbit, cat and pig have been reported to excrete 12-19% of the dose as urinary 7-HC (Gangolli *et al.*, 1974; Kaighen & Williams, 1961), whereas in two studies the baboon, an Old World primate, has been reported to be an extensive 7-hydroxylator of coumarin with 60-66% of the dose being rapidly excreted in the urine as 7-HC (Gangolli *et al.*, 1974; Waller & Chasseaud, 1981).

In the majority of human subjects studied coumarin is extensively metabolised to 7-HC. The measurement of urinary 7-HC following an oral dose of coumarin has been employed as a biomarker of human hepatic CYP2A6, the cytochrome P-450 (CYP) isoform which is responsible for coumarin 7-hydroxylation in human liver. While 7-hydroxylation is the major metabolic pathway of coumarin in most subjects, humans can also metabolise coumarin by the 3,4-epoxidation and possibly other pathways to o-HPAA. In a dermal administration study conducted in three subjects the 0-12 h urine contained 51% of the dose (range 44-57%) as total (free and conjugated) 7-HC, together with 1% (range 0-1.5%) of the dose as o-HPAA and 2.8% (range 2.1-3.5%) of the dose as other unknown metabolites (Huntingdon Life Sciences, 1996a).

The marked inter-individual variation in coumarin metabolism to 7-HC has led to studies to evaluate whether a genetic polymorphism exists in human CYP2A6. Work by Fernandez-Salguero *et al.* (1995) demonstrated variant alleles in the human CYP2A6 gene. Three alleles were identified namely the wild type, CYP2A6v1 and CYP2A6v2, which are now designated CYP2A6*1, CYP2A6*2 and CYP2A6*3, respectively (Gullstén *et al.*, 1997; Hadidi *et al.*, 1997). The relationship between CYP2A6 gene polymorphism and risk of liver cancer and cirrhosis has been recently investigated (Gullstén *et al.*, 1997). CYP2A6 function *in vivo*, as assessed by the urinary 7-HC excretion test, is impaired in patients with alcoholic liver disease (Sotaniemi *et al.*, 1995) and in both adults and children by hepatitis A infection (Pasanen *et al.*, 1997). In contrast, compared with normal subjects, urinary 7-HC excretion is enchanced in epileptic patients (Sotaniemi *et al.*, 1995). Such data suggest that anticonvulsant drugs such as carbamazepine and phenobarbitone can induce levels of hepatic CYP2A6 *in vivo*.

In vitro Metabolism Studies

Early studies demonstrated that coumarin was metabolized in rat liver microsomes by CYP-dependent pathways and that a number of metabolites were formed including 3-HC, o-HPLA and o-HPAA (Feuer 1970; Gibbs *et al.*, 1971). Depending on the coumarin substrate concentration employed, o-HPA is a major metabolite of coumarin in liver microsomes from several species (Fentem & Fry, 1992; Fentem *et al.*, 1991a; Lake *et al.*, 1992a, b; Peters *et al.*, 1991). Other coumarin metabolites that may be observed in incubations with liver microsomes include 4-HC, 5-HC, 6-HC, 7-HC, 8-HC, 6,7-diHC, o-CA and o-HPPA. Depending on the coumarin substrate concentration employed, o-HPA is a major metabolite of coumarin in liver microsomes from several species (Fentem & Fry, 1992; Fentem *et al.*, 1991a; Lake *et al.*, 1992a, b; Peters *et al.*, 1991). Other coumarin metabolites that may be observed in incubations with liver microsomes include 4-HC, 5-HC, 6-HC, 7-HC, 8-HC, 6,7-diHC, o-CA and o-HPPA.

Many studies have examined coumarin 7-hydroxylase activity, determined by fluorimetric or other procedures, in hepatic microsomal preparations (Cohen, 1979; Fentem & Fry, 1993; Pelkonen *et al.*, 1993, 1997). Rat liver microsomes have little or no coumarin 7-hydroxylase activity (Bogan *et al.*, 1996; Bullock *et al.*, 1998; Creaven *et al.*, 1965; Dominguez *et al.*, 1990; Fentem & Fry, 1991, 1992; Lake *et al.*, 1989b, 1992a; Pearce *et al.*, 1992; Pelkonen *et al.*, 1985, 1993; Raunio *et al.*, 1988b; Walters *et al.*, 1980). In contrast, coumarin 7-hydroxylase activity is readily detectable in liver microsomes from other species including the mouse, gerbil, guinea pig, rabbit and human (Cohen, 1979; Fentem & Fry, 1993; Pelkonen *et al.*, 1993, 1997).

In keeping with *in vivo* studies, marked strain differences have been reported in mouse hepatic microsomal coumarin 7-hydroxylase activity. Negishi *et al.* (1989) observed higher enzyme activity in female than in male 129/J strain mice.Other studies have also demonstrated both sex and strain differences in mouse coumarin 7-hydroxylase, with enzyme activity in high activity strains, such as DBA/2 and 129, being greater in female than in male mice (Lovell *et al.*, 1999; van Iersel *et al.*, 1994a).

The CYP isoform responsible for coumarin 7-hydroxylation in mouse liver is CYP2A5 (Chang & Waxman, 1996; Honkakoski & Negishi, 1997; Pearce *et al.*, 1992; Pelkonen *et al.*, 1993, 1997). In line with the sex difference in coumarin 7-hydroxylase activity, the expression of CYP2A5 is female predominant in mouse liver (Chang & Waxman, 1996; Honkakoski & Negishi, 1997; Pearce *et al.*, 1992; Squires & Negishi, 1990). Like mouse CYP2A5 and human CYP2A6, but unlike rat CYP2A1 and CYP2A2 and mouse CYP2A4, Syrian hamster CYP2A8, rabbit CYP2A10 and CYP2A isoforms from the baboon and cynomolgus monkey are all able to efficiently

catalyse the 7-hydroxylation of coumarin (Chang & Waxman, 1996; Honkakoski & Negishi, 1997).

While much attention has focused on the measurement of coumarin 7-hydroxylase activity in liver microsomes, it should be noted that this is only a minor pathway of coumarin metabolism in many species. Coumarin 7-hydroxylation also only represents a fraction of total coumarin metabolism in gerbil liver microsomes (Fentem & Fry, 1992). Moreover, in agreement with *in vivo* data (Lush & Andrews, 1978), coumarin 7-hydroxylation is not the major pathway of coumarin metabolism even in mouse strains with relatively high coumarin 7-hydroxylase activity (Lovell *et al.*, 1999).

Many studies have investigated coumarin 7-hydroxylase activity in human liver microsomes (Chang & Waxman, 1996; Forrester *et al.*, 1992; Kratz, 1976; Maurice *et al.*, 1991; Miles *et al.*, 1990; Pearce *et al.*, 1992; Pelkonen *et al.*, 1993, 1997; Shimada *et al.*, 1996; van Iersel *et al.*, 1994b; Wrighton & Stevens, 1992; Yamano *et al.*, 1990; Yun *et al.*, 1991). The major cytochrome P-450 isoform responsible for coumarin 7-hydroxylation in human liver is CYP2A6 (Chang & Waxman, 1996; Honkakoski & Negishi, 1997; Pelkonen *et al.*, 1993, 1997), whereas another CYP2A sub-family isoform present in human liver (Nelson *et al.*, 1996), namely CYP2A7, does not possess coumarin 7-hydroxylase activity (Ding *et al.*, 1995; Yamano *et al.*, 1990).

Compared to some other CYP isoforms, such as CYP1A2, CYP2C and CYP3A isoforms, levels of CYP2A6 are relatively low in human liver (Shimada *et al.*, 1994, 1996; Wrighton & Stevens, 1992). In one study with liver microsomes from 30 Caucasian and 30 Japanese subjects, CYP2A6 was reported to be 4.0% of the total cytochrome P-450 content (Shimada *et al.*, 1994). Unlike mouse liver CYP2A5, there appears to be a lack of any sex difference in levels of CYP2A6 in human liver (Chang & Waxman, 1996). Other studies suggest that CYP2A6 may be induced by various agents and also inhibited by known inhibitors of CYP isoforms (Chang & Waxman, 1996). For example, levels of CYP2A6 in cultured human hepatocytes are increased by treatment with phenobarbitone, dexamethasone and rifampicin, but not by b-naphthoflavone and pyrazole (Chang & Waxman, 1996; Dalet-Beluche *et al.*, 1992; Maurice *et al.*, 1991).

Many studies have demonstrated marked interindividual variation in levels of CYP2A6 protein, mRNA and associated coumarin 7-hydroxylase activity in human liver microsomes (Chang & Waxman, 1996; Forrester *et al.*, 1992; Maurice *et al.*, 1991; Miles *et al.*, 1990; Pearce *et al.*, 1992; Pelkonen *et al.*, 1993, 1997; Shimada *et al.*, 1996; van Iersel *et al.*, 1994b; Wrighton & Stevens, 1992; Yamano *et al.*, 1990; Yun *et al.*, 1991). While most studies with human liver microsomes have focused on 7-hydroxylation, the formation of other coumarin metabolites, including products of the 3,4-epoxidation and other pathway(s) (*i.e.* 3-HC, o-HPA, o-HPE and o-HPAA),

4-HC, 5-HC, 6-HC, 8-HC, 6,7-diHC, o-CA and o-HPPA have been observed (Fentem & Fry, 1992; Lake *et al.*, 1992a; van Iersel *et al.*, 1994b).

Apart from subcellular fractions the *in vitro* metabolism of coumarin has also been studied in hepatocytes and precision-cut liver slices from various species, and in precision-cut rat lung slices (den Besten *et al.*, 1990; Donato *et al.*, 1998; Lake *et al.*, 1995; Price *et al.*, 1995; Ratanasavanh *et al.*, 1996; Steensma *et al.*, 1994). Generally, the species differences in coumarin metabolism observed in hepatocytes and liver slices are in agreement with available literature data from *in vivo* studies and from investigations with liver microsomes.

In our research study, two coumarin derivatives, 7-ethoxy coumarin (7-EC) and 7-hydroxy coumarin (7-HC) were chosen as model compounds to study hepatic and extra-hepatic (intestine, kidneys, lungs and brain) metabolism in mice and rat tissue subcellular (S9 and microsomal) fractions and to scale the observed *in vitro* clearance to *in vivo* plasma clearance in rats. It was found that, both drugs showed significant metabolic degradation in rat liver subcellular fractions as compared to subcellular fractions obtained from intestine, kidney, lung and brain. The total *in vitro* metabolic clearance for 7-EC and 7-HC was determined by adding the individual *in vitro* organ clearance values obtained in hepatic and extra-hepatic microsomes or S9 fractions. The predicted *in vivo* clearance for 7-HC was 63.10 and 81.35 mL/min/kg by *in vitro* scaling from microsomes and S9 fractions, respectively. For 7-EC, the values were 78.36 and 76.72 mL/min/kg, respectively. The predicted clearance was found to be reasonably accurate with slight over and underprediction. Interestingly, the relative contribution of hepatic and extra-hepatic metabolism to total clearance of 7-EC and 7-HC was remarkably high, ranging from 62% to 77% and 22% to 38%, respectively, of the total metabolic clearance. It is concluded that, the model of multi-organ subcellular fractions is a useful *in vitro* tool for prediction of *in vivo* metabolic clearance, as it can provide information about the relative contribution of extra-hepatic metabolism in addition to hepatic metabolism to the total metabolic clearance (Behera *et al.*, 2008).

Genotoxicity Studies

A number of studies have examined the mutagenic and genotoxic potential of coumarin. Overall, the data suggest that coumarin is not a genotoxic agent. No evidence for coumarin-induced genotoxicity has been observed in *in vivo* studies (*Drosophila melanogaster* and mouse micronucleus tests). The findings from these *in vivo* studies are supported by data from some, but not all, of the *in vitro* studies. In *in vivo* studies coumarin did not induce sex-linked recessive lethal mutations in germ cells of male D. melanogaster exposed as adults either by feeding or by injection (NTP, 1993a; Yoon *et al.*, 1985), or as larvae by feeding (NTP, 1993a; Valencia *et al.*, 1989). The administration of 65 and 130 mg/kg/day coumarin by gavage

for 7 days did not induce micronuclei in the bone marrow of male and female immunocompromised (ICR) mice (Morris & Ward, 1992).

A number of *in vitro* genotoxicity studies have been conducted with coumarin. Recently the effect of coumarin on unscheduled DNA synthesis (UDS) in cultured precision-cut human liver slices has been evaluated. Previous work has demonstrated that like hepatocytes, cultured liver slices may also be employed to screen xenobiotics for effects on UDS (Lake *et al.*, 1996). No evidence for coumarin-induced UDS was obtained in experiments with cultured liver slices from four subjects employing coumarin concentrations of up to 5 mm (Beamand *et al.*, 1998). Some cytotoxicity was observed at high coumarin concentrations and the functional viability of the human liver slice preparations was confirmed by parallel studies with known genotoxins. Coumarin has also been reported not to induce UDS in a rat tracheal epithelium culture system (Ide *et al.*, 1981). No evidence of mutagenicity was obtained when coumarin was examined in *S. typhimurium* strains TA98, TA1535, TA1537 and TA1538, both with or without metabolic activation according to Rhodia studies.

The ability of coumarin to induce sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary (CHO) cells has been investigated for the NTP (Galloway *et al.*, 1987). Like certain other natural products, coumarin can have antimutagenic properties (Brown *et al.*, 1981; Edenharder & Tang, 1997; Grigg, 1977; Imanishi *et al.*, 1990; Sanyal *et al.*, 1997; Sasaki *et al.*, 1987, 1990). Coumarin has been reported to induce micronuclei in HepG2 cells at high (500 mg/mL) concentrations, but to protect against the effects of known chemical mutagens (namely 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and other cooked food mutagens) at low (≤5 μg/mL) concentrations (Sanyal *et al.*, 1997). Coumarin has also been shown to suppress UV- and X-ray-induced 6-thioguanine resistant mutations in Chinese hamster V79 cells (Imanishi *et al.*, 1990). Brown *et al.* (1981) reported that coumarin decreased the mutagenicity of benzo[a]pyrene in the Ames test, whereas Edenharder and Tang (1997) demonstrated that coumarin could inhibit the mutagenicity of 1-nitro-pyrene and 3-nitrofluoranthene. Other studies have shown that coumarin can bind to DNA and interact with excision repair processes (Grigg, 1977).

Genotoxicity studies have also been conducted on o-HPAA and 7-HC (*i.e.* the major metabolites of coumarin in the rat and human, respectively). Neither o-HPAA nor 7-HC at concentrations up to 5000 mg/plate showed any evidence of mutagenicity in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 either with or without metabolic activation (Microbiological Associates, 1994a). Similarly, neither o-HPAA nor 7-HC at concentrations of up to 150 and 50 mg/mL, respectively, induced unscheduled DNA synthesis in cultured rat hepatocytes (Microbiological Associates, 1994b). Higher concentrations (≤5000 μg/mL) of o-HPAA and

7-HC were cytotoxic to rat hepatocytes. In another study, o-HPAA at concentrations of up to 5000 mg/mL was reported not to produce chromosomal aberrations in CHO cells both with and without metabolic activation (Microbiological Associates, 1993a). However, a weak positive response was obtained in this test with 7-HC, but only at high, toxic concentrations and in the absence of metabolic activation (Microbiological Associates, 1993b).

Toxicity and Carcinogenicity Studies

A number of studies have examined the acute, chronic and carcinogenic effects of coumarin in the rat and mouse. In the rat, single oral or ip doses (*e.g.* 125-500 mg/kg) of coumarin result in a rapid depletion of hepatic non-protein sulfydryl groups [primarily reduced glutathione (GSH)], and after 24 h centrilobular hepatic necrosis and an elevation of plasma alanine and aspartate aminotransferase activities (Fentem *et al.*, 1992a; Lake, 1984; Lake & Evans, 1993; Lake *et al.*, 1989a, 1994a). Depending on the dose administered, coumarin treatment results in an increase in relative liver weight and changes in various hepatic biochemical parameters. Levels of total microsomal CYP and associated mixed-function oxidase enzymes tend to be reduced by coumarin administration especially after short periods of treatment. Microsomal glucose-6-phosphatase activity is also reduced. In contrast, coumarin administration leads to a sustained increase in non-protein sulfydryl groups and certain enzyme activities such as GSH S-transferase and gamma-glutamyltransferase. Coumarin-induced lysosomal changes in rat liver have been demonstrated by histochemical and ultrastructural techniques (Grasso *et al.*, 1974).

Morphological examination of liver sections from coumarin-treated rats reveals a variety of changes, which are dependent on the magnitude of the dose and the duration of treatment. Such changes include necrosis, apoptosis, vacuolation, fatty change and bile duct hyperplasia. An intriguing aspect of coumarin-induced hepatotoxicity in the rat is the shift in the site of liver damage from the centrilobular to the periportal region after acute and chronic administration, respectively. Coumarin has been reported to be hepatotoxic and nephrotoxic in the dog at an oral dose of 100 mg/kg given for periods of 8-22 days (Hagan *et al.*, 1967; Hazleton *et al.*, 1956). Morphological examination of liver sections revealed various lesions including cytoplasmic vacuolation, fatty change and necrosis. Hepatotoxic effects were also observed in dogs given 25 and 50 mg/kg coumarin for longer periods, but not in dogs given 10 mg/kg coumarin for up to 350 days (Hagan *et al.*, 1967).

A number of studies have examined the carcinogenicity of coumarin in the rat (Carlton *et al.*, 1996; Griepentrog, 1973; Hagan *et al.*, 1967; NTP, 1993a). Griepentrog (1973) reported that coumarin could produce bile duct carcinoma (cholangiocarcinoma) in rats given 0.5 and 0.6% coumarin, but

not in animals receiving 0.1% and 0.25% coumarin. Species differences in coumarin-induced toxicity *in vitro* have been investigated in cultured hepatocytes (Ratanasavanh *et al.*, 1996) and precision-cut liver slices (Price *et al.*, 1996). The treatment of male Sprague-Dawley rat hepatocytes with 0.1, 0.25 and 0.5 mm coumarin resulted in toxicity as demonstrated by morphological examination of the cultures and by lactate dehydrogenase leakage (Ratanasavanh *et al.*, 1996). Toxicity was also observed in hepatocyte cultures from male DBA/2J strain mice and rabbits, whereas with cultured human hepatocytes toxicity was only observed with 1 mm coumarin and not with coumarin concentrations of 0.5 mm or below. Coumarin has also been reported to produce toxicity in cultured gerbil hepatocytes (Fentem *et al.*, 1991b). Price *et al.* (1996) compared the toxicity of 0.5, 1 and 2 mm coumarin in 24-h cultured precision-cut male Sprague-Dawley rat, Dunkin-Hartley guinea pig, cynomolgus monkey and human liver slices. Based on the measurement of liver slice protein synthesis and potassium content coumarin produced concentration-dependent toxic effects in rat and guinea pig liver slices, whereas cynomolgus monkey and human liver slices were relatively resistant.

These studies provide evidence for species differences in coumarin-induced toxicity *in vitro*. The relative resistance of human and cynomolgus monkey liver slices and/or hepatocytes to coumarin toxicity correlates with coumarin 7-hydroxylation, the major pathway of coumarin metabolism in these species, being a detoxification pathway of coumarin metabolism.

Clinical Studies

Coumarin is being evaluated for the treatment of various clinical conditions, resulting in the employment of a variety of dosing regimens. Recommended doses range from 8 mg for the treatment of venous constriction to 7000 mg/day in antineoplastic therapies (Marshall *et al.*, 1994). While various mild side-effects have been reported following coumarin treatment, alterations to liver function have been noted in only a small proportion of patients receiving coumarin and tend to revert to normal after cessation of treatment. Reports of overt hepatotoxicity are rare (Cox *et al.*, 1989). In a study with 45 renal cell carcinoma patients receiving 100 mg coumarin daily in combination with cimetidine, Marshall *et al.* (1987b) found no evidence for liver toxicity in any patient. In other trials with similar dosing regimens, groups of 22 (Marshall *et al.*, 1989), 24 (Marshall *et al.*, 1987a), 50 (Dexeus *et al.*, 1990) and 17 (Nolte *et al.*, 1987) cancer patients showed no evidence of liver toxicity. Isolated cases of hepatotoxicity have been noted in other studies with patients receiving coumarin. Thus, Beinssen (1994) reported one possible case of hepatotoxicity due to coumarin treatment, and in another study Loprinzi *et al.* (1997) reported six cases of hepatotoxicity. Faurschou (1982) reported a case of toxic hepatitis in a patient given coumarin for 8 weeks, characterized by hepatomegaly and elevated serum

enzymes. All signs of liver toxicity returned to normal on cessation of treatment.

It is clear from the published clinical studies that there is no clear relationship between coumarin dose and hepatotoxicity. For some individuals, bio- chemical signs of hepatotoxicity, such as elevated serum transaminases, return to normal despite continued treatment and in others return to normal after cessation of treatment. It is possible that the polymorphism in the human population with respect to CYP2A6 enzyme activity may account for the differential effects seen. However, no link between coumarin-induced hepatotoxicity and 7-hydroxylation status has yet been established.

Assessment of Risk to Humans

The European Union Scientific Committee for Food has published an opinion on the levels of coumarin in natural flavouring source materials (Scientific Committee for Food, 1997). The limits for coumarin were less than 2 mg/kg in food and beverages with specific exceptions of 10 mg/kg in "special" caramels and in alcoholic beverages. While the Committee considered that further research on coumarin is desirable, it has recommended that the general limit for coumarin in food and beverages (due to the presence of natural source materials containing coumarin) should be lowered to the currently achievable limit of detection of 0.5 mg/kg.

In order to assess the safety of coumarin from dietary sources and from fragrance use in cosmetic products for human health, it is necessary to consider the likely human exposure and in view of the marked species differences in coumarin metabolism and toxicity, the relevance of the rodent toxicity data to humans. Based on food intake data, with coumarin containing foods only accounting for 5% of total solid foodstuffs, the maximum daily intake for a 60-kg consumer would be 1.235 mg coumarin/ day or 0.02 mg/kg/day. In addition, from fragrance use in cosmetic products the realistic daily exposure would be 2.289 mg/kg/day or 0.04 mg/kg/day for a 60-kg consumer. Both of these figs assume that all coumarin would be absorbed after either oral or dermal exposure and would be available to the systemic circulation. The total daily human coumarin exposure from the diet and from fragrance use in cosmetic products would thus be 0.06 mg/kg/day. This exposure level is over 2000 and over 3000 times lower, respectively, than those which produce liver tumours in rats (Carlton *et al.*, 1996) and lung tumours in mice (NTP, 1993a).

Unlike the rat and mouse, where high doses of coumarin can produce toxicity and carcinogenicity, there is little evidence of coumarin-induced toxicity in humans given therapeutic doses of coumarin which are up to 1900 times higher than those obtained from dietary sources and from fragrance use in cosmetic products. Such species differences are attributable

to differences in coumarin biotransformation and disposition. Unlike the rat and mouse, where the 3,4-epoxidation pathway predominates, the major pathway of coumarin metabolism in humans is by 7-hydroxylation. Studies in mouse strains and the Mongolian gerbil have demonstrated that the 7-hydroxylation pathway is a detoxification pathway of coumarin metabolism. However, while coumarin 7-hydroxylation is a detoxification pathway, this does not appear to be the only explanation for resistance of a species to coumarin-induced toxicity. For example, as demonstrated by *in vitro* (Lake *et al.*, 1992a; Pearce *et al.*, 1992) and limited *in vivo* (Gangolli *et al.*, 1974; Lake *et al.*, 1990) studies, the Syrian hamster is a poor 7-hydroxylator of coumarin, yet is resistant to chronic coumarin-induced liver injury. Unlike the rat, where large amounts of a coumarin dose appear in the faeces, species such as the Syrian hamster, baboon and humans excrete coumarin metabolites primarily in the urine. Such data suggest that the rat is an inappropriate animal model for the evaluation of the safety of coumarin for humans.

Overall, 7-hydroxylation status is not the only factor responsible for determining the susceptibility of a species to coumarin-induced toxicity. While high 7-hydroxylation status appears to confer resistance to coumarin-induced toxicity, some poor 7- hydroxylators are also not susceptible. The rat, mouse (*i.e.* B6C3F1 and CD-1 strains) and Syrian hamster are all poor 7-hydroxylators of coumarin. While the rat is susceptible to coumarin-induced toxicity, the Syrian hamster is resistant and toxicity was only observed in a chronic gavage study in the mouse and not after dietary administration. Further studies on the metabolism and disposition of coumarin in these species may help clarify the precise mechanisms of coumarin-induced toxicity.

A number of studies have examined the mutagenic and genotoxic potential of coumarin. Overall, the data suggest that coumarin is not a genotoxic agent. Indeed, the non-linear dose-response relationships for coumarin-induced tumour formation in rodents are typical of those of a non-genotoxic, rather than a genotoxic, carcinogen (Weisburger, 1994).

As coumarin does not appear to be a genotoxic carcinogen in rodents, other mechanisms are required to explain why high doses of this compound can produce liver and lung tumours in some chronic studies. From the available metabolism data, coumarin is metabolized by the rat and mouse strains by the 3,4-epoxidation pathway to yield potentially toxic metabolites. *In vitro* studies have demonstrated that o-HPA, the major metabolite of coumarin in the rat and mouse, is more toxic to rat hepatocytes than the parent compound and a number of coumarin metabolites (Born *et al.*, 1998b; Lake *et al.*, 1989a). Certainly various aldehydes are known to produce toxic effects in biological systems (Feron *et al.*, 1991). For example, formaldehyde carcinogenesis is a high-dose phenomenon in which cytotoxicity plays a crucial role (Feron *et al.*, 1991). Additional studies are required to identify which metabolite(s) are responsible for coumarin-induced toxicity in rodents.

Previous studies have demonstrated that while the majority of humans metabolize coumarin extensively to 7-HC, a deficiency in this pathway has been observed in some individuals which appears to be related to a genetic polymorphism in CYP2A6. The limited *in vivo* (Hadidi *et al.*, 1997) and *in vitro* (van Iersel *et al.*, 1994a) data available suggest that such deficient individuals will metabolize coumarin by the 3,4-epoxidation and possibly other pathways leading to the formation of o-HPAA. In clinical studies occasional but reversible changes in biochemical markers of liver function have been observed in response to coumarin therapy. However, no relationship between coumarin 7-hydroxylation status and toxicity has been demonstrated in humans. In any event, considering the no-observed-effect levels (NOELs) observed in studies with experimental animals, the low level of exposure to coumarin from the diet and from fragrance use in cosmetic products would not be expected to produce any hepatotoxicity even in individuals with deficient coumarin 7-hydroxylase activity.

CONCLUSIONS

Thus, by examination of available literature data, it can be concluded that coumarin exhibits marked species differences in both metabolism and toxicity and is not a genotoxic agent. Coumarin-induced hepatotoxicity has been found in the rat, mouse and dog, but not in humans may be due to predominance of 7-hydroxylation detoxification pathway. However, 7-hydroxylation status is not the only determinant of susceptibility of a species to coumarin-induced toxicity. Evidence of liver and lung carcinogenicity in rat and mouse is probably attributable to non-genotoxic mechanisms, which only operate when there is target organ damage. Also, comparison of the NOELs for coumarin-induced toxicity and carcinogenecity with estimated human exposure from the diet and cosmetic products provides acceptable safety margins, such that coumarin exposure from these sources should pose no health risk to humans.

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Naringin and its Aglycone, Research and Managements

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ABSTRACT

The grapefruit extract components, naringin and its aglycone naringinin are commonly used as health supplements; they exert a variety of pharmacological actions. This article attempts to review their pharmacokinetics, pharmacological actions and their uses in various managements, including effect on cardiovascular system; effect on skeletal system; effect on smooth muscle; effect on gastric intestinal system; effect on endocrine system; effect against tumour; protection against toxins in chemotherapy drugs and the environment; antioxidant effect; drug interactions; antiinflammatory effect and the newly discovered osteogenic and antibacterial actions.

Key words : Naringin, naringenin, antioxidant effect, drug interactions, osteogenic effect, antibacterial effect, anti-inflammatory effect

INTRODUCTION

Naringin is a flavonoid compound found in grapefruit and other citrus fruit, which gives grapefruit its characteristic bitter flavor. Naringin is believed to enhance our perception of taste by stimulating the taste buds.

Naringin and its aglycone naringinin, are commonly used as health supplements; they exert a variety of pharmacological actions. For example,

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it may be instrumental in inhibiting cancer-causing compounds and thus may have potential chemotherapeutic value. On the other hand, studies have also shown that naringin interferes with enzymatic activity in the intestines and, thus, with the breakdown of certain drugs, resulting in higher blood levels of the drug. In order to summarize their actions on health, a MEDLINE search covering the period 1977-2007 was performed to identify review articles, studies, and case reports referencing the biological properties of naringin and naringenin. The aim/method, major results and conclusion/actions were tabulated in the following sections for easy reference:

Pharmacokinetics
Effects on cardiovascular system
Effects on skeletal system
Effects on smooth muscle
Effects on gastric intestinal system
Effects on endocrine system
Effects against tumour
Protections against toxins in chemotherapy drugs and the environment
Antioxidant effects
Antimicrobial effects
Drug interactions
Antiinflammatory effects
Other effects

RESEARCH ON NARINGIN AND NARENGININ SHOWS THE FOLLOWINGS:

Pharmacokinetics

Action/Conclusion	Aim/Method	Results
Absorbed citrus flavanones may undergo glucuronidation before urinary excretion (Ameer *et al.*, 1996).	Disposition of citrus flavonoids was evaluated after single oral doses of pure compounds (500 mg naringin and 500 mg hesperidin) and after multiple doses of combined grapefruit juice and orange juice and of once-daily grapefruit.	Cumulative urinary recovery indicated low bioavailability (<25%) of naringin and hesperidin. The aglycones naringenin and hesperidin were detected in urine and plasma.

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Action/Conclusion	Aim/Method	Results
Extensive glucuronidation/ sulfation of naringenin occurred during the first pass at gut wall (Hsiu *et al.*, 2002).	Naringenin was administered intravenously and orally to rabbits, and naringin was administered orally. The concentration of naringenin in serum prior to and after enzymatic hydrolysis was determined by HPLC method.	The absolute bioavailability of oral naringenin was only 4%, whereas after taking the conjugated naringenin into account, it increased to 8%. When naringin was administered orally, only little naringenin and predominantly its glucuronides/ sulfates were circulating in the plasma
Oral administration of the flavanone aglycones, hesperetin and naringenin, lead to their rapid absorption as their conjugated forms. The cumulative urinary recovery data indicated low bioavailability for both flavanone aglycones, owing to extensive first-pass metabolism partly by cleavage of the C-ring by the enzymes of intestinal bacteria leading to degradation products such as phenolic acids (Kanaze *et al.*, 2007).	Six healthy volunteers received orally 135 mg of each compound, hesperetin and naringenin, under fasting conditions. Blood samples were collected at 14 different time points over a 12 h period. Urine was collected over 24 h, in five sequential timed intervals.	Pharmacokinetic analysis showed that both hesperetin and naringenin were rapidly absorbed and their concentrations in plasma observed 20 min after dosing and reached a peak in 4.0 and 3.5 h, respectively. The mean peak plasma concentration (C(max)) for hesperetin and naringenin were 825.78 + /- 410.63 ng/mL (2731.8 + /- 1358.4 nmol/l) and 2009.51 + /-770.82 ng/mL (7386.6 + /- 2833.4 nmol/l), respectively and the mean AUC(0-infinity) values were 4846.20 + /- 1675.99 ng h/mL and 9424.52 + /-2960.52 ng h/mL for hesperetin and naringenin, respectively. The elimination half-life for hesperetin was found to be 3.05+/ -0.91 h and for naringenin 2.31 + /-0.40 h, respectively. The mean values of the relative cumulative urinary excretion, as percentage of the administered dose, for hesperetin and naringenin, were found to be 3.26 + /-0.44 and 5.81 + /-0.81%, respectively.

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Action/Conclusion	Aim/Method	Results
The results of the present study demonstrate the absorption of grapefruit flavanones via the presence of their metabolites in plasma (Mata-Bilbao Mde *et al.*, 2007).	Ten healthy beagles were administered 70 mg citrus flavonoids as a grapefruit extract contained in capsules, while two additional dogs were used as controls and given an excipient.	Naringin reached its maximun plasma concentration at around 80 min, whereas naringenin and naringenin glucuronide reached their maximun plasma concentrations at around 20 and 30 min, respectively. Maximum plasma concentrations of naringin, naringenin and naringenin glucuronide (medians and ranges) were 0.24 (0.05-2.08), 0.021 (0.001-0.3) and 0.09 (0.034-0.12) micromol/l, respectively. The areas under the curves were 23.16 l (14.04-70.62) min x micromol/for narininingin, 1.78 (0.09-4.95) min x micromol/l for naringenin and 22.5 (2.74-99.23) min x micromol/l for naringenin glucuronide. The median and range values for mean residence time were 3.3 (1.5-9.3), 2.8 (0.8-11.2) and 8.0 (2.3-13.1) h for naringin, naringenin and naringenin glucuronide, respectively.

Effects on cardiovascular system

Action/Conclusion	Aim/Method	Results
Ingestion of grapefruit lowers elevated hematocrits in human subjects (Robbins *et al.*, 1988).	The effect on hematocrits of adding grapefruit to the daily diet was determined using 36 human subjects (12 F, 24 M) over a 42-day study.	The hematocrits ranged from 36.5 to 55.8% at the start and 38.8% to 49.2% at the end of the study.

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Action/Conclusion	Aim/Method	Results
Plasma and hepatic cholesterol and hepatic activities of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and acyl CoA: cholesterol transferase (ACAT) are lower in rats fed citrus peel extract or a mixture of citrus bioflavonoids (Bok *et al.*, 1999).	The cholesterol-lowering effects of tangerine peel extract and a mixture of two citrus flavonoids were tested.	The inhibition of HMG-CoA reductase and ACAT activities resulting from either tangerine-peel extract or its bioflavonoids could account for the decrease in fecal neutral sterol.
Hypocholesterolemic effect of naringin associated with hepatic cholesterol regulating enzyme changes in rats (Shin *et al.*, 1999).	The effects of the citrus bioflavonoid naringin were tested by using it as a supplement in a high-cholesterol diet.	The combination of the inhibited HMG-CoA reductase (-24.4%) and ACAT (-20.2%) activities as a result of naringin supplementation could account for the decrease of fecal neutral sterols.
Anti-atherogenic effect of citrus flavonoids, naringin and naringenin (Lee *et al.*, 2001).	The anti-atherogenic effects of the citrus flavonoids, naringin and naringenin, were evaluated in high cholesterol-fed rabbits.	The anti-atherogenic effect of the citrus flavonoids, naringin and naringenin, is involved with a decreased hepatic ACAT activity and with the downregulation of VCAM-1 and MCP-1 gene expression.
Interactive effect of naringin and vitamin E on cholesterol biosynthesis (Choi *et al.*, 2001).	The interactive effect of naringin and vitamin E was studied with respect to cholesterol metabolism and antioxidant status in high-cholesterol-fed rats.	Naringin lowers the plasma lipid concentrations when the dietary vitamin E level is low. The HMG-CoA reductase-inhibitory effect of naringin was more potent when dietary vitamin E was at a normal level.
Naringin has an antiatherogenic effect with the inhibition of intercellular adhesion molecule-1 in hypercholesterolemic rabbits (Choe *et al.*, 2001).	This study evaluated the effect of naringin on blood lipid levels and aortic fatty streaks, and its action mechanism in hypercholesterolemic rabbits.	Naringin treatment inhibited hypercholesterolemia-induced intercellular adhesion molecule-1 (ICAM-1) expression on endothelial cells. Hypercholesterolemia caused fatty liver and elevation of liver enzymes, which was prevented by naringin but not by lovastatin.

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Action/Conclusion	Aim/Method	Results
Hypocholesterolemic effect of naringin and rutin flavonoids (da Silva *et al.*, 2001).	The effect of naringin and rutin on the metabolism lipidic of chicks hypercholesterolemic was evaluated.	Naringin and rutin reduced the levels of total cholesterol significantly, cholesterol-LDL, cholesterol-VLDL and triglycerols, not presenting, however, reductions in the levels of cholesterol-HDL.
Flavonoids prevent *in vitro* LDL oxidation and probably would be important to prevent atherosclerosis (Naderi *et al.*, 2003).	The susceptibility of LDL to *in vitro* oxidation was assessed. LDL oxidation were monitored by change in 234-absorbance in the presence and absence of pure flavonoids.	Genistein, morin and naringin have stronger inhibitory activity against LDL oxidation than biochanin A or apigenin.
Naringin may play an important role in lowering plasma cholesterol and regulating the antioxidant capacity in hypercholesterolemic subjects (Jung *et al.*, 2003).	The effect of naringin on hypercholesterolemic subjects was studied. A hypercholesterolemic group (n=30) and healthy control group (n=30) were established.	Naringin supplementation was found to lower the plasma total cholesterol by 14% and low-density lipoprotein cholesterol concentrations by 17%, apolipoprotein B levels were significantly lowered, erythrocyte superoxide dismutase and catalase activities were significantly increased.
Naringin lowers the plasma cholesterol level via the inhibition of hepatic HMG-CoA reductase activity and improve the activities of hepatic antioxidant enzymes against oxidative stress (Kim *et al.*, 2004).	The lipid lowering and antioxidant capacity of naringin was evaluated in LDL receptor knockout (LDLR-KO) mice fed a cholesterol (0.1 g/100 g) diet.	The hepatic HMG-CoA reductase activity was significantly lower in the naringin and lovastatin supplemented groups than in the control group, the superoxide dismutase, catalase, and glutathione reductase activities were all significantly higher in the naringin-supplemented group than in the control group.
Flavonoids that showed strong increase of ocular blood flow also showed marked increase of retinal function recovery (Chiou & Xu, 2004).	Effects of flavonoids to improve retinal function recovery after ischemic insult were studied. Electroretinography was used to measure the b-wave recovery as an indication of retinal function recovery.	Naringenin, hesperetin, and rutin were found to produce marked positive effects on b-wave recovery, whereas naringin, hesperidin, and quercetin showed poor recovery of b-wave after ischemic insult of the retina.

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Action/Conclusion	Aim/Method	Results
Reactive oxygen species (ROS) play a causal role in renal ischemia/ reperfusion (I/R) induced renal injury and naringin exert renoprotective effects probably by the radical scavenging and antioxidant activities (Singh & Chopra, 2004).	The protective effect of naringin against the damage inflicted by ROS during renal I/R was investigated in Sprague-Dawley rats using histopathological and biochemical parameters.	Pretreatment of animals with naringin markedly attenuated renal dysfunction, morphological alterations, reduced elevated TBARS levels and restored the depleted renal antioxidant enzymes.
Both naringin and lovastatin contributed to hypocholesterolemic action. Naringin seemed to preserve tissue morphology from damages induced by high cholesterol diet (Jeon *et al.*, 2004).	To confirm the hypocholesterolemic role of naringin, male rabbits were fed 0.5% high-cholesterol diet or high-cholesterol diet supplemented with either 0.05% naringin or 0.03% lovastatin for 8 weeks.	The naringin and lovastatin supplements significantly lowered plasma total- and LDL-cholesterol and hepatic lipids levels, while significantly increasing HDL-C/total-C ratio compared to the control group.
The vasorelaxant effects of (+/-)-naringenin seem to be basically related to the inhibition of phosphodiesterase (PDE)1, PDE4 and PDE5 activities (Orallo *et al.*, 2005).	The potential vasorelaxant, antioxidant and cyclic nucleotide PDE inhibitory effects of the citrus-fruit flavonoids naringin and (+/-)-naringenin were comparatively studied.	(+/-)-naringenin relaxed, in a concentration-dependent manner, the contractions elicited by phenylephrine (PHE, 1 microM) or by a high extracellular KCl concentration (60 mM) in intact rat aortic rings.
The active ingredients associated with the antihypertensive effect of sweetie juice are the flavonoids naringin and narirutin (Reshef *et al.*, 2005).	Patients with stage I hypertension, the antihypertensive effect of juice of the so-called sweetie fruit (a hybrid between grapefruit and pummelo) with and without high flavonoid content were studied.	The high-flavonoid (HF) sweetie juice was more effective than LF sweetie juice in reducing diastolic blood pressure.
Hesperidin and naringin are beneficial for improving hyperlipidemia and hyperglycemia in type-2 diabetic animals (Jung *et al.*, 2006).	The effect of the flavonoids hesperidin and naringin on glucose and lipid regulation in C57BL/KsJ-db/db mice was studied.	Hesperidin and naringin effectively lowered the plasma free fatty acid and plasma and hepatic triglyceride levels, and simultaneously reduced the hepatic fatty acid oxidation and carnitine palmitoyl transferase activity.

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Action/Conclusion	Aim/Method	Results
Naringin has a lipid-lowering effect in ISO-induced MI rats (Rajadurai & Prince, 2006).	The preventive effect of naringin in isoproterenol (ISO)-induced myocardial infarction (MI) in rats was studied.	Pretreatment with naringin significantly decreased the levels of total, ester, and free cholesterol, triglycerides, and free fatty acids in serum and heart and increased phospholipids in heart.
Naringin possess anti-lipoperoxidative and antioxidant activity in experimentally induced cardiac toxicity (Rajadurai & Prince, 2006).	The cardioprotective potential of naringin on lipid peroxides, enzymatic and nonenzymatic antioxidants and histopathological findings in isoproterenol (ISO)-induced myocardial infarction (MI) in rats were evaluated.	Oral administration of naringin to ISO-induced rats showed a significant decrease in the levels of lipid peroxidative products and improved the antioxidant status. Histopathological findings of the myocardial tissue showed the protective role of naringin in ISO-induced rats.
Naringin possess cardioprotective effect in ISO-induced MI in rats (Rajadurai & Prince, 2007).	The preventive role of naringin on cardiac troponin T (cTnT), lactate dehydrogenase (LDH)-isoenzyme, cardiac marker enzymes, electrocardiographic (ECG)-patterns and lysosomal enzymes in isoproterenol (ISO)-induced myocardial infarction (MI) in male Wistar rats were investigated.	Pretreatment with naringin positively altered the levels of cTnT, intensity of the bands of the LDH1 and LDH2-isoenzyme and the activities of cardiac marker enzymes, ECG-patterns and lysosomal hydrolases in ISO-induced rats.
Naringin time-dependently lowers hepatic cholesterol biosynthesis and plasma cholesterol in rats fed high-fat and high-cholesterol diet (Kim *et al.*, 2006).	Naringin was investigated for its differential effects on hepatic cholesterol regulation when supplemented for 3 weeks and 6 weeks in Sprague-Dawley rats.	Supplementation with naringin did not exhibit a hypolipidemic effect when given with a HFHC diet. Naringin can, however, be beneficial for lowering hepatic cholesterol biosynthesis and levels of plasma lipids in this animal model.
Naringin has cardioprotective role in ISO-induced MI in rats (Rajadurai & Prince, 2007).	The preventive role of naringin on heart weight, blood glucose, total proteins, albumin/globulin (A/G) ratio, serum uric acid, serum iron, plasma iron binding capacity and membrane bound enzymes and glycoproteins such as hexose, hexosamine, fucose and sialic acid in isoproterenol (ISO)-induced myocardial infarction (MI) in rats and *in vitro* free radical scavenging assay were studied.	Pretreatment with naringin exhibited a significant effect and altered these biochemical parameters positively in ISO-induced rats. Naringin also scavenges 1,1-diphenyl-2-picrylhydrazyl, 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) and nitric oxide radicals *in vitro*.

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Action/Conclusion	Aim/Method	Results
(Rajadurai & Prince, 2007).	The preventive role of naringin on mitochondrial enzymes in isoproterenol (ISO)-induced myocardial infarction in male albino Wistar rats was studied.	Oral pretreatment with naringin to ISO-induced rats daily for a period of 56 days significantly minimized the alterations in all the biochemical parameters and restored the normal mitochondrial function. Transmission electron microscopic observations also correlated with these biochemical findings.

Effects on skeletal system

Action/Conclusion	Aim/Method	Results
Rutin, quercetin, and naringin supplementation reduce molar crestal alveolar bone-cemento-enamel junction distance in young rats (Wood, 2004).	The influence of dietary bioflavonoid (rutin [R], quercetin [Q] and naringin [N]) supplementation on physiological molar crestal alveolar bone (CAB)-cemento-enamel junction (CEJ) distances in young male albino rats was studied.	The N group demonstrated the lowest CAB-CEJ distance, followed by the R and Q groups (P<.001-.05) except in the mandibular lingual region where the Q group had a lower CAB-CEJ distance than the N and R groups (P <.05). The control group showed the largest CAB-CEJ distances.
Naringin in collagen matrix have the effect of increasing new bone formation locally and can be used as a bone graft material (Wong & Rabie, 2006).	The amount of new bone produced by naringin in collagen matrix to that produced by bone grafts and collagen matrix in rabbits was compared.	A total of 284% and 490% more new bone was present in defects grafted with naringin in collagen matrix than those grafted with bone and collagen respectively.
Besides statin, this provided another example of HMG-CoA reductase inhibition that increases the bone cell activities (Wong & Rabie, 2006).	The effect of naringin, which was also a HMG-CoA reductase inhibitor was studied in UMR 106 osteoblastic cell line *in vitro*.	Naringin significantly increased bone cell activities *in vitro*.
Two active constituents were isolated and identified as naringin and neoeriocitrin (Li *et al.*, 2006).	The osteoblastic activity of extracts of *Drynaria fortunei* (Kunze) J. Sm. rhizome was assayed in the UMR106 cell line cultured *in vitro*.	The ethanol extract, and its ethyl acetate and n-butanol fractions exhibited stimulating activity.

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Action/Conclusion	Aim/Method	Results
These outcomes suggest that naringin offer a potential in the management of osteoporosis *in vitro* (Wei *et al.*, 2007).	A retinoic acid-induced osteoporosis model of rats was used to assess whether naringin has similar bioactivity against osteoporosis *in vitro*.	A blood test showed that naringin-treated rats experienced significantly lower activity of serum alkaline phosphatase and had higher femur bone mineral density, compared to untreated rats.

Effects on smooth muscle

Action/Conclusion	Aim/Method	Results
Naringin and naringinin increased contractions induced by noradrenaline in rat vas deferens (Herrera & Marhuenda, 1993).	Effect of naringin and naringenin on contractions induced by noradrenaline in rat vas deferens was studied.	Naringin significantly increased contractions induced by noradrenaline in rat vas deferens. Naringenin increased the contractile effect of noradrenaline and was dose dependent.
Relaxant effects of flavonoids on vascular smooth muscle of the isolated rat thoracic aorta (Ajay *et al.*, 2003).	The potency, structure-activity relationship and mechanism of vasorelaxation of flavonols: fisetin, rutin, quercetin; flavones: chrysin, flavone, baicalein; flavanones: naringenin, naringin; isoflavones: diadzein and flavanes: epigallo catechin gallate were examined in the isolated rat aorta.	Most of the flavonoids tested showed concentration dependent relaxant effects against K+(80 mm) and phenylephrine (PE, 0.1 microM)-induced contractions with a greater inhibition of the responses to the alpha1-adrenoceptor agonist.
The vasorelaxant effect of the naturally-occurring flavonoid (+/-)-naringenin on endothelium-denuded vessels was due to the activation of BK(Ca) channels in myocytes (Saponara *et al.*, 2006).	The mechanical and electrophysiological effects of (+/-)-naringenin were investigated in vascular smooth muscle cells.	(+/-)-Naringenin induced concentration-dependent relaxation in endothelium-denuded rat aortic rings.

Effects on gastric intestinal system

Action/Conclusion	Aim/Method	Results
Naringenin, a specific histidine decarboxylase inhibitor, has gastric anti-ulcer activity (Parmar, 1983).	The gastric anti-ulcer activity of a specific histidine decarboxylase inhibitor naringenin, has been studied on the various types of ulcers experimentally induced in rats, *viz.*, pylorus-ligated (Shay method) and restraint ulcers, and on the gastric mucosal damage induced by aspirin, phenylbutazone or reserpine.	Naringenin possessed significant anti-ulcer activity in all these models, manifesting a dose-dependent anti-ulcer effect.
Naringin has a 'cytoprotective' effect against ethanol injury in the rat, but this property appears to be mediated by non-prostaglandin-dependent mechanisms (Martín *et al.*, 1994).	To determine the gastroprotective properties of naringin on and the involvement of endogenous prostaglandins in mucosal injury produced by absolute ethanol.	Oral pretreatment with the highest dose of naringin (400 mg/kg), 60 min before absolute ethanol was the most effective antiulcer treatment.
Flavonoids promote cell migration in nontumorigenic colon epithelial cells differing in Apc genotype (Fenton & Hord, 2004).	Whether specific flavonoids induce cell migration in colon epithelial cells either wild type or heterozygous for Apc genotype was studied.	Naringin and hesperidin induced the greatest migratory response in IMCE cells at 1 microM and induced migration greater than untreated control cells.

Effects on endocrine system

Action/Conclusion	Aim/Method	Results
Dietary flavonoids inhibit thyroid peroxidase (Divi & Doerge, 1996).	A structure-activity study of 13 commonly consumed flavonoids was conducted to evaluate inhibition of thyroid peroxidase (TPO), the enzyme that catalyzes thyroid hormone biosynthesis.	Inhibition by the more potent fisetin, kaempferol, naringenin, and quercetin, was consistent with mechanism-based inactivation of TPO as previously observed for resorcinol and derivatives. Myricetin and naringin inhibited TPO by different mechanisms.

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Action/Conclusion	Aim/Method	Results
Naringinin interacts with human sex hormone-binding globulin (Déchaud *et al.*, 1999).	This study reports on some environmental chemicals with estrogenic activity (xenoestrogens) and their binding interaction for human plasma sex-hormone binding globulin (hSHBG).	The flavonoid phytoestrogens genistein and naringenin were also identified as hSHBG ligands, whereas their glucoside derivatives, genistin and naringin, had no binding activity for hSHBG.
Plants containing flavonoids may have preventive effects in diabetic complications (Asgary *et al.*, 2002).	Several flavonoids, such as rutin, kaempferol, quercetin, apigenin, naringin, morin and biochanin A were selected to determine their antioxidant effects on *in vitro* insulin, hemoglobin and albumin glycosylation.	Biochanin A, the best inhibitor of insulin and hemoglobin glycosylation, inhibits their glycosylation 100% and 60%, respectively. Glycosylation of albumin was inhibited 100% by both biochanin A and apigenin.
Hesperidin and naringin prevent the progression of hyperglycemia, by increasing hepatic glycolysis and glycogen concentration and/or by lowering hepatic gluconeogenesis (Jung *et al.*, 2004).	The effect of citrus bioflavonoids on blood glucose level, hepatic glucose-regulating enzymes activities, hepatic glycogen concentration, and plasma insulin levels was studied, and assessed the relations between plasma leptin and body weight, blood glucose, and plasma insulin.	Hesperidin and naringin supplementation significantly reduced blood glucose compared with the control group. Naringin also markedly lowered the activity of hepatic glucose-6-phosphatase and phosphoenolpyruvate carboxykinase compared with the control group.
Naringin provided a significant amelioration of hypoglycaemic and antioxidant activity in STZ-induced diabetic rats (Ali & Kader, 2004).	The effect of various doses of naringin was studied on streptozotocin (STZ)-induced hyperglycaemic rats to evaluate the possible hypoglycaemic and antioxidant activity of naringin in diabetes.	Exogenous administration of naringin to hyperglycaemic rats causes a dose-dependent decrease of the glucose level, an increase of the insulin concentration, a decrease of the H_2O_2 and TBARS levels, as well as the increase of the total antioxidant status.
Inhibition of intestinal glucose uptake and renal glucose reabsorption explains, the antihyperglycemic action of naringenin and its derivatives (Li *et al.*, 2006).	Using purified intestinal brush border membrane vesicles and everted intestinal sleeves, glucose uptake in intestine was studied with naringinin and naringin.	Naringenin, but not naringin, significantly inhibited glucose uptake in the intestine.

Effects against tumour

Action/Conclusion	Aim/Method	Results
Citrus flavonoids are effective inhibitors of human breast cancer cell proliferation *in vitro*, especially when paired with quercetin (So *et al.*, 1996).	Two citrus flavonoids, hesperetin and naringenin, and four noncitrus flavonoids, baicalein, galangin, genistein, and quercetin, were tested singly and in one-to-one combinations for their effects on proliferation and growth of a human breast carcinoma cell line, MDA-MB-435. These compounds, were tested for their ability to inhibit development of mammary tumors in female Sprague-Dawley rats.	IC_{50} values for the one-to-one combinations ranged from 4.7 micrograms/mL (quercetin + hesperetin, quercetin + naringenin) to 22.5 micrograms/mL (naringenin + hesperetin). Rats given orange juice had a smaller tumor burden than controls, although they grew better than any of the other groups.
The antimutagenic properties the Citrus flavonoids, especially tangeretin and nobiletin, might prevent cancer (Calomme *et al.*, 1996).	The antimutagenicity of the Citrus flavonoids naringin, hesperidin, nobiletin, and tangeretin against the mutagens benzo[a]pyrene, 2-aminofluorene, quercetin, and nitroquinoline N-oxide was investigated in the Salmonella/microsome assay.	Naringin and hesperidin showed a weak antimutagenic activity against benzo[a]pyrene.
Foods rich in certain flavonoids may protect against certain forms of lung cancer. Decreased bioactivation of carcinogens by inhibition of CYP1A1 should be explored (Le Marchand *et al.*, 2000).	To investigate the possible relationship between intake of flavonoids-powerful dietary antioxidants that may also inhibit P450 enzymes-and lung cancer risk, we conducted a population-based, case-control study in Hawaii.	Authors found statistically significant inverse associations between lung cancer risk and the main food sources of the flavonoids quercetin (onions and apples) and naringin (white grapefruit).
Bioflavonoids as antiradicals, antioxidants and DNA cleavage protectors(Russo *et al.*, 2000).	Authors investigated the free-radical scavenging capacity of bioflavonoids (rutin, catechin, and naringin) and the effects of these polyphenols on xanthine oxidase activity, spontaneous lipid peroxidation, and DNA cleavage.	The bioflavonoids under examination showed a dose-dependent free-radical scavenging effect, a significant inhibition of xanthine oxidase activity, and an antilipoperoxidative capacity. In addition, they showed a protective effect on DNA cleavage.

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Action/Conclusion	Aim/Method	Results
Inhibitory effects of naringenin on tumor growth in human cancer cell lines and sarcoma S-180-implanted mice (Kanno *et al.*, 2005).	The effect of naringenin on tumor growth in various human cancer cell lines and sarcoma S-180-implanted mice was studied.	Naringenin inhibited tumor growth in sarcoma S-180-implanted mice, following intraperitoneal or peroral injection once a day for 5 d. Naringin also inhibited tumor growth by peroral injection but not intraperitoneal injection.
The tested flavonoids were weak apoptosis inducers on multidrug-resistant (MDR) and parent cells (Ugocsai *et al.*, 2005).	The effects of various flavonoids and carotenoids on Rhodamine 123 accumulation in MDR Colo 320 human colon cancer cells expressing MDR1/LRP were studied. The Colo 205 cell line was used as a drug-sensitive control.	Catechin, Neohesperidin, Naringin, Robinin, Phloridzin, Dihydrobinetin and Sakuranetin, had only marginal effects on Rhodamine 123 accumulation.
Consumption of grapefruit or limonin may help to suppress colon cancer development (Vanamala *et al.*, 2006).	The hypothesis that untreated and irradiated grapefruit as well as the isolated citrus compounds naringin and limonin would protect against azoxymethane (AOM)-induced aberrant crypt foci (ACF) by suppressing proliferation and elevating apoptosis through anti-inflammatory activities was examined.	Lower levels of iNOS and COX-2 are associated with suppression of proliferation and upregulation of apoptosis, which may have contributed to a decrease in the number of high multiplicity ACF in rats provided with untreated grapefruit and limonin.
Glycosylated flavonoids (*i.e.* naringin, a constituent of citrus fruits, and rutin, a constituent of cranberries) induced the greatest response to treatment at the lowest concentration in MDA human breast cancer cells (Schindler & Mentlein, 2006).	Whether secondary plant constituents, *i.e.* flavonoids, tocopherols, curcumin, and other substances regulate VEGF in human tumor cells *in vitro* was studied by measuring VEGF release by ELISA from MDA human breast cancer cells and, for comparison, U-343 and U-118 glioma cells.	The rank order of VEGF inhibitory potency was naringin > rutin > alpha-tocopheryl succinate > lovastatin > apigenin > genistein > alpha-tocopherol >or= kaempferol > gamma-tocopherol; chrysin and curcumin were inactive except at a concentration of 100 micromol/L. Glioma cells were similarly sensitive, with U343 more than U118, especially for alpha-TOS and tocopherols.

Protections against toxins in chemotherapy drugs and the environment

Action/Conclusion	Aim/Method	Results
Naringin and other okadaic acid-antagonistic flavonoids could have potential therapeutic value as protectants against pathological hyperphosphorylations, environmental toxins, or side effects of chemotherapeutic drugs (Gordon *et al.*, 1995).	55 different flavonoids were tested for their effect on okadaic acid-inhibited autophagy, measured as the sequestration of electroinjected [3H] raffinose.	Naringin (naringenin 7-hesperidoside) and several other flavanone and flavone glycosides (prunin, neoeriocitrin, neohesperidin, apiin, rhoifolin, kaempferol 3-rutinoside) offered virtually complete protection against the autophagy-inhibitory effect of okadaic acid.
Action of naringin is mediated through suppression of lipopolysaccharide-induced TNF production (Kawaguchi *et al.*, 1999).	Suppressive effects of naringin on lipopolysaccharide-induced tumor necrosis factor (TNF) release followed by liver injury were investigated.	Treatment with naringin 3 h prior to lipopolysaccharide challenge resulted in complete protection from lipopolysaccharide lethality in D-galactosamine-sensitized mice.
Prevention of toxin-induced cytoskeletal disruption and apoptotic liver cell death by the grapefruit flavonoid, naringin (Blankson *et al.*, 2000).	The protein phosphatase-inhibitory algal toxins, okadaic acid and microcystin-LR, induced overphosphorylation of keratin and disruption of the keratin cytoskeleton in freshly isolated rat hepatocytes. In hepatocyte cultures, the toxins elicited DNA fragmentation and apoptotic cell death within 24 h.	All these toxin effects could be prevented by the grapefruit flavonoid, naringin. The cytoprotective effect of naringin was apparently limited to normal hepatocytes, since the toxin-induced apoptosis of hepatoma cells, rat or human, was not prevented by the flavonoid.
Naringenin inhibits the activity of cytochrome P450 (CYP) isoforms that activate NNK (Bear & Teel, 2000a).	Authors investigated the effects of five citrus phytochemicals on the *in vitro* metabolism of the tobacco-specific nitrosamine NNK and on the dealkylation of methoxyresorufin (MROD) and pentoxyresorufin (PROD) in liver and lung microsomes of the Syrian golden hamster.	Results suggest that naringenin and quercetin from citrus fruits inhibit the activity of cytochrome P450 (CYP) isoforms that activate NNK and may afford protection against NNK-induced carcinogenesis.

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Action/Conclusion	Aim/Method	Results
Diosmin, naringin, naringenin and rutin are chemoprotective towards CYP1A2 mediated mutagenesis of heterocyclic amines (HCA's) (Bear & Teel, 2000b).	Using Aroclor 1254 induced rat liver S9, four citrus flavonoids: diosmin, naringenin, naringin and rutin were tested for their effects on the mutagenicity of HCA's MeIQx, Glu-P-1*, IQ and PhIP in *Salmonella typhimurium* TA98.	MeIQx induced mutagenesis and PhIP induced mutagenesis in *S. typhimurium* were significantly inhibited by all four flavonoids. Glu-P-1 induced mutagenesis was inhibited by rutin and naringenin. IQ induced mutagenesis was significantly inhibited by each flavonoid except diosmin.
Naringin protects against the radiation-induced genomic instability in the mice bone marrow (Jagetia & Reddy, 2002).	The effect of various doses of naringin was studied on the alteration in the radiation-induced micronucleated polychromatic (MPCE) and normochromatic (MNCE) erythrocytes in mouse bone marrow exposed to 2 Gy of 60Co gamma-radiation.	Naringin is able to protect mouse bone marrow cells against the radiation-induced DNA damage and decline in the cell proliferation as observed by a reduction in the micronucleus frequency and an increase in PCE/NCE ratio, respectively, in the naringin-pretreated irradiated group.
Naringin supplement regulate lipid and ethanol metabolism (Seo *et al.*, 2003).	The effect of naringin supplements on the alcohol, lipid, and antioxidant metabolism in ethanol-treated rats was investigated.	Naringin would appear to contribute to alleviating the adverse effect of ethanol ingestion by enhancing the ethanol and lipid metabolism as well as the hepatic antioxidant defense system.
Naringin from natural products is a useful drug having antioxidant and anti-apoptopic properties (Kanno *et al.*, 2003).	The effects of naringin on H_2O_2-induced cytotoxicity and apoptosis in mouse leukemia P388 cells were investigated. H_2O_2-induced cytotoxicity was	significantly attenuated by naringin or the reduced form of glutathione, a typical intracellular antioxidant. Naringin suppressed chromatin condensation and DNA damage induced by H_2O_2.
Naringin can protect mouse bone marrow cells against radiation-induced chromosomal damage (Jagetia *et al.*, 2003).	The radioprotective action of 2 mg/kg naringin in the bone marrow of mice exposed to different doses of (60)Co gamma-radiation was studied by scoring the frequency of asymmetrical chromosomal aberrations.	Naringin at 5 μM scavenged the 2,2-azino-bis-3-ethyl benzothiazoline-6-sulphonic acid cation radical very efficiently, where a 90% scavenging was observed.

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Action/Conclusion	Aim/Method	Results
Naringin and naringenin inhibit nitrite-induced methemoglobin formation (Kumar *et al.*, 2003).	The effect of naringin and naringenin protect hemoglobin from nitrite-induced oxidation to methemoglobin was studied.	Naringenin was more effective than naringin, probably because of the extra phenolic group in the aglycone.
Suppression of infection-induced endotoxin shock in mice by naringin (Kawaguchi *et al.*, 2004).	The protective effect of the naringin was studied in an endotoxin shock model based on *Salmonella* infection. Intraperitoneal (*i.p.*) infection with 10 (8) CFU *Salmonella typhimurium* aroA caused lethal shock in lipopolysaccharide (LPS) -responder but not LPS-non-responder mice.	Administration of 1 mg naringin 3 h before infection resulted in protection from lethal shock, similar to LPS-non-responder mice. Also resulted not only in a significant decrease in bacterial numbers in spleens and livers, but also in a decrease in plasma LPS levels.
Naringin blocked apoptosis caused by Ara-C-induced oxidative stress, resulting in the inhibition of the cytotoxicity of Ara-C (Kanno *et al.*, 2004).	The effect of naringin on the cytotoxicity and apoptosis in mouse leukemia P388 cells treated with Ara-C. Ara-C caused cytotoxicity in a concentration and time-dependent manner in the cells was examined.	Naringin remarkably attenuated the Ara-C-induced apoptosis and completely blocked the DNA damage caused by Ara-C treatment at 6 h using the Comet assay.
There is a protective effect of naringin on Fe-NTA-induced nephrotoxicity in rats(Singh *et al.*, 2004a).	The effect of naringin, a bioflavonoid with anti-oxidant potential, was studied on Fe-NTA-induced nephrotoxicity in rats.	Pre-treatment of animals with naringin, 60 min before Fe-NTA administration, markedly attenuated renal dysfunction, morphological alterations, reduced elevated TBARS, and restored the depleted renal antioxidant enzymes.
There is a protective effect of naringin in glycerol-induced renal failure in rats (Singh *et al.*, 2004b).	The effect of naringin, a bioflavonoid with anti-oxidant potential, was studied in glycerol-induced ARF in rats.	Pretreatment of animals with naringin 60 min prior to glycerol injection markedly attenuated renal dysfunction, morphological alterations, reduced elevated thiobarbituric acid reacting substances (TBARS), and restored the depleted renal antioxidant enzymes.

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Action/Conclusion	Aim/Method	Results
Enhanced antioxidant status by naringin could compensate the oxidative stress and may facilitate an early recovery from iron-induced genomic insult *in vitro* (Jagetia *et al.*, 2004).	Whether naringin treatment may help to overcome the iron-induced toxic effects *in vitro* was studied.	Pretreatment of HepG2 cells with naringin resulted in an elevation in all the antioxidant enzymes.
Naringin protects mouse liver and intestine against the radiation-induced damage by elevating the antioxidant status and reducing the lipid peroxidation (Jagetia & Reddy, 2005).	The alteration in the antioxidant status and lipid peroxidation was investigated in Swiss albino mice treated with 2 mg/kg b.wt. naringin, a citrus flavoglycoside, before exposure to 0.5, 1, 2, 3, and 4 Gy gamma radiation.	The alteration in the antioxidant status and lipid peroxidation was investigated in Swiss albino mice treated with 2 mg/kg b.wt. naringin, a citrus flavoglycoside, before exposure to 0.5, 1, 2, 3, and 4 Gy gamma radiation.
A combination of beta-carotene with naringin, rutin or quercetin may increase the safety of beta-carotene (Yeh *et al.*, 2005).	The interaction of beta-carotene with three flavonoids-naringin, rutin and quercetin-on DNA damage induced by ultraviolet A (UVA) in C3H10T1/2 cells was studied	All three flavonoids had some absorption at the UVA range, but the effects were opposite to those on DNA damage and beta-carotene oxidation.
Possible mechanism for the antiocular inflammatory effect may be the suppression of PGE_2 and NO by naringin and naringenin (Shiratori *et al.*, 2005).	The efficacy of naringin and naringenin on endotoxin- induced uveitis (EIU) in rats was studied. EIU was induced in male Lewis rats by a footpad injection of lipopolysaccharide (LPS).	40 microM/kg of naringin and naringenin suppressed increases in cell count owing to LPS treatment by 31% and 38%, respectively.
Suppression of the LPS-induced mortality and production of NO by NG is due to inhibition of the activation of NF-kappaβ (Kanno *et al.*, 2006).	The effect of naringin, on LPS-induced endotoxin shock in mice and NO production in RAW 264.7 macrophages was studied.	Naringin suppressed LPS -induced production of NO and the expression of inflammatory gene products such as iNOS, TNF-alpha, COX-2 and IL-6 as determined by RT-PCR assay.
Naringin reduced the genotoxic effects of bleomycin and consequently increased the cell survival and therefore may act as a chemoprotective agent in clinical situations (Jagetia *et al.*, 2005).	The effect of naringin, a grapefruit flavonone was studied on bleomycin-induced genomic damage and alteration in the survival of cultured V79 cells.	Treatment of cells with naringin before exposure to different concentrations of bleomycin arrested the bleomycin-induced decline in the cell survival accompanied by a significant reduction in the frequency of micronuclei when compared with bleomycin treatment alone.

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Action/Conclusion	Aim/Method	Results
Among the plant ingredients tested in the current study, morin, naringin, and quercetin were found to be desirable protectors against B[a]P phototoxicity-(Hori *et al.*, 2007).	A diversity of antioxidants and plant ingredients were examined for their protective effect in cultured Balb/c 3T3 cells against ultraviolet A (UVA)-induced cytotoxicities of extracted air pollutants and benz[a]pyrene (B[a]P).	The B[a]P phototoxicity was not eliminated by well-known antioxidants but was markedly diminished by diversity of plant ingredients.

Antioxidant effects

Action/Conclusion	Aim/Method	Results
Some flavonoids have inhibitory action on enhanced spontaneous lipid peroxidation following glutathione depletion (Younes & Siegers, 1981).	Depletion of hepatic glutathione in phenobarbital-induced rats by phorone (diisopropylidene acetone) led to an enhancement of spontaneous lipid peroxidation *in vitro*. Addition of exogenous glutathione, dithiocarb or one of the flavonoids (+)-catechin, (-)-epicatechin, 3-O-methylcatechin, quercetin, taxifolin, rutin, naringin or naringenin led in every case to a dose-dependent inhibition of this peroxidative activity.	The concentration values yielding 50% inhibition (I (50)) varied from 1.0 x 10 (-6) M for glutathione to 1.9 x 10 (-5) M for naringenin.
Citrus fruit peels have antioxidant activity (Kroyer, 1986).	The antioxidant properties of freeze-dried citrus fruit peels (orange, lemon, grapefruit) and methanolic extracts from the peel were studied.	Freeze-dried orange peel showed the highest, lemon peel somewhat less and grapefruit peel the lowest but still remarkable antioxidant activity.
Flavonoids have protective effect against lipid peroxidation of erythrocyte membranes (Affany *et al.*, 1987).	Cumene hydroperoxide induces *in vitro* the peroxidation of erythrocyte membrane. The protective effect of various flavonoids was compared to that of butylated hydroxytoluene (BHT). Protective effect was evaluated by the inhibition of peroxidation product formation.	Quercetin and catechin showed a protective effect against lipid peroxidation as high as that of BHT. Morin, rutin, trihydroxyethylrutin, and naringin were active but to a lesser degree.

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Action/Conclusion	Aim/Method	Results
Polyhydroxylated substitutions on rings A and B, a 2,3-double bond, a free 3-hydroxyl substitution and a 4-keto moiety confer antiperoxidative properties (Ratty & Das, 1988).	The *in vitro* effects of several flavonoids on nonenzymatic lipid peroxidation in the rat brain mitochondria was studied. The lipid peroxidation was indexed by using the 2-thiobarbituric acid test.	The flavonoids, apigenin, flavone, flavanone, hesperidin, naringin, and tangeretin promoted the ascorbic acid-induced lipid peroxidation.
Flavonoids are superoxide scavengers and antioxidants (Chen *et al.*, 1990).	The superoxide anions scavenging activity and antioxidation of seven flavonoids were studied. The superoxide anions were generated in a phenazin methosulphate-NADH system and were assayed by reduction of nitroblue tetrazolium.	The scavenging activity ranked: rutin was the strongest, and quercetin and naringin the second, while morin and hispidulin were very weak.
Aromatic hydroxyl group is very important for antioxidative effects of the compounds. None of the compounds tested exerted an obvious pro-oxidant effect (Ng *et al.*, 2000).	A variety of flavonoids, lignans, an alkaloid, a bisbenzyl, coumarins and terpenes isolated from Chinese herbs was tested for antioxidant activity as reflected in the ability to inhibit lipid peroxidation in rat brain and kidney homogenates and rat erythrocyte hemolysis. The pro-oxidant activities of the aforementioned compounds were assessed by their effects on bleomycin-induced DNA damage.	The flavonoid rutin and the terpene tanshinone I manifested potent antioxidative activity in the lipid peroxidation assay but no inhibitory activity in the hemolysis assay. The lignan deoxypodophyllotoxin, the flavonoid naringin and the coumarins columbianetin, bergapten and angelicin slightly inhibited lipid peroxidation in brain and kidney homogenates.
Antioxidative activity of naringin and lovastatin in high cholesterol-fed rabbits (Jeon *et al.*, 2001).	To determine the antioxidative effects of the citrus bioflavonoid, naringin, a potent cholesterol-lowering agent, compared to the cholesterol-lowering drug, lovastatin, in rabbits fed a high cholesterol diet.	Naringin regulate antioxidative capacities by increasing the SOD and catalase activities, up-regulating the gene expressions of SOD, catalase, and GSH-Px, and protecting the plasma vitamin E. Lovastatin exhibited an inhibitory effect on the plasma and hepatic lipid peroxidation and increased the hepatic catalase activity.

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Action/Conclusion	Aim/Method	Results
Antioxidant effects of naringin and probucol in cholesterol-fed rabbits (Jeon *et al.*, 2002).	Twenty male rabbits were served a high-cholesterol diet or high-cholesterol diet supplemented with naringin or probucol for 8 weeks to compare the antioxidative effects of the naringin and antioxidative cholesterol-lowering drug (probucol).	The probucol supplement was very potent in the antioxidative defense system, whereas naringin exhibited a comparable antioxidant capacity based on increasing the gene expressions in the antioxidant enzymes, increasing the hepatic SOD and CAT activities, sparing plasma vitamin E and decreasing the hepatic mitochondrial H_2O_2 content.
Several structural features were linked to the strong antioxidant activity of flavonoids (Yu *et al.*, 2005).	A variety of *in vitro* models such as beta-carotene-linoleic acid, 1,1-diphenyl-2-picryl hydrazyl (DPPH), superoxide, and hamster low-density lipoprotein (LDL) were used to measure the antioxidant activity of 11 citrus bioactive compounds.	Flavonoids, which contain a chromanol ring system had stronger antioxidant activity as compared to limonoids and bergapten, which lack the hydroxy groups.
Naringin is a powerful plasma lipid lowering and plasma antioxidant activity increasing flavonone. However, fresh red grapefruit is preferable than naringin (Gorinstein *et al.*, 2005).	The influence of naringin versus red grapefruit juice on plasma lipid levels and plasma antioxidant activity in rats fed cholesterol-containing and cholesterol-free diets was compared.	After 30 days of different feeding, it was found that diets supplemented with red grapefruit juice and to a lesser degree with naringin improved the plasma lipid levels mainly in rats fed cholesterol and increased the plasma antioxidant activity.
Induction of cell apoptosis in 3T3-L1 pre-adipocytes by flavonoids is associated with their antioxidant activity- (Hsu & Yen 2006).	The relationship between the influence of flavonoids on cell population growth and their antioxidant activity was studied.	The inhibition of flavonoids (naringenin, rutin, hesperidin, resveratrol, naringin and quercetin) on 3T3-L1 pre-adipocytes was 28.3, 8.1, 11.1, 33.2, 5.6 and 71.5%, respectively.
The Naringin-Cu (II) complex 1 showed higher antioxidant, anti-inflammatory and tumor cell cytotoxicity activities than free naringin without reducing cell viability- (Pereira *et al.*, 2007).	In order to understand the contribution of the metal coordination and the type of interaction between a flavonoid and the metal ion, in this study a new metal complex of Cu (II) with naringin was synthesized and characterized by FT-IR, UV-VIS, mass spectrometry (ESI-MS/MS), elemental analysis and 1H-NMR.	The results of these analyses indicate that the complex has a Cu (II) ion coordinated via positions 4 and 5 of the flavonoid.

Antimicrobial effects

Action/Conclusion	Method	Results
Naringin inhibited *P. aeruginosa* (Ng *et al.*, 1996).	Coumarins, flavonoids and polysaccharopeptide were tested for antibacterial activity. The bacteria used for this study included clinical isolates of *Staphylococcus aureus, Shigella flexneri, Salmonella typhi, Escherichia coli* and *Pseudomonas aeruginosa.*	When tested at the dose of 128 mg/l, the flavonoids (rutin, naringin and baicalin) inhibited 25% or less of *P. aeruginosa* and only baicalin was active against *S. aureus.*
Anti-Sindbis activity of flavanones hesperetin and naringenin (Paredes *et al.*, 2003).	The effect of hesperetin, naringenin and its glycoside form on the Sindbis neurovirulent strain (NSV) replication *in vitro* was studied. All flavanones tested were not cytotoxic on Baby Hamster cells 21 clone 15 (BHK-21).	Hesperetin and naringenin had inhibitory activity on NSV infection. However their glycosides, hesperidin and naringin did not have inhibitory activity. Implying that the presence of rutinose moiety of flavanones blocks the antiviral effect.
There exist antimicrobial activity of grapefruit seed and pulp ethanolic extract(Cvetnić & Vladimir-Knezević, 2004).	Antibacterial and antifungal activity of ethanolic extract of grapefruit (*Citrus paradisi* Macf., Rutaceae) seed and pulp was examined against 20 bacterial and 10 yeast strains.	Ethanolic extract exibited the strongest antimicrobial effect against *Salmonella enteritidis* (MIC 2.06%, m/V). Other tested bacteria and yeasts were sensitive to extract concentrations ranging from 4.13% to 16.50% (m/V).
Selected bioflavonoids may show promise as an alternative means of reducing dental caries (Wood, 2007).	The present study evaluates two separate, but related, dietary trials-trial 1, dietary naringenin (NAR) supplementation; and trial 2, dietary rutin (R), quercetin (Q), and naringin (N) supplementation-on dental caries formation in 40 different male albino rats, at the expense of dextrose, for periods of 42 days.	An inverse dose-dependent relationship was established among the NAR experimental groups and control group. In dietary trial 2, statistically significant reductions in occlusal caries were observed for R, Q, and N in the maxillary molars and for Q and N in the mandibular molars compared with the control group.
Naringin possesses significant antimicrobial properties on periodontal pathogens *in vitro*. It also has an inhibitory effect on some common oral microorganisms in low concentrations (Tsui *et al.*, 2007).	The effects of naringin on the growth of periodontal pathogens such as *A. actinomycetemcomitans* and *P. gingivalis* were studied *in vitro*. For comparison, the effects of naringin on several oral microbes were also studied.	Naringin also had an inhibitory effect against all bacteria and yeasts tested.

Drug interactions

Action/Conclusion	Method	Results
Grapefruit juice and naringenin inhibit CYP1A2 activity in man (Fuhr *et al.*, 1993).	The effects of grapefruit juice and naringenin on the activity of the human cytochrome P450 isoform CYP1A2 were evaluated using caffeine as a probe substrate.	*In vitro* naringin was a potent competitive inhibitor of caffeine 3-demethylation by human liver microsomes (Ki = 7-29 microM). *In vivo* grapefruit juice decreased the oral clearance of caffeine and prolonged its half-life.
Grapefruit juice produces a marked and variable increase in felodipine bioavailability (Bailey *et al.*, 1993a).	The pharmacokinetics of felodipine and its single primary oxidative metabolite, dehydrofelodipine, were studied after drug administration with 200 mL water, grapefruit juice, or naringin in water at the same concentration as the juice in a randomized crossover trial of nine healthy men.	Grapefruit juice produces a marked and variable increase in felodipine bioavailability.- Naringin solution produced much less of an interaction, showing that other factors were important.
The bioavailability of some dihydropyridine calcium antagonists can be augmented by grapefruit juice but does not involve naringin (Bailey *et al.*, 1993b).	The pharmacokinetics of nisoldipine coat-core tablet were studied in a Latin square-designed trial in which 12 healthy men were administered the drug with water, grapefruit juice, or encapsulated naringin powder at the same amount as that assayed in the juice.	The bioavailability of some dihydropyridine calcium antagonists can be markedly augmented by grapefruit juice. The naringin capsule did not change nisoldipine pharmacokinetics.
As naringin alone is ineffective, the inhibitory effect of grapefruit juice on the metabolism of coumarin is caused by at least one compound other than naringin (Runkel *et al.*, 1997).	To investigate whether the presence of naringin is demanded for the inhibition of the coumarin 7-hydroxylase in man or other compounds are responsible for it.	While increasing amounts of grapefruit juice delay the excretion of 7-hydroxycoumarin by 2 h, increasing doses of naringin in water up to twofold do not cause any alteration in the time course of excretion.
To avoid the interaction, nimodipine should not be taken with grapefruit juice (Fuhr *et al.*, 1998).	A randomized crossover interaction study on the effects of grapefruit juice on the pharmacokinetics of nimodipine and its metabolites.	Grapefruit juice increases oral nimodipine bioavailability.

Table *Contd.*

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Action/Conclusion	Method	Results
Naringin and 6',7'-dihydroxybergamottin are not the major active ingredients, although they may contribute to the grapefruit juice-felodipine interaction (Bailey *et al.*, 1998).	To test whether naringin or 6',7'-dihydroxybergamottin is a major active substance in grapefruit juice-felodipine interaction in humans.	The findings show the importance of *in vivo* testing to determine the ingredients in grapefruit juice responsible for inhibition of cytochrome P450 3A4 in humans.
In vitro inhibition of simvastatin (SV) metabolism in rat and human liver by naringenin (NRG) (Ubeaud *et al.*, 1999).	NRG's inhibition of the metabolism of SV in rat hepatocytes (the intrinsic clearance of SV) was studied.	Naringenin present in grapefruit juice inhibits *in vitro* the metabolism of simvastatin, a HMG-CoA reductase inhibitor.
These effects may indicate a chemopreventive role of naringin against protoxicants activated by P450 1A2 (Ueng *et al.*, 1999).	*In vitro* and *in vivo* effects of naringin on microsomal monooxygenase were studied to evaluate the drug interaction of this flavonoid.	Naringenin is a potent inhibitor of benzo(a)pyrene hydroxylase activity *in vitro* and naringin reduces the P450 1A2 protein level *in vivo*.
Patients taking drugs which are P-glycoprotein substrates may need to restrict their intake of bioflavonoid-containing foods and beverages, such as grapefruit juice (Mitsunaga *et al.*, 2000).	To see whether grapefruit juice bioflavonoids alter the permeation of vincristine across the blood-brain barrier, we conducted experiments with cultured mouse brain capillary endothelial cells (MBEC4 cells) *in vitro* and ddY mice *in vivo*.	The *in vivo* brain-to-plasma concentration ratio of [3H]vincristine in ddY mice was decreased by coadministration of 0.1 mg/kg quercetin, but increased by 1.0 mg/kg quercetin. Kaempferol had a similar biphasic effect. Cchrysin, flavon, hesperetin, naringenin increased [3H]vincristine uptake in the 10-50 microM range and glycosides (hesperidin, naringin, rutin) were without effect.
Flavonoids with EGF-receptor tyrosine kinase inhibitory activities enhance the intestinal absorption of the beta-lactam antibiotic cefixime in Caco-2 cells.	33 flavonoids, occurring ubiquitously in foods of plant origin were tested for their ability to alter the transport of the beta-lactam antibiotic cefixime via the H+-coupled intestinal peptide transporter PEPT1 in the human intestinal epithelial cell line Caco-2.	Quercetin, genistein, naringin, diosmin, acacetin and chrysin increased uptake of [14C] cefixime dose dependently by up to 60%.

Table *Contd.*

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Action/Conclusion	Method	Results
Bergapten appears to be a potent inhibitor of CYP3A4 and may therefore be primarily responsible for the effect of grapefruit juice on CYP3A4 activity (Ho *et al.*, 2001).	To evaluate the inhibition of CYP3A4 activity in human liver microsomes by flavonoids, furanocoumarins and related compounds and investigate possibly more important and potential inhibitors of CYP3A4 in grapefruit juice.	Bergapten (5-methoxypsoralen) with the lowest IC_{50} value (19-36 microM) was the most potent CYP3A4 inhibitor.
Enhanced paclitaxel bioavailability after oral coadministration of paclitaxel prodrug with naringin to rats (Choi & Shin, 2005).	The effect of naringin on the bioavailability and pharmacokinetics of paclitaxel after oral administration of paclitaxel or its prodrug coadministered with naringin to rats was studied.	The bioavailability of paclitaxel coadministered as a prodrug with or without naringin was remarkably higher than the control.
The metabolism of verapamil and the formation of norverapamil were inhibited by naringin possibly by inhibition of CYP3A in rabbits (Kim & Choi, 2005).	The pharmacokinetics of verapamil and one of its metabolites, norverapamil were investigated after oral administration of verapamil at a dose of 9 mg/kg without or with oral naringin at a dose of 7.5 mg/kg in rabbits.	With naringin, the total area under the plasma concentration-time curve (AUC) of verapamil was significantly greater, the AUC(verapamil)/AUC(norverapamil) ratio was considerably greater.
The concomitant use of naringin significantly enhanced the oral exposure of diltiazem in rats (Choi & Han, 2005).	Pharmacokinetic parameters of diltiazem and desacetyldiltiazem were determined in rats following an oral administration of diltiazem to rats in the presence and absence of naringin.	Absolute and relative bioavailability values of diltiazem in the presence of naringin were significantly higher than those from the control group.
Verapamil dosage should be adjusted when given with naringin or a naringin-containing dietary supplement (Yeum & Choi, 2006).	The effect of naringin on the pharmacokinetics of verapamil and its major metabolite, norverapamil in rabbits were studied.	Pretreatment of naringin enhanced the oral bioavailability of verapamil.
The inhibition of hepatic P-gp by oral naringin could also contribute to the significantly greater AUC of intravenous paclitaxel by oral naringin (Lim & Choi, 2006).	The effects of oral naringin on the pharmacokinetics of intravenous paclitaxel in rats were studied.	After intravenous administration of paclitaxel, the AUC was significantly greater and Cl was significantly slower than controls.

Table *Contd.*

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Action/Conclusion	Method	Results
Naringin is a major and selective clinical inhibitor of organic anion-transporting polypeptide 1A2 (OATP1A2) in grapefruit juice (Bailey *et al.*, 2007).	Inhibition of OATP1A2 transport by flavonoids in grapefruit (naringin) and orange (hesperidin) was conducted *in vitro*. Two randomized crossover pharmacokinetic studies were performed clinically.	Naringin most probably directly inhibited enteric OATP1A2 to decrease oral fexofenadine bioavailability. Inactivation of enteric CYP3A4 was probably not involved.
Kaempferol and naringenin are shown to mediate pharmacokinetic drug interaction with the prodrugs lovastatin and enalapril due to their capability of esterase inhibition (Li *et al.*, 2007).	The esterase-inhibitory potential of 10 constitutive flavonoids and furanocoumarins toward p-nitrophenylacetate (PNPA) hydrolysis was investigated.	In Caco-2 cells, demonstrated to contain minimal CYP3A activity, the permeability coefficient of the prodrugs lovastatin and enalapril was increased in the presence of the active flavonoids kaempferol and naringenin, consistent with inhibition of esterase activity.
The *in vitro* data suggest that compounds present in grapefruit juice are able to inhibit the P-gp activity modifying the disposition of drugs that are P-gp substrates such as talinolol (de Castro *et al.*, 2007).	The potential interaction between selected ingredients of grapefruit juice and, the transport of talinolol, a P-gp substrate, across Caco-2 cells monolayers was determined in the absence and presence of distinct concentrations of grapefruit juice, bergamottin, 6',7'-dihydroxybergamottin, 6',7'-epoxybergamottin, naringin and naringenin.	The flavonoid aglycone naringenin was around 10-fold more potent than its glycoside naringin with IC_{50} values of 236 and 2409 microM, respectively.
Some flavonoids appeared to be competitive inhibitors of monocarboxylate transporter 1 (MCT1) (Shim *et al.*, 2007).	The cellular uptake of benzoic acid was examined in the presence and the absence of naringin, naringenin, morin, silybin and quercetin in Caco-2 cells.	All the tested flavonoids except naringin significantly inhibited the cellular uptake of [(14)C]-benzoic acid. Particularly, naringenin and silybin exhibited strong inhibition effects.

Antiinflammatory effects

Action/Conclusion	Method	Results
Flavonoids with antioxidant action (naringin and rutin) prevent the release of mastocytic and nonmastocytic histamine (Lambev *et al.*, 1980a).	Experiments are carried out on 35 male albino rats. The effect of the flavonoids naringin and rutin on the level of mastocytic and nonmastocytic histamine is studied, as well as on its release induced by compound 48/80 (2 mg/kg i. p.). The histamine content is determined fluorimetrically.	Naringin and rutin have no effect on the levels of mastocytic and nonmastocytic histamine. They prevent the release of mastocytic histamine, induced by compound 48/80.
Naringin has antiexudative effect of in experimental pulmonary edema and peritonitis (Lambev *et al.*, 1980b).	The authors examined antiexudative activity of bioflavonoids naringin and rutin in comparative aspect in two models of acute inflammation. The experiments were carried out on 180 male white rats and 24 guinea pigs.	The two flavonoids manifested marked antiexudative effect in rats with experiments peritonitis.
Flavonoid inhibited human basophil histamine release stimulated by various agents (Middleton & Drzewiecki, 1984).	Eleven flavonoids included flavone, quercetin, taxifolin, chalcone, apigenin, fisetin, rutin, phloretin tangeretin, hesperetin and naringin were studied for their effects on human basophil histamine release triggered by six different stimuli.	The flavonols, quercetin and fisetin, and the flavone, apigenin, exhibited a predilection to inhibit histamine release stimulated by IgE-dependent ligands (antigen, anti-IgE and con A). The flavanone derivatives, taxifolin and hesperetin, were inactive as were the glycosides, rutin and naringin. The open chain congeners chalcone and phloretin, also possessed inhibitory activity.
Flavanone glycosides can be activated by intestinal bacteria, and may be effective toward IgE-induced atopic allergies (Park *et al.*, 2005).	The passive cutaneous anaphylaxis-inhibitory activity of the flavanones isolated from the pericarp of *Citrus unshiu* (Family Rutaceae) and the fruit of *Poncirus trifoliata* (Family Rutaceae) was studied.	Naringenin, hesperetin and ponciretin potently inhibited IgE-induced beta-hexosaminidase release from RBL-2H3 cells and the PCA reaction.

Other effects

Action/Conclusion	Method	Results
Naringin and naringenin have been identified as prooxidants independent of transition metal catalysed autoxidation reactions (Chan *et al.*, 1999).	Flavonoids containing phenol B rings, *e.g.* naringenin, naringin, hesperetin and apigenin, were studied if they formed prooxidant metabolites that oxidised NADH upon oxidation by peroxidase/H_2O_2.	Prooxidant phenoxyl radicals formed by these flavonoids cooxidise NADH to form NAD radicals which then activated oxygen.

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19

Biotransformation of Drugs

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ABSTRACT

Biotransformation is the termination or alteration of biologic activity of a drug or xenobiotic. In general, lipophilic drugs are transformed to more polar and hence readily excretable products. Most metabolic biotransformations occur at a point between absorption of a drug into general circulation and its renal elimination. The major sites for biotransformation are liver, gastrointestinal tract, the lungs, skin and the kidneys. The enzymes involved in the metabolism are either microsomal or nonmicrosomal. In general all the reactions can be assigned to two major categories called phase I and phase II. The phase I or non synthetic reactions involve oxidation, reduction, hydrolysis, cyclization and decyclization. The phase II or synthetic reactions involve glucuronide conjugation, acetylation, methylation, sulfate conjugation, glycine conjugation, glutathione conjugation and ribonucleoside/nucleotide synthesis. Certain drugs are inactivated spontaneously e.g. the Hoffman elimination of atracurium. Some drugs like the lithium and streptomycin do not under go any metabolic transformation.

Key words : Xenobiotic, metabolism, conjugation, hydrolysis, renal, synthetic, reaction

INTRODUCTION

Living organisms metabolize drugs and other xenobiotics through a natural process that involves the same enzymatic processes that are utilized for metabolism of dietary substances (Jenner & Testa, 1981). The capacity to metabolize unusual xenobiotics and other food sources is important for

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survival of living beings. Drugs are considered as xenobiotics and most of them undergo extensive metabolism in humans.

A large plethora of diverse enzymes have evolved in animals with the sole function of metabolizing foreign chemicals. Enzymes that metabolize xenobiotics have been called as drug-metabolizing enzymes, though they are involved in the metabolism of many foreign chemicals to which humans are exposed (Correia, 2006).

For example, lipophilic barbiturates such as thiopental and pentobarbital would have extremely long half lives if it were not for their metabolic conversion to water soluble compounds. The lipophilic nature of renal tubular membranes also facilitates the reabsorption of hydrophobic compounds following their glomerular filtration. Consequently, most drugs would have a prolonged duration of action if termination of their action depended solely on renal excretion.

Lastly, drug metabolizing enzymes have been exploited through the design of pharmacologically non active prodrugs that are converted *in vivo* to pharmacologically active drugs.

SITES OF DRUG METABOLISM

Drug metabolizing enzymes are found in most of the tissues in the body with highest levels in liver and the gut. The liver is considered as the major site for degradation of endogenous chemicals for *e.g.* cholesterol and steroid hormones. Other organs that contain significant xenobiotic – metabolizing enzymes include the kidneys and lung and nasal mucosa, the latter two playing significant roles in first-pass metabolism of drugs that are administered through aerosol sprays. Other tissues that display considerable activity include skin.

Although drug metabolism within a living body can occur by spontaneous, uncatalyzed chemical reactions, the majority of transformations are catalyzed by specific cellular enzymes (Gonzalez & Tukey, 2006). At the subcellular level these enzymes may be situated in the mitochondria, endoplasmic reticulum, cytosol, lysosomes or even the nuclear envelope or plasma membrane.

PHASES OF DRUG BIOTRANSFORMATION

- Biochemical alteration of the drug in the body
- It is needed to render non polar (lipid soluble) compounds, to polar (lipid insoluble) so that they are not reabsorbed in the renal tubules
- Hydrophilic drugs *e.g.* streptomycin, Neostigmine, decamethonium etc. are not biotransformed

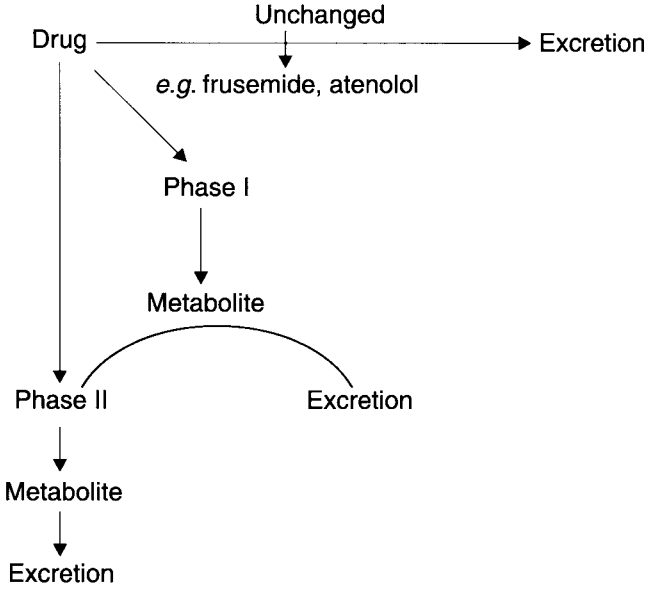
CONSEQUENCES OF BIOTRANSFORMATION

Active drug to inactive metabolite (inactivation)	Active drug to active metabolite		Inactive drug to active metabolite (prodrug)	
e.g. Morphine, chloramphenicol, pentobarbitone, propranolol	*e.g.* Codeine - Morphine		*e.g.* Levodopa - Dopamine	
	Phenacetin - Paracetamol		Enalapril - Enalaprilat	
	Digitoxin - Digoxin		α-methyldopa - α methyl norepinophrine	
	Diazepam - Oxazepam		Prednisone - Prednisolone	
	Desmethyl - Diazepam			
	Amitriptyline - Nortriptyline		Bacampicillin - Ampicillin	
			Sulfasalazine - 5 - Amino salicylic acid	

PHASES IN METABOLISM OF DRUGS

The chemical reaction of biotransformation can take place in two phases

1. Phase I/Non – synthetic reactions
   - Metabolite may be active or inactive
2. Phase II/Synthetic reactions
   - Metabolite is mostly inactive



Note: If the metabolite of phase I is not sufficiently polar to be excreted, it undergoes phase II reactions

Phase I Reactions

Non-Synthetic Reactions

- Phase I reactions convert the drug to a more polar metabolite by oxidation, reduction, hydrolysis, cyclization and decyclization
- Phase I reactions introduce or expose a functional group on the parent compound
- The enzyme systems involved in phase I reactions are located primarily in endoplasmic reticulum (phase II conjugation enzymes are mainly cytosolic)

OXIDATION

Oxidation is the process of addition of oxygen (or a negatively charged radical) to a drug molecule or removal of hydrogen (or a positively charged radical) from a drug molecule. Oxidation reactions are the most of monooxygenases in the liver, which in the final step involve a cytochrome P-450 haemoprotein, NADPH, cytochrome P-450 reductase and O_2 (Ortiz de Montellano & Corriea, 1995).

More than 100 cytochrome P-450 isoenzyme differing in their affinity for various substrates (drugs) have been identified. The cytochrome P-450 isoenzymes important in human beings are given as following:

1) *CYP3A 4/5*

- Carryout biotransformation of largest number of drugs (nearly 50%) present in liver and also expressed in intestine and kidney. This isoenzyme is inhibited by erythromycin, clarithromycin, ketoconazole, itraconazole, verapamil, diltiazem, ritonavir, a constituent of grape fruit juice – induced by – rifampicin, barbiturates, anticonvulsants (Thummel & Wilkinson, 1998)

2) *CYP2D6*

- Next most important CYP isoform
- Metabolizes nearly 20% of drugs, including tricyclic antidepressants, selective serotonin receptake inhibitors, neuroleptics, antiarrhythmics, β-blockers and opiates (Guengrich, 1997)
- Isoenzyme inhibited by quinidine, results in failure of conversion of codeine to morphine analgesic effect of codeine is lost

3) *CYP2C8/9*

- >15% of drugs are biotransformed by this isoenzyme
- Drugs metabolized include – phenytoin and warfarin

4) *CYP2C19*

- >12% of drug
- Drugs metabolized include omeprazole, lansoprazole
- Isoenyzme induced by rifampicin and carbamazepine

5) *CYP1A1/2*

- Participates in the metabolism of only few drugs
- This isoenzyme is important for activation of procarcinogens
- Inducers include
  (1) Rifampicin
  (2) Carbamazepine
  (3) Polycyclic hydrocarbons
  (4) Cigarette smoke
  (5) Smoked meat (charbroiled meat)

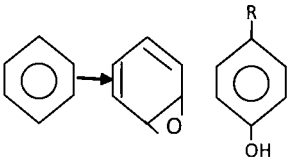
6) *CYP2E1*

- Catalyses formation of minor metabolites of few drugs, especially the hepatotoxic N-acetyl benzoquinonemine from paracetamol
- Chronic alcoholism induces the isoenzyme

The relative amount of different cytochrome P-450 differs among species and among individuals of the same species. These differences largely account for marked interspecies and inter individual differences in rate of metabolism of drugs

Various oxidation reactions are

1. N-dealkylation
2. O-dealkylation
3. Aliphatic hydroxylation
4. Aromatic hydroxylation
5. N-oxidation
6. S-oxidation
7. Deamination

S. No.	Oxidative reactions	Reaction	Examples
1.	N-dealkylation	$RNCH_3 \longrightarrow RNH_2 + CH_2O$	Imipramine, diazepam, codeine, erythromycin, morphine, tamoxifen, theophylline,caffeine
2.	O-dealkylation	$ROCH_3 \longrightarrow ROH + CH_2O$	Codeine, indomethacin, dextromethorphan
3.	Aliphatic hydroxylation	$RCH_2CH_3 \longrightarrow R{-}\underset{\displaystyle OH}{\underset{\displaystyle |}{CH}}CH_3$	Tolbutamide, ibuprofen, pentobarbital, meprobamate, cyclosporine, midazolam
4.	Aromatic hydroxylation		Phenytoin, phenobarbital, propranolol, phenylbutazone, ethinylestradiol, amphetamine, warfarin
5.	N-oxidation	$RNH_2 \longrightarrow RNHOH$ $\begin{matrix} R_1 \\ R_2 \end{matrix}\!\!>\!NH \longrightarrow \begin{matrix} R_1 \\ R_2 \end{matrix}\!\!>\!N{-}OH$	Chlorpheniramine, dapsone, meperidine, quinidine, acetaminophen
6.	S-oxidation	$\begin{matrix} R_1 \\ R_2 \end{matrix}\!\!>\!S \longrightarrow \begin{matrix} R_1 \\ R_2 \end{matrix}\!\!>\!S{=}O$	Cimetidine, chlorpromazine, thioridazine, omeprazole
7.	Deamination	$RCHCH_3 \longrightarrow R{-}\underset{\displaystyle NH_2}{\underset{\displaystyle |}{CH}}{-}\underset{\displaystyle OH}{\underset{\displaystyle |}{C}}{-}CH_3 \longrightarrow R{-}\overset{\displaystyle O}{\overset{\displaystyle ||}{C}}{-}CH_3 + NH_2$	Diazepam, amphetamine

REDUCTION

This reaction is the converse of oxidation and involves cytochrome P-450 enzymes working in opposite directions. Drugs primarily reduced are chloralhydrate, chloramphenicol, halothane.

There are many types of reduction reactions.

1. Nitro reduction - *e.g.* chloramphenicol -Arylamine
2. Keto reduction - *e.g.* cortisone – hydrocortisone

Note: Reduction may be catalysed by non microsomal enzymes also. *e.g.* disulfiram and nitrates

HYDROLYSIS

This is the cleavage of drug molecule by taking up a molecule of water.

$$\text{Ester} + H_2O \xrightarrow{\text{Esterase}} \text{Acid} + \text{Alcohol}$$

Similarly amides and polypeptides are hydrolysed by amidases and peptidases.

Hydrolysis occurs in liver, intestine, plasma and other tissues.

Hydrolysis reactions

	Reaction	Example
(i)	$R_1\overset{\overset{O}{\|}}{C}OR_2 \longrightarrow R_1COOH + R_2OH$	Procaine, aspirin, clofibrate, meperidine, enalapril, cocaine
(ii)	$R_1\overset{\overset{O}{\|}}{C}OR_2 \longrightarrow R_1COOH + R_2NH_2$	Lidocaine, procainamide, indomethacin

CYCLIZATION

This is formation of ring structure from a straight chain compound *e.g.* proguanil

DECYCLIZATION

This is the opening up of ring structure of the cyclic drug molecule. This is a minor pathway *e.g.* barbiturates, phenytoin

Phase II Reactions

Phase II reactions are carried out by enzymes that are synthetic in nature. The metabolites of phase I reaction are conjugated to endogenous substances in phase II reaction. These endogenous substances are usually derived from carbohydrate or aminoacid source. The formation of conjugate involves high-energy intermediates and specific transfer enzymes, which requires high energy. Such transferase enzymes are either located in microsomes or in the cytosol. This results in termination of biological activity of the drug. However, certain conjugation reaction (*i.e.* acyl glucuronidation of NSAIDS, N-acetylation of INH) may lead to formation of reactive species responsible for the hepatotoxicity of the drug.

The various phase II reactions include, glucuronidation, acetylation, methylation, sulfate conjugation, glycine conjugation and glutathione conjugation.

Glucuronidation

This reaction is catalysed by UDP-Glucuronic transferases (UGTs). It is one of the most important reactions in synthetic reactions. These enzymes catalyse the transfer of glucuronic acid from the co-factor UDP – glucuronic acid. Compunds with hydroxyl or carboxylic acid group are easily conjugated with glucuronic acid *e.g.* aspirin, acetaminophen, morphine, digoxin (Kroemer & Klotz, 1992; Dutton, 1980).

Some of the endogenous substrates including, bilirubin, steroid hormones and thyroxine utilizes this pathway for its metabolism.The UGTs are present in high concentrations in specific tissues like GI tract and liver. Glucuronides are either excreted by the kidneys into the urine or through active transport of liver hepatocytes into the bile. These acids are re-absorbed to the liver by entero-hepatic re-circulation. Many drugs metabolized by liver utilizes the same process and re-enter circulation. The commensals located in the lower GI tract releases β-D-glucopyrano-siduronic acid and liberates the free drug into the intestinal lumen. Similar to water re-absorption, the drug also gets absorbed passively from the intestinal lumen and re-enter circulation.

Acetylation

N-acetyl transferases (NATs) are located in the cytosol and they are involved in addition of acetyl group from the co-factor acetyl coenzyme A. Compounds having amino or hydrazine residues undergoes acetylation *e.g.* Dapsone, sufonamides, clonazepam, INH (Vatsis *et al.*, 1995).

The N-acetyl tranferases in human shows extreme genetic polymorphism ranging from fast or rapid acetylators to slow acetylators. The administration of therapeutic dose of drugs like hydralazine or INH leads to toxicity of the drug. Slow acetylation are also predisposed to idiosyncratic hypersensitivity reactions and drug-induced auto-immune disorders.

Methylation

Some xenobiotics and drugs undergo O-, N- and S-methylation. Methionine and cystiene acts as methyl donor. Commonly the amines and phenols undergo methylation is carried out by group of enzymes known as N-methyl tranferases (Weinshilboum *et al.*, 1999). They are catechol-O-methyl transferase (COMT), phenolromethyl transferase (POMT), a thiopurine-S-methyl transferase (TPMT) and thiol-methyl transferase includes TMT, which catalyses S-methylation of azathioprine, 6-mercaptopurine and thioguamine. Dopamine, epinephrine, histamine and thiouracil are some of the drugs which undergo methylation.

Sulfate conjugation

Aromatic and aliphatic compounds are acted upon by sulfotransferases (SULTS) (Glatt, 2000). These enzymes are located in the cytosol and conjugate sulfate to hydroxyl groups of aromatic and aliphatic compounds. SULTS are responsible for wide variety of xenobiotic metabolism and many isoforms of this enzyme have been identified (Negishi *et al.*, 2001).The drugs which undergo sulfation include chloromphenicol, adrenal and sex steroids. SULTs also play an important role in normal human homeostasis. *e.g.* SULT (B) is expressed in skin and brain and carries out catalysis of cholesterol and thyroid hormones. A significant proportion of catecholamines and sex hormones exist in sulfated form, due to the action of SULT.

Glycine conjugation

This reaction is carried out by Acyl-CoA glycine tranferase. These enzymes are located in the mitochondria of hepatocyte. Salicylic acid, benzoic acid, nicotinic acid and cinnamic acids undergo glycine conjugation, although this is not a major pathway of metabolism.

Glutathione conjugation

Glutathione-S-tranferase are located in the microsome and cytosol (Hayes *et al.*, 2004). This enzyme catalyzes the transfer of glutathione to reactive electrophiles. Glutathione exist either as oxidised form (GSSG) or as reduced form (GSH) in the cell (Townsend & Tew, 2003). The GSH: GSSG ratio is important for maintaining cellular environment in reduced state. A reduced intracellular level of GSH can lead to severe oxidative stress and damage to the cell. In this pathway a mercapturate is formed, which serves to inactivate highly reactive quinone or epoxide intermediate formed during metabolism of certain drugs *e.g.* paracetamol. In condition like paracetamol overdose, large amounts of quinines are formed. This leads to reduced level of intracellular glutathione, which causes cell damage. However, this can be presented by supplying enogenous glutathione in the form of N-acetyl cysteine.

Almost all xenobiotics are metabolized by many pathways, either simultaneously or sequentially. As a result one drug can produce variety of metabolites. However, enzymes of intermediary metabolism like alcohol dehydrogenase, xanthine onidase, plasma choline esterase and monoamine oxidase also metabolise compounds like alcohol, allopurinol, succinylcholine and adrenaline respectively.

Some drugs undergo spontaneous molecular re-arrangement without any enzyme. Classical example is metabolism of atracurium and this is called as Hofmann elimination (Zheng *et al.*, 1997).

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Pharmacokinetics and Drug Interactions of Glycyrrhizin/Glycyrrhiza

A.K. AGGARWAL[1] AND D.K. MAJUMDAR[1,*]

ABSTRACT

Glycyrrhiza glabra (licorice) root contains a triterpenoid saponin called glycyrrhizin. Glycyrrhizin is being used in the treatment of hepatitis. Pharmacokinetics of glycyrrhizin has been evaluated in rats following intravenous (iv) and oral administration. Glycyrrhizin is a glycoside containing 2 glucuronic acid molecules attached to glycyrrhetic acid, the aglycone. After intravenous administration in rats, glycyrrhizin is secreted from liver into the bile and comes to intestine where it is hydrolyzed by intestinal bacteria to glycyrrhetic acid which is absorbed into the systemic circulation. Glycyrrhetic acid also is secreted from the liver as unchanged glycyrrhetic acid and glycyrrhetic acid monoglucoronide which is hydrolyzed in the intestine, reabsorbed and undergoes enterohepatic cycling thereby reducing clearance of the drug and prolonging its residence in the body. Thus decline in plasma concentration of glycyrrhizin, following iv administration is biexponential. Since secretion is an active process involving carriers, the transport of drug from liver to bile is a saturable process and pharmacokinetics of glycyrrhizin is nonlinear. Glycyrrhizin showed a dose-dependent pharmacokinetics. Glycyrrhizin and glycyrrhetic acid both being actively secreted and having molecular weight >250, would have high biliary clearance. Oral absorption of glycyrrhizin is very poor (bioavailability 1%), it is slowly metabolized by the intestinal bacteria to

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glycyrrhetic acid and absorbed. Intraperitoneal and nasal administration in rats has been found to improve bioavailability of glycyrrhizin. Studies in human subjects indicate oral glycyrrhizin is partly absorbed from gastrointestinal tract in intact form; rest of the dose is metabolized to glycyrrhetic acid and absorbed. The decline in plasma concentration following iv administration of glycyrrhizin is biexponential and no dose dependency of drug disposition has been observed. Glycyrrhizin iv administration in cirrhosis/hepatitis patients showed a monoexponential decline of drug in plasma indicating a linear pharmacokinetics of the drug and the pharmacokinetics are closely related to the extent of liver function. Similarly pharmacokinetics of glycyrrhizin (iv), in patients with chronic hepatitis C infection, has also been linear but no correlation between hepatic function and pharmacokinetics is observed which may be explained by the patients having milder liver disease. Oral administration of amoxicillin and metronidazole could destroy intestinal bacteria and thereby make coadministered glycyrrhizin ineffective. Glycyrrhizin could inhibit hepatic metabolism of corticosteroids like prednisolone resulting in toxicity. Licorice (glycyrrhizin) if concurrently administered with anticoagulant could cause bleeding due to its antiplatelet/antithrombin effect. Concurrent administration of licorice with potassium-losing diuretics, laxatives cause excessive potassium loss and may need potassium supplement. Licorice induced hypokalemia may cause digoxin toxicity. Licorice in combination with contraceptive may cause sodium and fluid retention and elevated blood pressure.

Key words : AUC, CLtotal, enterohepatic, exponential, glycyrrhizin, glycyrrhetic acid, MRT, hypokalemia

INTRODUCTION

Glycyrrhiza glabra (Leguminosae) is a plant of about 1.5 meter height having purplish blue flower. The dried roots and stolons of the plant are used as drug. *Glycyrrhiza* is also known as licorice root. *Glycyrrhiza* contains glycyrrhizin, a triterpenoid saponin and its sweet principle, and other bioactive components *e.g.* saponins, flavones, isoflavones, coumarins, triterpene sterols etc. Glycyrrhizin is a glycoside of glycyrrhetic (glycyrrhetinic) acid (Fig 1). On hydrolysis the glycoside loses its sweet taste and is converted to the aglycone glycyrrhetic acid and two molecules of glucuronic acid (Vyas & Majumdar, 2005).

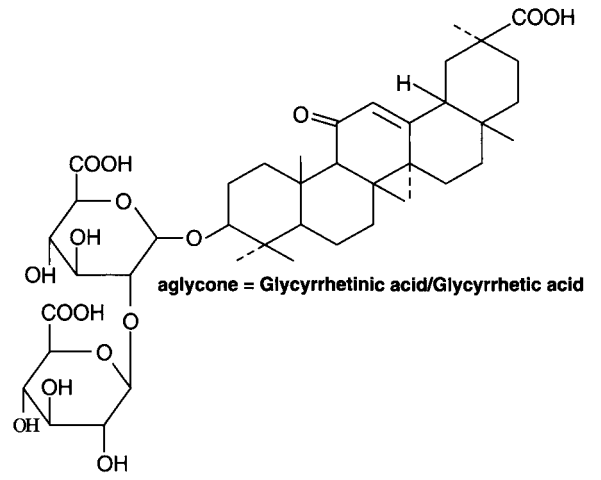

Fig 1. Glycyrrhizin

IUPAC NAME

(3-β,20-β)-20-Carboxy-11-oxo-30-norolean-12-en-3-yl 2-O-β-D-glucopyranuronosyl-α-D-glucopyranosiduronic acid.

Glycyrrhizin (glycyrrhizic acid, glycyrrhizinic acid, glycyrrhetinic acid glycoside) (C_{42} H_{62} O_{16}), mol weight 822.93, C=61.30%, H=7.59%, O=31.11 %, has been crystallized from glacial acetic acid. It has intensely sweet taste. It is freely soluble in hot water, alcohol and practically insoluble in ether. Ammonium glycyrrhizinate pentahydrate (C_{42} H_{65} NO_{16}· 5 H_2O) has been crystallized as needles from 75% aqueous ethanol.

The salt melts around 212-217 degree Celsius with decomposition. It is soluble in ammonia water and glacial acetic acid. It has UV absorption maximum at 248 nm. Dipotassium salt of glycyrrhizinic acid (C_{42} H_{60} K_2 O_{16}), mol wt 899.11 has also been reported (Merck Index, 2006).

USES

Traditionally licorice is used as demulcent and expectorant. The liquid extract is used in cough mixtures and as a flavoring agent in mixtures containing nauseous medicines *e.g.* ammonium chloride, the alkali iodide, quinine and cascara liquid extract. A decoction with linseed has been used as a domestic treatment of cough and bronchitis. Due to its mild anti-inflammatory and mineralocorticoid activities, licorice has been used as a substitute to corticosteroids. Deglycyrrizinated licorice having reduced mineralocorticoid activity has been used in peptic ulcer (Reynolds, 1989).

Recent studies indicate anti-inflammatory, antiallergic, antioxidant, antithrombin, antiviral, hepatoprotective, immunomodulatory, and antitumour activities of glycyrrhizin, an active constituent of *Glycyrrhiza glabra* (Vyas & Majumdar, 2005).

In Japan and India glycyrrhizin is being used in the treatment of hepatitis. Injection and tablet formulations of monoammonium glycyrrhizinate are available in the market.

Studies in the recent past have helped in better understanding of the metabolism/pharmacokinetics and adverse reaction of glycyrrhizin and glycyrrhiza. The relevant articles has been retrieved from the Medline and presented in the following section.

METABOLISM AND PHARMACOKINETICS

Ichikawa *et al.* (1986) studied biliary excretion of glycyrrhizin using rat with or without biliary fistulization. The plasma decay in the control rats without fistulization following an intravenous (*iv*) dose of 100 mg/kg of glycyrrhizin was generally biphasic. However, secondary peaks were observed in all rats in the elimination phase *i.e.* 0.5 to 12 h following dosing. The plasma concentration in the rats with biliary fistulization administered same dose showed a biexponential decline. The area under the plasma concentration-time curve (AUC) and total clearance (CLtotal) were significantly higher and lower in control rats respectively. The biliary excretion was 80.6 ± 9.9% of the administered dose and intestinal absorption was confirmed by using the bile collected after *iv* dosing. It was concluded that the glycyrrhizin was predominantly secreted from the liver into the bile and that the secondary peak in the elimination phase, the higher AUC and the lower total clearance in the control rats were due to the effects of the enterohepatic cycling of glycyrrhizin. Furthermore the transport of the drug from liver to the bile appears to be a saturable process

For appreciable biliary excretion the drug should be polar. It should be actively secreted and its molecular weight should be more than 250. Glycyrrhizin is actively secreted in the bile and its molecular weight is around 800. Thus it seems likely that glycyrrhizin will have high biliary clearance.

In a subsequent study pharmacokinetics of glycyrrhetic acid, a major metabolite of glycyrrhizin was evaluated in rats after bolus *iv* injection at a dose of 2, 5 or 12 mg/kg. The decline in plasma concentration was generally biexponential at each dose but the terminal disposition became much slower with increase in dose. A greater than proportional increase in plasma glycyrrhetic acid concentration was observed with increase of dose, suggesting a dose dependent glycyrrhetic acid disposition. Apparent total body clearance decreased significantly, with increase of dose. On the other

hand apparent steady state distribution volume after *iv* administration was unaffected by the dose. The plasma disposition at each dose fitted well to a two compartment pharmacokinetic model with Michaelis-Menten elimination. It was concluded that the pharmacokinetics of glycyrrhetic acid in the rat is dose dependent owing to a saturable elimination rate. The plasma level of glycyrrhetic acid after *iv* dosing (100 mg/kg) of glycyrrhizin in the control rats (without biliary fistulation) sustained the concentration range of 1.5-3 mg/mL during 1-48 h but that in the rats with biliary fistulation declined with time. It was suggested that the sustained plasma level of glycyrrhetic acid is accounted for by the intestinal absorption of glycyrrhetic acid produced from glycyrrhizin and glycyrrhetic acid conjugates during the enterohepatic cycling of both (Ishida *et al.*, 1989).

Tsai *et al.* (1992) studied pharmacokinetics of glycyrrhizin in rat after bolus *iv* administration at a dose of 20, 50 or 100 mg/kg. Concentration of glycyrrhizin was measured by HPLC. The decline in the concentration of glycyrrhizin in plasma was generally biexponential at each dose but the terminal disposition became much slower as the dose was increased. A greater than proportional increase in the glycyrrhizin concentration in plasma was observed with an increase in the dose, a result suggesting dose-dependent glycyrrhizin disposition. The disposition of drug in plasma at each dose level fitted well to a two compartment pharmacokinetic model. The apparent total body clearance decreased significantly with increase in the dose. On the other hand the apparent volume of distribution after *iv* administration was unaffected by the dose. The results indicate that the pharmacokinetics of glycyrrhizin is nonlinear.

Ishida *et al.* (1992) also investigated dose dependent pharmacokinetics of glycyrrhizin by measuring drug disappearance from plasma and biliary excretion in rats. The decline in plasma concentration was biexponential after *iv* dose of 5, 10, 20 or 50 mg/kg. Dosage however, had a marked effect on the pharmacokinetics with greater than proportional increase in area under the plasma concentration curve (AUC) at doses 20 and 50 mg/kg, even though the increase was proportional at doses of 5 and 10 mg/kg. There was also a significant increase of the steady state distribution volume (Vdss) as well as significant decreases in total body clearance (CL total) and biliary clearance at 20 and 50 mg/kg from those at 5-10 mg/kg and 5-20 mg/kg respectively. The AUC, Vdss and renal clearance (CLR) at a given dose showed no significant difference between rats with or without bile fistulas. The plasma unbound fraction (0.006-0.026) increased with increasing plasma glycyrrhizin concentration over the observed range (2-900 microgram/mL). No significant difference in Vdss for unbound glycyrrhizin was observed between the doses which indicate that the distribution of glycyrrhizin into tissues is not changed by an increase of dose. On the other hand a dose dependency of CL total for unbound glycyrrhizin was observed and confirmed to be attributable to

dose dependency in CLB for unbound glycyrrhizin since there was no significant difference in CLR or metabolic clearance for unbound glycyrrhizin between the doses.

In another study the role of enterohepatic circulation of glycyrrhetic acid in rats, was determined by kinetic analysis of glycyrrhetic acid. The concentration of glycyrrhetic acid in the plasma of control rat (without bile duct cannulation) during the first 5 h after *iv* administration of glycyrrhetic acid (2, 5, 10 and 20 mg/kg) were similar to those in the bile duct cannulated rat at each dose. No significant difference was observed in the values of the terminal half lives, total body clearance, the distribution volume at steady state, and the area under the curve of concentration in plasma versus time and the mean residence time (MRT) in each dose between both groups. When glycyrrhetic acid (2, 5, 10 and 20 mg/kg) was administrated *iv* to the bile duct cannulated rat, excretion of unchanged glycyrrhetic acid in bile was <1% of each dose, and that of glycyrrhetic acid 3-O-glucuronide was 1-2%. In the control rat, a secondary peak of glycyrrhetic acid was observed 12 h after *iv* administration of glycyrrhetic acid (20 mg/kg). The enterohepatic circulation of glycyrrhetic acid was confirmed by the link-rat method in which bile of the donor rat after *iv* administration of glycyrrhetic acid (20 mg/kg) was allowed to flow directly into the duodenum of the recipient rat. Glycyrrhetic acid was found in the plasma of the recipient rat after 6 h and its concentration reached maximum (approximately 0.5 mg/mL) 8-12 h after dosing the donor rat (Kawakami *et al.*, 1993).

Akao *et al.* (1994) prepared gnotobiote rats by infecting germ free rats with *Eubacterium* sp. strain GLH, a human bacterium capable of hydrolyzing glycyrrhizin to 18-beta-glycyrrhetic acid. Glycyrrhizin (100 mg/kg) was administered orally to conventional, germ free and gnotobiote rats but no glycyrrhizin could be detected in plasma 4 or 17 h after the administration. Plasma 18-beta-glycyrrhetic acid was not detected 4 or 17 h neither after the administration of glycyrrhizin to germ free rats nor could this compound be detected in caecal contents or in the faeces. However 18-beta-glycyrrhetic acid (0.6-2.6 nmole/mL) was detected in the plasma of the conventional and gnotobiote rats 4 and 17 h after the administration and the caecal contents after 4 h and the cumulative faeces up to 17 h of the conventional and the gnotobiote rats contained considerable amount of 18-beta glycyrrhetic acid. These findings indicate that orally administered glycyrrhizin is poorly absorbed from the gut but is hydrolyzed to 18-beta-glycyrrhetic acid by intestinal bacteria such as *Eubacterium* sp. strain GLH and the resulting 18-beta-glycyrrhetic acid is absorbed.

To clarify the metabolic fate of glycyrrhizin when orally ingested, a subsequent study investigated the bioavailability of glycyrrhetic acid after intravenous or oral administration of glycyrrhetic acid (5.7 mg/kg equimolar

to glycyrrhizin) or glycyrrhizin (10 mg/kg) at a therapeutic dose in rats. Plasma concentration of glycyrrhetic acid rapidly declined after its *iv* administration with AUC of 9200 ± 1050 ng.h/mL and MRT of 1.1 ± 0.2 h. The AUC and MRT values after oral administration were 10600± 1090 ng.h/mL and 9.3 ± 0.6 h respectively. After oral administration of glycyrrhizin the parent compound was not detectable in plasma at any time but glycyrrhetic acid was detected at a considerable concentration with AUC of 11700 ± 1580 ng.h/mL and MRT of 19.9 ± 1.3 h while glycyrrhetic acid was not detected in the plasma of germ free rats at 12 h after oral administration of glycyrrhizin. The AUC value of glycyrrhetic acid after oral administration of glycyrrhizin was comparable with those after intravenous and oral administration of glycyrrhetic acid indicating a complete biotransformation of glycyrrhizin to glycyrrhetic acid by intestinal bacteria and a complete absorption of the resulting glycyrrhetic acid from the intestine. Plasma glycyrrhizin rapidly decreased and disappeared in 2h after intravenous administration. AUC and MRT values were 2410 ± 125 microgram min/mL and 29.8 ± 0.5 min respectively. Plasma concentration of glycyrrhetic acid showed two peaks, a small peak at 30 min and a large peak at 11.4 h after intravenous administration of glycyrrhizin, with an AUC of 15400 ± 2620 ng.h/mL and an MRT of 18.8 ± 1.0 h. The plasma concentration profile of the later large peak was similar to that of glycyrrhetic acid after oral administration of glycyrrhizin, which slowly appeared and declined. The difference of MRT values (19.9 and 9.3 h) for plasma glycyrrhetic acid after oral administration of glycyrrhizin and glycyrrhetic acid suggests slow conversion of glycyrrhizin to glycyrrhetic acid in the intestine (Takeda *et al.*, 1996).

Pharmacokinetic behavior of glycyrrhizin after intravenous (*iv*), oral and intraperitoneal (*ip*) administration was compared in rats. The elimination half life, total body clearance and volume of distribution at steady state of glycyrrhizin were not significantly different among doses (2, 10, 50 mg/kg. *iv*).Glycyrrhizin was only detected in plasma (maximum level 1.3 mg/mL) after oral administration of 50 mg/kg. From comparison of the area under the plasma concentration-time curve after *iv* and oral administration of 50 mg/kg, the bioavailability of glycyrrhizin was estimated to be approximately 1%. Glycyrrhizin was stable for at least 3h in gastric juice. The plasma concentration of glycyrrhizin after oral administration to neomycin treated rats was not significantly different from that after administration to untreated rats. From the results it appeared that the extremely low bioavailability by the oral route may be due to poor absorption of glycyrrhizin from the intestinal tract. On the other hand, the plasma concentration of glycyrrhizin rapidly increased after *ip* administration of doses of 2, 10 and 50 mg/kg and reached a maximum level (4.7, 33, 238.9 microgram/mL respectively) with in 30 min. The bioavailability (65-90%) of glycyrrhizin after *ip* administration was enhanced dramatically. The *ip* route of

administration may thus improve the bioavailability of glycyrrhizin (Yamamura *et al.*, 1995).

A recent study has reported improved bioavailability of glycyrrhizin in rat after nasal administration. The absolute availability has been reported to be 20% which was much higher than that observed after oral administration (Sasaki *et al.*, 2003).

Pharmacokinetic profile of glycyrrhizin and its metabolites after oral and intravenous administration of glycyrrhizin to healthy human volunteers has also been reported. An improved HPLC method was used to monitor glycyrrhizin and its metabolites in plasma, and glycyrrhizin could be detected at 500 ng/mL level. After oral administration of glycyrrhizin (100 mg) to three normal subjects, the major metabolite of glycyrrhizin (*i.e.* glycyrrhetic acid) appeared in plasma (<200 ng/mL) but glycyrrhizin was not found. On the other hand glycyrrhizin was found in urine and the amount excreted was 1.1-2.5% of the dose. This finding suggests that glycyrrhizin is partly absorbed in the intact form from the gastro-intestinal tract. The concentration of glycyrrhizin in plasma after *iv* administration of glycyrrhizin (40, 80, 120 mg) showed biexponential profile during the 24 h period after administration of each dose, which suggests a two compartment model of distribution of the drug. The glycyrrhizin metabolite, glycyrrhetic acid and glycyrrhetic acid -3-O-glucuronide were not detected in either plasma or urine. The terminal half life of glycyrrhizin, the apparent volume of central compartment, the steady state distribution volume and the total body clearance in three dosing experiments were 2.7-4.8 h, 37-64 mL/kg, 59-98 mL/kg and 16-25 mL/kg/h respectively. Glycyrrhizin was not detected in plasma after oral administration of the usual therapeutic dose of glycyrrhizin and no dose dependency of the drug was observed in dose range of 40-120 mg (Yamamura *et al.*, 1992).

The effect of components of aqueous licorice root extract (LE) on the pharmacokinetics of glycyrrhizin and glycyrrhetic acid was investigated in rats and humans. The aim of the work was to define the role of pharmacokinetics on glycyrrhizin toxicity. Significantly lower glycyrrhizin and glycyrrhetic acid plasma levels were found in rats and humans treated with LE compared to the levels obtained with those in which glycyrrhizin alone was present. The pharmacokinetic curve showed significant differences in the area under the plasma-time curve (AUC), C_{max} and T_{max} parameters. The data obtained from urine samples were in agreement with the above results and confirm a reduced availability of glycyrrhizin present in LE compared to pure glycyrrhizin. This should be attributed to the interaction during intestinal absorption between the glycyrrhizin constituent and the several constituent of LE. The modified bioavailability could explain the

various clinical adverse effects resulting from the chronic administration of glycyrrhizin alone as opposed to LE (Cantelli-Forti *et al.*, 1994)

The pharmacokinetic behavior of glycyrrhizin in four patients with acute hepatitis (hepatitis group) and six patients with liver cirrhosis (cirrhosis group) receiving chronically an *iv* administration of a 120 mg dose once a day or once every other day of glycyrrhizin was investigated. The plasma concentration of glycyrrhizin declined monoexponentially in both groups. The elimination half life for glycyrrhizin in hepatitis and cirrhosis group varied significantly in the range of 2.7-7.6 h and 6.2-40.1 h and total body clearance (CL total) in the range of 2.8-23.2 mL/h/kg and 1.4-12.9 mL/h/kg respectively. The half life for glycyrrhizin in the hepatitis and cirrhosis group was about twice and eight times that in normal subjects respectively and CL total values were about 0.7 and 0.23 times that in normal subjects respectively. There was significant correlation between CL total and hepatic functions (serum aspartate aminotransferase and alanine aminotransferase) in both patient groups. With improvements of the liver function the CL total for glycyrrhizin increased from 2.8 mL/h/kg to 11.4 mL/h/kg and the half life shortened from 7.6 h to 3.4 h. These findings indicated that the variation of pharmacokinetic behavior of glycyrrhizin in both groups was closely related to the extent of the liver function (Yamamura *et al.*, 1995).

In a subsequent study pharmacokinetics of *iv* glycyrrhizin was evaluated after single and multiple doses in European patients with chronic hepatitis C infection. Patients received 80, 160 or 240 mg glycyrrhizin three times a week or 200 mg glycyrrhizin six times a week. Glycyrrhizin level was determined by HPLC. Decline in plasma glycyrrhizin concentration was monoexponential. Glycyrrhizin exhibited linear pharmacokinetics up to 200 mg dose. No significant differences in CL total and half life were observed between cirrhotic and non cirrhotic patients. No correlation between hepatic function and pharmacokinetics was found, probably because patients with severe liver disease were excluded in the study (Van Rossum *et al.*, 1999).

The intestinal bacteria, *Eubacterium* sp. and *Bifidobacterium* sp., participate in the metabolism of glycyrrhizin which is an active ingredient of kampo-medicines. Most antibiotics have activity against intestinal bacteria. This means that antibiotics may lower the metabolism of glycyrrhizin when administered in combination. On the other hand, it is also highly possible that bacterial preparations such as *Bifidobacterium longum, Clostridium butyricum* and *Streptococcus faecalis* increase the number of *Eubacterium* sp. and *Bifidobacterium* sp., resulting in enhanced metabolism of glycyrrhizin when they are used concomitantly with kampo-medicines. The studies suggested that the drug interactions of kampo-medicines with antibiotics and bacterial preparations should be confirmed in clinical studies (Ishihara *et al.*, 2002).

The influence of synthetic drugs prescribed for peptic ulcer on the pharmacokinetic fate of glycyrrhizin from Shaoyao-Gancao-tang (SGT, a traditional Chinese formulation) was investigated in rats. Co-administration of histamine H2-receptor antagonist (cimetidine) and anticholinergic drug (scopolamine butyl bromide) with SGT didn't influence the area under the plasma concentration-time curves (AUC) of glycyrrhetic acid, an active metabolite derived from glycyrrhizin in SGT. The AUC of glycyrrhetic acid from SGT were significantly reduced by co-administration of synthetic drugs commonly used for peptic ulcer in a triple therapy (OAM), a combination of a proton pump inhibitor (omeprazole) and two antibiotics (amoxicillin and metronidazole). The reduction of AUC in OAM treatment was due to the antibacterial effect of amoxicillin and metronidazole on intestinal bacteria in rat which lead to the decrease of glycyrrhizin hydrolysis activity. The study suggests that it may not be a proper way to use triple therapy containing antibiotics simultaneously with SGT for healing of chronic ulcers (He *et al.*, 2001).

Another study indicates that, the bioavailability of glycyrrhizin in SGT is severely reduced by a single administration of amoxicillin and metronidazole (AMPC-MET), and the reducing effect continues for 12 days. In order to reduce the negative effect of AMPC-MET on the bioavailability of glycyrrhizin, the optimum scheduling of the medications was examined. It was found that the reduction in the plasma glycyrrhetic acid concentration and the glycyrrhizin -metabolizing activity in faeces caused by a single dose of AMPC-MET could be sharply attenuated by the repetitive administration of SGT for 4 days. The glycyrrhetic acid concentration and the glycyrrhizin-metabolizing activity were strongly enhanced by further continuous administration of SGT. These findings suggest that repetitive administration of SGT starting 1 or 2 days after the administration of AMPC-MET speeds the recovery of the bioavailability of glycyrrhizin in SGT. Such strategies for administering medications may also be useful for combination therapy of antibiotics with other traditional Chinese formulations containing bioactive glycosides (He *et al.*, 2003).

The pharmacokinetics of total and free prednisolone (PSL) in six healthy men with or without pretreatment with oral glycyrrhizin was investigated to confirm whether oral administration of glycyrrhizin influences the metabolism of PSL in man. Each subject received an *iv* administration of 0.096 mg/kg of prednisolone hemisuccinate (PSL-HS) with or without pretreatment with 50 mg oral glycyrrhizin four times. Total PSL and free PSL in plasma were measured by HPLC and isocolloid osmolar equilibrium dialysis method. The pharmacokinetic parameters of PSL were determined by non-compartmental analysis. Oral administration of glycyrrhizin was found to significantly increase the concentration of total PSL at 6, 8 h and of the free PSL at 4, 6 and 8 h after PSL-HS. After oral administration of glycyrrhizin the area under the curve (AUC) was

significantly increased, total plasma clearance (CLtotal) was significantly decreased and the mean residence time (MRT) was significantly prolonged. However the volume of distribution (Vdss) showed no evident change. This suggests that oral administration of glycyrrhizin increases the plasma PSLconcentrations and influences its pharmacokinetics by inhibiting its metabolism but not by affecting its distribution (Chen *et al.*, 1991).

Prolonged intake of licorice extract (LE) or glycyrrhizin on murine liver cyp catalyzed drug metabolism was studied. Results indicate that the induction of cytochrome P450 dependent activities by prolonged intake of high LE or glycyrrhizin doses may result in accelerated metabolism of coadministred drug with important implications for their disposition (Paolini *et al.*, 1998).

Pharmacokinetics of glycyrrhizin following oral and *iv* administration has been summarized below (Fig 2).

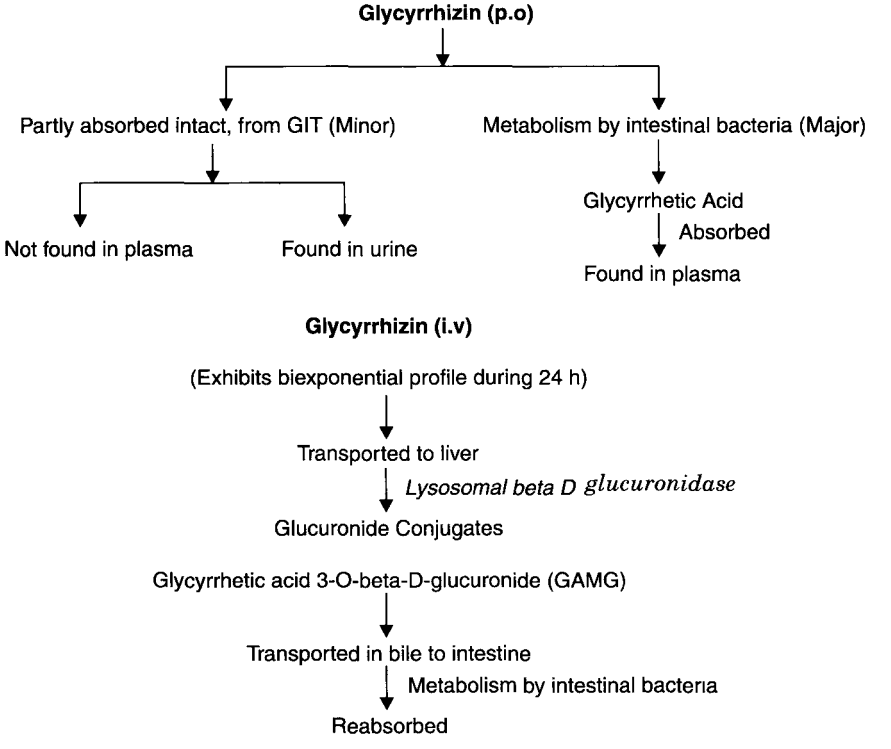

Fig 2. Pharmacokinetics of glycyrrhizin following oral and *iv* administration

LICORICE DRUG INTERACTIONS

Licorice has been found to interact with a number of drugs leading to effects varying from increased risk of bleeding, hypokalemia, elevated blood

pressure, increased risk of corticosteroid adverse effects and increased risk of digoxin toxicity.

The following table (Table 1) gives an overview of the various dimensions of licorice interactions along with the probable mechanisms involved and clinical management of the adverse effects.

Table 1. Licorice Drug Interactions

Drugs	Effect of the interaction with licorice	Summary of the interaction	Management	Mechanism
ALTEPLASE (for the treatment of acute ischemic stroke), ASPIRIN (NSAID), DIPYRIDAMOLE,(inhibits thrombus formation) PRAVASTATIN SODIUM (HMG-coA reductase inhibitor), CLOPIDOGREL (potent oral antiplatelet agent), DICUMAROL (Vitamin K antagonist).	The risk of bleeding increases.	Licorice may potentiate the effects of antiplatelet drugs (Norred & Brinker, 2001; Heck *et al.*, 2000). Licorice root contains coumarins and derivatives of coumarin which are anticoagulants (Heck *et al.*, 2000). Glycyrrhizin demonstrated selective thrombin inhibition activity *in vitro* (Francischetti *et al.*, 1997).	Caution is primarily advised if licorice is to be taken along with an antiplatelet agent. The signs and symptoms of excessive bleeding should be monitored and dose of drug adjusted accordingly.	Licorice causes inhibition of thrombin and platelet aggregation.
ANTACIDS, CASTOR OIL (strong laxative), PERU BALSAM, BISACODYL.	Hypokalemia may increase due to concurrent administration.	With concurrent use of licorice and laxatives hypokalemic paralysis has been reported (Hussain, 2003; Lin *et al.*, 2003; Shintani *et al.*, 1992; Corsi *et al.*, 1983) Licorice alone could cause hypokalemia, hypertension, and cardiac arrhythmia. (Dellow *et al.*, 1999; Eriksson *et al.*, 1999; Kageyama *et al.*, 1997; Bernardi *et al.*, 1994; Blachley & Knochel, 1980; Wash & Bernard, 1975).	Concomitant use of licorice and laxatives should be avoided.	Excessive potassium loss.

Table *Contd.*

Table 1. *Contd.*

Drugs	Effect of the interaction with licorice	Summary of the interaction	Management	Mechanism
ORAL CONTRACEPTIVE (Estrogen and progestogen combination).	Elevated blood pressure and increased risk of fluid retention.	Elevated blood pressure and increased fluid retention is quite common with simultaneous use of licorice and the oral contraceptives (Bernardi *et al.*, 1994) which may be associated with the estrogen and/or progesterone. 3-monoglucuronyl-glycyrrhetinic acid (3MGA), a metabolite of glycyrrhetinic acid inhibits 11-beta-hydroxysteroid dehydrogenase and reduces breakdown of cortisol, resulting in a hypermineralocorticoid effect (Kato *et al.*, 1995; Walker & Edwards, 1994).	If licorice is used with oral contraceptives and the patient develops fluid retention or hypertension, concurrent use should be discontinued.	Mineralo-corticoid effect increased.
TESTOSTERONE (steroid hormone).	Testosterone effectiveness decreases.	Endogenous testosterone levels in healthy men and in women with polycystic ovary disease were significantly reduced when licorice was administered (Armanini *et al.*, 1999; Takahashi & Kitao, 1994). It may inhibit conversion of androstenedione to testosterone through inhibition of 17-beta-hydroxysteroid dehydrogenase and 17, 20-lyase (Armanini *et al.*, 1999; Sakamoto	Concomitant use of licorice and testosterone. should be avoided.	The inhibition of 17-beta-hydroxy-steroid dehydrogenase and 17, 20-lyase responsible for the catalytic conversion of androstenedione to testosterone; increase in the estradiol synthesis due to aromatase stimulation.

Table *Contd.*

Table 1. *Contd.*

Drugs	Effect of the interaction with licorice	Summary of the interaction	Management	Mechanism
		& Wakabayashi, 1988; Takeuchi *et al.*, 1991). Some studies suggest that licorice may also stimulate aromatase activity and increase the estradiol to testosterone ratio.		
BETAMETHASONE, CORTICOTROPIN, CORTISONE, DEXAMETHASONE.	Chances of adverse effects of corticosteroid increase	Glycyrrhizin increased the AUC of prednisolone and decreased the clearance in healthy subjects (Homma *et al.*, 1994; Chen *et al.*, 1990) The activity of topically applied hydrocortisone was potentiated by glycyrrhetic acid in healthy subjects (Teelucksingh *et al.*, 1990).	Lower corticosteroid dose may be required to avoid adverse effects if licorice is to be used.	Corticosteroid metabolism inhibition by licorice.
DIGOXIN	Digoxin toxicity may increase	The symptoms of congestive heart failure and hypokalemia appeared in a patient taking licorice, furosemide, and digoxin (Harada *et al.*, 2002).	Concurrent use of licorice and digoxin must be avoided.	Hypokalemia caused by licorice increase the risk of digoxin toxicity.
BENDROFLUMETHIAZIDE, BENZTHIAZIDE, BUMETANIDE, CHLOROTHIAZIDE, CHLORTHALIDONE, CYCLOTHIAZIDE (Potassium-losing diuretics).	Reduced effectiveness of the diuretic and increased risk of hypokalemia.	Some studies reported that patients experienced hypokalemia and hypertension with concomitant use of licorice and diuretics (Harada *et al.*, 2002; Folkerson *et al.*, 1996; Farese *et al.*, 1991).	Concurrent use of licorice and potassium- losing-diuretics need to be avoided. Concurrent administration may need potassium supplement.	Licorice ingestion causes pseudoaldosteronism resulting in hypokalemia.

SUMMARY

Glycyrrhiza glabra (licorice) root contains a triterpenoid saponin called glycyrrhizin. Glycyrrhizin is being used in the treatment of hepatitis. Pharmacokinetics of glycyrrhizin has been evaluated in rats following intravenous *iv* and oral administration. Glycyrrhizin is a glycoside containing 2 glucuronic acid molecules attached to glycyrrhetic acid, the aglycone. After intravenous administration in rats, glycyrrhizin is secreted from liver into the bile and comes to intestine where it is hydrolyzed by intestinal bacteria to glycyrrhetic acid which is absorbed into the systemic circulation giving rise to a secondary peak in the elimination phase. Glycyrrhetic acid also is secreted from the liver as unchanged glycyrrhetic acid and glycyrrhetic acid monoglucoronide which is hydrolyzed in the intestine, reabsorbed and undergoes enterohepatic cycling thereby reducing clearance of the drug and prolonging its residence in the body. Thus decline in plasma concentration of glycyrrhizin, following *iv* administration is biexponential. Since secretion is an active process involving carriers, the transport of drug from liver to bile is a saturable process and pharmacokinetics of glycyrrhizin is nonlinear. Glycyrrhizin showed a dose-dependent pharmacokinetics. Glycyrrhizin and glycyrrhetic acid both being actively secreted and having molecular weight >250, would have high biliary clearance. Oral absorption of glycyrrhizin is very poor (bioavailability 1%) probably due to its high molecular weight and polarity, it is slowly metabolized by the intestinal bacteria to glycyrrhetic acid and absorbed. Prolonged intestinal transit could increase the extent of absorption of glycyrrhetic acid from the gut. Intraperitoneal and nasal administration in rats has been found to improve bioavailability of glycyrrhizin. Studies in human subjects indicate oral glycyrrhizin is partly absorbed from gastrointestinal tract in intact form; rest of the dose is metabolized to glycyrrhetic acid and absorbed. The decline in plasma concentration following *iv* administration of glycyrrhizin is biexponential and no dose dependency of drug disposition has been observed. Glycyrrhizin *iv* administration in cirrhosis/hepatitis patients showed a monoexponential decline of drug in plasma indicating a linear pharmacokinetics of the drug and the pharmacokinetics are closely related to the extent of liver function. Similarly pharmacokinetics of glycyrrhizin *iv*, in patients with chronic hepatitis C infection, has also been linear but no correlation between hepatic function and pharmacokinetics is observed which may be explained by the patients having milder liver disease. Oral administration of amoxicillin and metronidazole could destroy intestinal bacteria and thereby make coadministered glycyrrhizin ineffective by inhibiting its hydrolysis to glycyrrhetic acid. Glycyrrhizin could inhibit hepatic metabolism of corticosteroids like prednisolone resulting in toxicity. Licorice (glycyrrhizin) if concurrently administered with anticoagulant could cause bleeding due to its antiplatelet/antithrombin effect. Concurrent administration of licorice

with potassium-losing diuretics, laxatives cause excessive potassium loss and may need potassium supplement. Licorice induced hypokalemia may cause digoxin toxicity. Licorice in combination with contraceptive may cause sodium and fluid retention and elevated blood pressure. In human subjects, oral absorption of glycyrrhizin from aqueous licorice root extract has been found to be lower than that obtained after administration of pure glycyrrhizin. Thus prolonged administration of pure glycyrrhizin carries more chances of adverse effects as opposed to crude licorice root extract.

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21

Determination of Total Antioxidant Status and Rosmarinic Acid from *Orthosiphon stamineus* in Rat Plasma

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ABSTRACT

A simple high-performance liquid chromatography (HPLC) method was developed to determine the content of rosmarinic acid (RA) in plasma of rat collected at different time (0-8 h) after oral administration of Orthosiphon stamineus leaf extract. The features of the assay include protein precipitation using acetonitrile, isocratic elution and reverse phase C-18 column with ultraviolet (UV) detection. The maximum concentration of RA in the plasma was 1.10 ± 0.34 μg/mL at 1 h after oral administration of the extract. The extract showed significant reduction ($p<0.05$) of malondialdehyde (MDA) levels in plasma samples of rats fed with the methanol extract compared with a control group. The total antioxidant status in plasma was significantly ($p<0.05$) elevated in rats treated with the methanol extract compared to normal controls.

Key words : Antioxidant status, lipid peroxidation, *Orthosiphon stamineus*, plasma, rosmarinic acid

INTRODUCTION

Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, which are important natural bioactive substances occurring widely in food plants (Ho *et al.*, 1992). It is a well

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known natural product extracted from rosemary plant (*Rosmarinus officinalis*), and other members of Labiatae (Litvinenko *et al.,* 2001). It occurs throughout the Boraginaceae, Lamiaceae, Zosteraceae (Takeda *et al.,* 1990) and in lower plants such as the hornworts (Ravn *et al.,* 1994). RA has a various medicinal values and well characterized physiological functions. The main activities are astringent, antioxidative, anti-inflammatory, antimutagen, antibacterial and antiviral (Parnham & Kesselring, 1985). RA obtained from plants is a multi-active substance used in cosmetics to maintain healthy skin due to its antioxidant qualities which is superior to that of vitamin E (Leung & Foster, 1996).

Generally, after administration of a drug, large amount distributes throughout the body and resided in the body at various times, some leaving soon after absorption whilst others taking a longer time to eliminate. Polyphenolic substances such as phenolic acids and flavonoids are reported to be degraded by gut flora and then metabolized in tissues such as kidneys, liver and intestine (Williamson *et al.,* 2000; Rechner *et al.,* 2002). *Orthosiphon stamineus* has several chemically active constituents including RA, the most abundant phenolic acid in the leaves. There is scarcely any report on the absorption studies of bioactive polyphenolic acid constituents in *O. stamineus* leaf due to the complexity of these components in the leaf hence this report describes a simple, rapid and validated HPLC method for the determination of RA from *O. stamineus* leaf extract in rat plasma after oral administration. The study also includes evaluation of plasma lipid peroxidation inhibition and total plasma antioxidant status in rats.

MATERIALS AND METHODS

Chemical and Reagents

Rosmarinic acid was purchased from Sigma Chemical Company (St. Louis MO, USA). 2,2′-azino-di[3-ethylbezthiazoline sulfonate (ABTS), thiobarbituric acid (TBA), trichlroacetic acid, 1,1,3,3-tetraethoxypropane were obtained from Sigma (USA). Methanol, acetonitrile, and water (acidified to pH 3 with phosphoric acid), were obtained from Merck (Darmstadt, Germany). Membrane filters (0.45 μm pore size) from Millipore were used for filtration of the mobile phase and the samples. All solvents were of analytical grade or HPLC grade.

Plant Samples

Plants were grown from cuttings using standard agronomic practices at Kepala Batas (Pinang, Malaysia). The cultivated leaves were collected in late afternoon, from 30- to 45-day-old plants. Voucher specimen of the plant material was deposited at Bilik Herba, School of Pharmaceutical Sciences, Universiti Sains Malaysia.

Preparation of Extracts

Powdered dried leaves of *A. paniculata* (1.0 kg) were extracted with methanol using soxhlet. The extract was filtered (Whatman No. 1) with a Buchner filter and concentrated under vacuum. 100 g of the methanol extract was freeze-dried. The content of RA in the freeze dried extract was determined by HPLC before oral administration.

Animals and Dosage

Experimental animals used were Sprague-Dawley rats, weighing 220-250 g, from the Animal House of University Sains Malaysia. The animals were kept in the animal room (25 ± 2°C) under 12 h light/dark cycle and fed with standard diet and free access to distilled water prior to the start of the study. Animals were maintained and handled according to the recommendations of the local ethical committee which approved the design of the animal experiments.

Sampling of Blood

Six overnight fasted rats were treated orally with single dose of *O. stamineus* freeze-dried extract at 1 g/kg body weight in 10 mL of the extract suspension. The freeze-dried extract was dissolved in distilled water and vortex prior to oral administration. The animals were put under anaesthesia with sodium pentobarbital (50 mg/kg *i.p.*) and remained anesthetized throughout the experimental period. The jugular vein was cannulated with Pe-50 tubing to collect blood with an established heparin-lock using 100 U/mL heparin in saline. Blood samples (0.3 mL) were taken using a disposable syringe at 0 min (pre-dose) 0.5, 1, 2, 3, 4, 6 and 8 h post dose. Blood samples were immediately transferred to a heparinized microcentrifuge tube and centrifuged at 3000 rpm for 10 min at 5°C. The resulting plasma sample (0.2 mL supernatant) was transferred into 1.5 mL tubes and stored at –80 °C until assayed.

Preparation of Standard Solutions of Plasma

The stock solution 0.1 mg/mL of the reference RA was freshly prepared in acetonitrile. Solutions of RA in the range of 0.1 – 10 μg/mL were prepared in blank plasma samples for calibration curve. Spiked plasma samples were vortexed for 15 seconds and centrifuged at 3000 × g for 5 min. at 5°C. The resulting supernatants were used for injection. Quality control (QC) samples at the concentration of 0.1 μg/mL, 1 μg/mL and 10 μg/mL were prepared by the same procedures as described.

HPLC Conditions for RA in Plasma

The plasma (0.2 mL) was precipitated with acetonitrile (0.3 mL) and centrifuged at 3000 × g for 10 min. at 5°C. The organic layer was transferred into an empty tube and dried under a stream of nitrogen at 40°C. The

residue was reconstituted in acetonitrile to give a 0.5 mL solution. The solution was passed through membrane filter (pore size 0.5 μm,) prior to HPLC analysis. HPLC analysis was performed with a Gilson HPLC pump (Model 305), a Gilson UV/VIS detector (Gilson Medical Electronics, Villiersss-le-Bel, France) connected to a Hitachi D-2500 Chromato-integrator (Hitachi, Tokyo, Japan) and a Rheodyne sample injector valve fitted with a 50 μl sample loop. The detector was operated using a sensitivity range of 0.005 AUFS. A LiChrosorb RP-18 column (250 mm × 4.6 *i.d.* mm, 10 μm particle size) (Merck) was used. The chromatographic conditions used for analyzing plasma samples were; Flow-rate of 1 mL/min; Injection volume of 20 μl; UV detection at 340 nm and ambient temperature for all HPLC analysis. The mobile phase for elution of RA was methanol: water (pH = 3) (8:2; v/v).

Validation of HPLC Method

Linearity and sensitivity: The linearity of the responses was determined for seven concentrations in plasma by three injections. The contents of RA were calculated using regression parameters obtained from the standard curve for blank plasma samples. The limit of quantification (LOQ) was established at signal to noise ratio (S/N) of 10.

Precision

Intra- and inter-day precision of the assay were determined five times on the same day and continuously for 5 days at the concentration of the QC solutions. The intra- and inter-assay relative standard deviation was used to validate the precision of the assay by determining standard samples of RA in plasma.

Accuracy

Accuracy was determined by recovery studies. Recoveries of RA from plasma were estimated by comparing the area obtained from injections of standard solutions (QC solutions) in blank plasma to those in acetonitrile. The mean recoveries were determined at the concentration of the QC solutions in triplicates.

Stability

The stability of the reference compound was studied in the plasma and acetonitrile precipitated plasma with the QC samples. The samples were put aside at room temperature for 12 h, or stored in a refrigerator at 4°C and –20°C for 1, 2, and 3 days; –80°C for 1 month. The content of RA was calculated. The compound was considered stable if the variation of the content was less than 10% of initial time concentration or response (Hu & Morris, 2003).

Data Analysis

Calibration Curves

Standard calibration curve for RA was prepared and the weight was computed with equation obtained from linear regression analysis;

$$y = mx + b$$

where y = relative peak area of analyte, m = slope of the line generated by a standard curve; x = concentration of analyte found (μg/mL); b = intercept of the line generated by the standard curve.

Amount of RA in plasma: The amount of analyte found in plasma (ng/mL) was calculated as follows: $A = C \times V_f / V_p$

where A = ng/mL of the analyte found in test sample; C = concentration in μg/mL of the analyte found in test samples from standard curve; V_p = test portion (0.2 mL, plasma); volume V_f = final volume (0.5 mL, plasma).

Pharmacokinetics Parameters

The following pharmacokinetic parameters were determined for the period of 0-8 h: the area under plasma concentration time curve from time zero to the last measurable RA sample time ($AUC_{0\text{-}8\ h}$); maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were determined directly from the data. The elimination rate constant Kel (h^{-1}) was calculated by log /linear regression using terminal phase of the plasma concentration time plot. The half-life, $t_{1/2}$, (h) was calculated by dividing 0.693 by Kel.

Lipid Peroxidation (MDA assay) and Plasma Antioxidant Status

Experimental animals used were Sprague-Dawley rats, weighing 220-250 g. The rats were separated into 2 groups with each group containing six animals (n = 6). The animals in the first group were treated orally with single dose of 1 g/kg body weight in 10 mL of the extract suspension per day for two weeks. The animals in the second group served as a control were given appropriate amount of distilled water. 24 h after the administration of the last dose of the extract, the blood samples were collected from the orbital plexus. Blood samples were immediately transferred to a heparinized microcentrifuge tube and centrifuged at 3000 rpm for 10 min. at 5°C. The resulting plasma sample (supernatant) stored at –80°C until assayed. Rats were not deprived of feed before obtaining their blood samples to prevent possible changes in plasma antioxidant status cause by latent stresses of food deprivation.

Thiobarbituric acid reactive substances (TBARS) content of the plasma samples was determined by the method of Drapper *et al.* (1993). The amount of TBARS produced was calculated using 1,1,3,3, tetraethoxypropane as a standard. Lipid peroxidation was expressed in nmoles of TBARS formed per mL of the sample. Plasma antioxidant status of the samples was determined

by the method of described by Miller *et al.* (1993). Trolox was used as a standard. The result was expressed as nmoles of trolox equivalent antioxidant capacity (TEAC) per litre of sample.

Data are presented as means ± S.D. (n=6). Student's *t-test* was used for statistical significance between groups. P values<0.05 were considered statistically significant.

RESULTS

HPLC Assay Development and Validation

The RA (Fig 1) content of the freeze dried extract determined prior to oral administration to the animals was 4.10% w/w. External standard HPLC method was used to analyze RA in plasma samples following the oral administration of *O. stamineus* (1 g/kg body weight) to rat. As shown in Fig 2, RA (t_R =14.8 min) in the plasma was well resolved by using a mixture of methanol: water (pH = 3) (8:2). Deproteinization of plasma samples with acetonitrile resulted in less interference from endogenous compounds, best accuracy, precision and recovery. The HPLC method was validated; parameters included in the method validation were linearity, accuracy, precision and limit of quantification. The calibration curves were linear over the concentration range of 0.1–10 µg/mL with correlation coefficient greater than 0.998 in all standard curves. The limit of quantification (LOQ) of RA determined in blank rat plasma samples at signal to noise ratio of 10 was 50 ng/mL. The intra- and inter-assay relative standard deviation was used to validate the precision of the assay. Table 1 shows the results of inter- and intra-day precision and accuracy for RA in rat plasma. Acceptable precision was achieved with the method as revealed by the R.S.D. data, which did not exceed 10%. The mean recoveries determined at the QCs in triplicates were between 90.64 - 95.12%. The R.S.D. of recovery from plasma was less than 10%. The compound was stable at room temperature, -20°C and 4°C in plasma. No peaks corresponding to degradation products were observed.

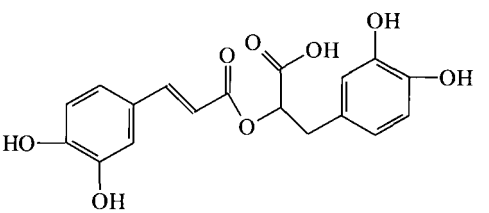

Fig 1. Structure of rosmarinic acid

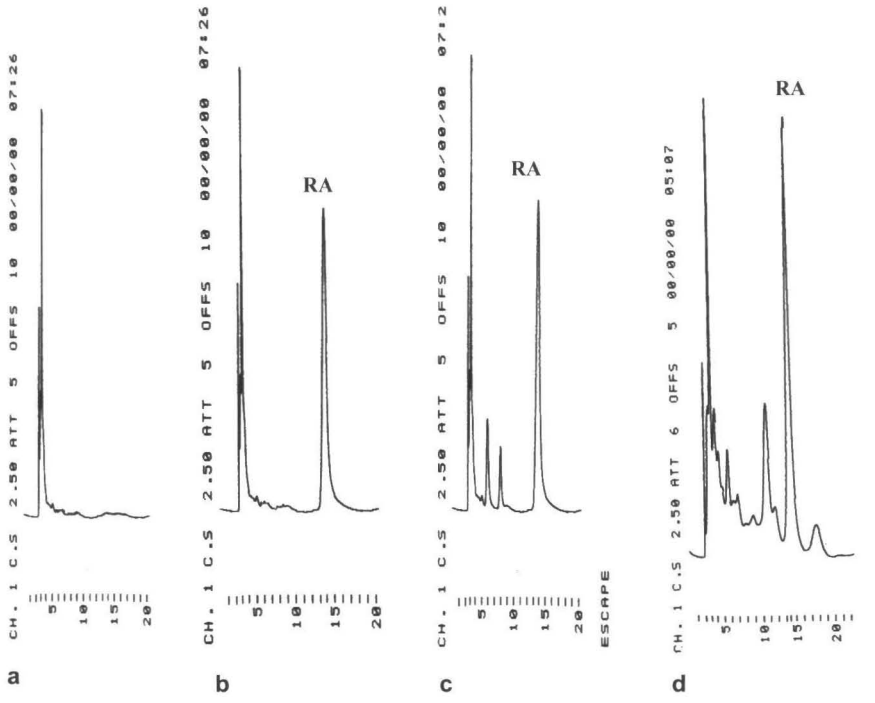

Fig 2. Chromatograms for rat plasma samples obtained from the analyses of rosmarinic acid (RA) from *O. stamineus*, [a] blank plasma, [b] blank plasma spiked with RA, [c] a rat plasma sample obtained after an oral dose of 1g/kg of the extract. (d) Methanol leaf extract. Chromatographic peaks were identified with the aid of pure reference standard based on retention time t_R

Table 1. Precision and accuracy of rosmarinic acid in rat plasma

Concentration added(µg/mL)	Precision (n=5)		Accuracy (n=3)	
	Intra-day (RSD, %)	Inter-day (RSD, %)	Recovery[a] (%)	RSD[b] (%)
0.1	5.12	5.60	90.64	9.65
1	4.39	3.85	92.25	8.74
10	2.73	2.47	95.12	6.89

RSD: relative standard deviation in percentage

[a] Recovery = (calculated conc/spike conc) x 100.

[b] RSD = (SD/conc) x 100

The validated method was used for determination of RA in rat plasma samples after oral administration of *O. stamineus* extracts. The time course of plasma concentrations of RA after oral administration of the extract is shown in Fig 3. The parameters estimated from the curve included, $t_{1/2}$ = 2.28 ± 0.30 h, T_{max} = 1 h, C_{max} = 1.10 ± 0.35 µg/mL and $AUC_{0\text{-}8\ h}$ = 4.87 ± 1.56 µg/mL h.

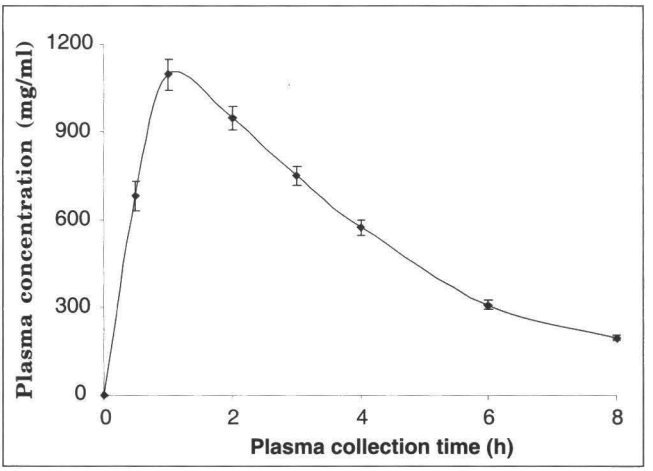

Fig 3. Plasma concentration-time curve of rosmarinic acid after oral administration of *Orthosiphon stamineus* with a single dose of 1 g/kg in rats. Data are means ± S.E. (*n=6*)

Lipid Peroxidation (MDA assay) and Total Antioxidant Status

Fig 4 shows the effect of 14 days treatment of rats with single dose of the extract (1 g/kg of body weight) per day on malondialdehyde (MDA) levels and antioxidant status in plasma. The MDA levels were significantly reduced ($p<0.05$) in plasma samples of rats fed with the methanol extract compared with the control group. Significant lipid peroxidation inhibition of 30.2% as compared to the control group was observed in rats treated orally with the extract. The total antioxidant status in plasma was significantly

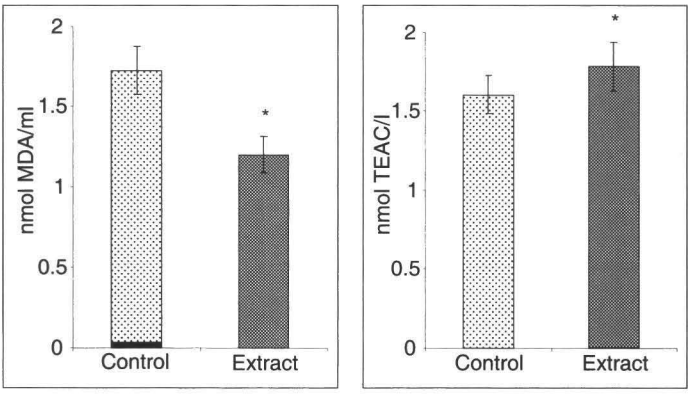

Fig 4. Effect of methanolic extract of *Orthosiphon stamineus* on plasma MDA levels (A) and plasma total antioxidant status (B) of rats treated orally with single dose of 1 g/kg body weight in 10 mL of the extract suspension for two weeks. Data are means ± S.D. (n=6). Student's *t-test* was used for statistical significance between groups. * $p<0.05$ as compared to control group

elevated in rats treated with the methanol extract compared to normal controls. An average increase of 11.3% in total antioxidant status was observed in plasma of rats treated orally with the extract.

DISCUSSION

In our earlier studies, the methanolic extract of *O. stamineus* leaves was reported to exhibit significant radical scavenging activity in many *in vitro* models (Akowuah *et al.,* 2005). In the present study, the methanol extracts showed lipid peroxidation inhibition and plasma antioxidant effect which may be ascribed to its polyphenol content. The aqueous methanolic extract of *O. stamineus* leaf has been reported to contain 67% of total identified phenolics (Sumaryono *et al.,* 1991). Polyphenols have been observed to be very important in prevention of tissue damage by activated oxygen species due to their antioxidant effects including reactive oxygen species scavenging, metal chelation and enzyme modulation (Pietta *et al.,* 1998). Naturally occurring polyphenols were shown to be effective in reducing oxidative stress (Holman & Katan, 1999). Rosmarinic acid, the most abundant polyphenolic acid in *O. stamineus* leaf extract (Akowuah *et al.,* 2004), was reported to show antioxidant qualities superior to that of vitamin E and butylated hydroxytoluene (Leung & Foster, 1996).

The increase in total antioxidant status and decrease in lipid peroxidation of rat plasma after 14 days treatment with extract of *O. stamineus* suggests that active constituents in the extract were absorbed. Absorption of RA from the *O. stamineus* leaf extract was studied in order to gain more understanding on the oral administration of the extract in relation to the *in vivo* antioxidant effect. The bioavailability of RA is due to its hydrophilic character which facilitated its absorption and rapid eliminated from the blood circulation after intravenous administration (Parnham & Kesselring, 1985). RA and other polyphenolics have the tendency to bind to proteins and polysaccharide by hydrogen bonding because of the overall strength derived from multiplicity of hydrogen bonds radiating from the polyphenolic substrate. That is, absorption of RA depends on factors related to the basic structure and since it is susceptible to degradation by gut microorganisms, the enzymes in body tissues after absorption, and other metabolic transformations distribution in the liver, bile, lung etc, only small proportion of RA reached the blood stream (Bravo *et al.,* 1994; Baba *et al.,* 2005). The result obtain in the present study agrees with these reports. The values of the pharmacokinetic parameters observed in this study may indicate absorption of some amount of free RA into the blood stream and hence distribution into tissues. The absorption of free RA into the blood circulation may account for the effective *in vivo* activity of the *O. stamineus* extract observed in the study. That is, some of the antioxidant activity of free RA remained during the metabolic process and may have affected the

plasma lipid peroxidation inhibition and antioxidant status as shown by our *in vivo* data.

Metabolic transformations of drugs are believed to produce significantly less active metabolites compared to the parent drug. However, modifications of polyphenols by enterohepatic circulation mechanism do produce metabolites with considerable antioxidant properties. For instance, RA has been reported to be present as free, degraded and conjugated forms such as m-hydroxyphenylpropionic acid, m-coumaric acid, and sulfated form of caffeic acid and ferulic acid after oral administration of RA in rats and humans (Nakazawa & Ohasawa, 1988; Baba Osakabe *et al.,* 2004). These metabolites are important natural antioxidant substances present in food plants hence they may have contributed substantially to the *in vivo* antioxidant activity.

CONCLUSIONS

Antioxidant activity of herbals depends on the absorption of specific active constituents; therefore understanding of the *in vivo* antioxidant activity of *O. stamineus* leaf extract was attained by studying the absorption of the active ingredient (RA) in the extract after oral administration to rats. Most herbal medicines are administered orally in the form of crude extract in clinical use hence the absorption of RA in the gastrointestinal tract could be used as suitable reference in clinical application of herbal medicinal products from *O. stamineus.*

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22

Green Tea: Molecular Targets in Glucose Uptake, Inflammation, and Insulin Signaling Pathways

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ABSTRACT

Obesity and related disorders including diabetes and cardiovascular diseases have been studied extensively, but the prevention and treatment of these health conditions remains inadequate. Green tea has anti-inflammatory, anti-diabetic, and anti-obesity activities, but the molecular mechanisms of these effects have not been fully elucidated. Quantitative real-time PCR assays were used to investigate the effects of green tea extract on the expression of anti-inflammatory tristetraprolin family genes, pro-inflammatory genes, glucose transporter family genes, and insulin signaling pathway genes in liver and muscle of rats fed a high-fructose diet known to induce insulin resistance, oxidative stress, and inflammation. In this chapter, we review the experimental approaches used, the basal level expression of the various genes analyzed, and the transcriptional regulation of genes coding for the tristetraprolin family, glucose transporter family, pro-inflammatory and insulin signal transduction pathway components. The results show that anti-inflammatory tristetraprolin mRNA levels are increased and pro-inflammatory tumor necrosis factor mRNA levels are reduced in the liver and skeletal muscle of rats fed a high-fructose diet given 1 g of green tea extract/kg of diet. Additional results suggest that green tea consumption

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regulates gene expression in glucose uptake and insulin signaling pathways in the same rats. These animal studies suggest that intake of adequate amounts of green tea can effect mRNA message levels for genes that regulate inflammation and glucose metabolism in the liver and skeletal muscle.

Key words : Gene expression, glucose transporter, green tea, high-fructose diet, inflammation, insulin signaling pathway, metabolic syndrome, rat, tristetraprolin, tumor necrosis factor

INTRODUCTION

The extensive studies of obesity and related disorders including diabetes and cardiovascular diseases have not led to generally effective prevention and treatment of these health conditions (Dandona *et al.*, 2004; Stumvoll *et al.*, 2005; Eckel *et al.*, 2005; Hotamisligil, 2006; Greenberg & Obin, 2006). Diet plays an important role in disease development, and the diets widely consumed in developed countries may increase the incidence of diabetes (Carter *et al.*, 1996). The combination of higher content of refined sugars and fats and lower intake of traditional herbs, spices, and other plant products may contribute to the higher incidences of diabetes and obesity in the U.S. (Gross *et al.*, 2004). Drug treatment for obesity and diabetes is not feasible for the majority of people and alternative and inexpensive therapies need to be evaluated.

Medicinal plants have been used for the treatment of diabetes and related disorders for thousands of years (Gray & Flatt, 1997). Herbal medicines are playing a role in the prevention of obesity and control of type 2 DM for people with elevated levels of blood glucose and glucose intolerance who have a greater risk of developing diabetes. Plant seeds, fruits, leaves, and bark contain polyphenolic compounds. These compounds are the end products of the plant flavonoid biosynthetic pathway and are used by plants for protection against predators (Dixon *et al.*, 2005). Plant polyphenols are also widely present in the diet (Prior & Gu, 2005) and are believed to be important for human health (Yang *et al.*, 2001; McKay & Blumberg, 2002; Rodriguez *et al.*, 2006).

Tea (*Camellia sinensis*) is a popular beverage worldwide. Recent studies indicate that tea has a wide range of effects on animal and human health. A number of studies have indicated that green tea has anti-inflammatory properties (Dona *et al.*, 2003). Tea has been reported to have beneficial effects in conditions such as collagen-induced arthritis (Haqqi *et al.*, 1999), inflammatory bowel disease (Varilek *et al.*, 2001) and carrageenan-induced paw edema (Das *et al.*, 2002). However, the molecular mechanisms of tea's anti-inflammatory properties have not been completely elucidated.

Other studies indicate that tea can be used to prevent diabetes and obesity for animal and human health (Wolfram *et al.*, 2006; Kao *et al.*, 2006). Several studies suggest that green tea extract (GTE) mimics insulin action. First, rat epididymal adipocyte assays indicate that GTE has insulin-potentiating activity on utilization of glucose (Broadhurst *et al.*, 2000; Anderson & Polansky, 2002), and that the predominant active ingredient is epigallocatechin gallate (EGCG) (Broadhurst *et al.*, 2000; Anderson & Polansky, 2002), the major polyphenol in green tea. Second, green tea powder and its polyphenols decrease fasting plasma levels of glucose, insulin, triglycerides, and free fatty acids, and increase the insulin-stimulated glucose uptake in rats fed green tea extract and polyphenols for 12 weeks (Wu *et al.*, 2004a). Third, EGCG inhibits β-2-aminobicycle (2.2.1)-heptane-2-carboxylic acid-stimulated insulin secretion and glutamate dehydrogenase (Li *et al.*, 2006a). Fourth, EGCG induces forkhead transcription factor family O phosphorylation by a similar but not identical mechanism to insulin and insulin growth factor I (Anton *et al.*, 2007). A recent study suggests, however, that green tea consumption does not improve blood glucose, lipid profiles, insulin resistance or serum adiponectin levels in patients with type 2 diabetes (Ryu *et al.*, 2006). Clearly, more studies are needed to demonstrate tea's insulin-like activity and the underlining molecular mechanisms.

A high-fructose diet has been used as a model for the study of insulin resistance, oxidative stress (Faure *et al.*, 1999), and inflammation (Kelley *et al.*, 2004). Green tea was shown to decrease fasting plasma levels of glucose, insulin, triglycerides, and free fatty acids, and increase the insulin-stimulated glucose uptake and glucose transporter 4 (GLUT4) protein in rats fed a fructose-rich diet for 12 weeks (Wu *et al.*, 2004b). However, that study did not address more potential targets in the GLUT family and the insulin signal transduction pathway (Cao *et al.*, 2007c). Overproduction of free radicals under oxidative stress is associated with inflammation. Pro-inflammatory tumor necrosis factor alpha (TNF-α) levels are increased in rat muscle by high-fructose diet, which is one of the determinants of insulin resistance in skeletal muscle (Togashi *et al.*, 2000; Yamaguchi *et al.*, 2005). A high-fructose diet was also shown to activate inflammatory pathways in the liver (Kelley *et al.*, 2004). Since liver and muscle are two of the most insulin-responsive organs and anti-inflammatory tristetraprolin/zinc finger protein 36 (TTP/ZFP36) gene expression is induced by insulin (Lai *et al.*, 1990), we hypothesized that TTP and/or its homologues might be involved in the inflammatory response induced by a high-fructose diet in rats, and that green tea might possess beneficial effects in response to inflammation.

We used quantitative real-time PCR to investigate the effects of GTE on the expression of numerous genes (Table 1) including the anti-inflammatory TTP family mRNAs and some of the pro-inflammatory mRNAs

known to be regulated by TTP family proteins, and the GLUT family (Shepherd & Kahn, 1999) and insulin signal transduction pathway family genes (Taha & Klip, 1999; Cao *et al.*, 2007c). In this chapter, we review the experimental approaches used, the basal level expression of the various genes analyzed, and the transcriptional regulation of genes in the TTP family, GLUT family, pro-inflammatory and insulin signaling pathway components. Our results show that TTP mRNA levels are increased and TNF mRNA levels are reduced in the liver and skeletal muscle of rats fed a high-fructose diet given 1 g of GTE/kg of diet (Cao *et al.*, 2007b). Results also suggest that green tea consumption regulates the expression of genes coding for the glucose uptake and insulin signaling pathway proteins in the same rats (Cao *et al.*, 2007a). These studies suggest that intake of adequate amounts of green tea can effect mRNA message levels for genes that regulate inflammation and glucose metabolism in the liver and skeletal muscle that could lead to palliative effects on inflammation induced by high fructose in the diet.

Table 1. Categories of the molecular targets analyzed by PCR assays

Category	mRNA targets
Anti-inflammation	TTP/ZFP36, ZFP36L1, ZFP36L2, ZFP36L3
Pro-inflammation	TNF, GM-CSF, COX2, HUR, VEGF
Glucose transport	GLUT1, GLUT2, GLUT3, GLUT4
Insulin signaling	INS1, INS2, INSR, IRS1, IRS2, AKT1, GRB2, IGF1, IGF2, IGF1R, IGF2R, GSK3B, GYS1, PIK3CB, PIK3R1, SHC1, SOS1

EXPERIMENTAL APPROACHES

Animals, Fructose-rich Diet, and Green Tea Extract

Male Wistar rats (6 weeks old, ~ 150 g) were housed individually in thermoformed polystyrene cages. The rats were kept under the conditions with a 12 h light: 12 h dark schedule, at 21 ± 1°C, and a relative humidity of 55%. All procedures were in accord with guidelines of the U.S. National Institutes of Health. The composition of the fructose-rich diet (60% fructose, w/w) (SAFE, 89290, Augis, France) is given in Table 2. Green tea extract (GTE) (Unilever France) was prepared by boiling leaves in hot water. The dried water-soluble extract was added to the diet at 1 or 2 g/kg of diet. GTE contained 12.75% (w/w) epigallocatechin-3-gallate (EGCG), 9.21% epigallocatechin (EGC), 3.73% epicatechin gallate (ECG), 2.4% epicatechin (EC), 5.94% caffeine, and 0.195% L-theanine (Table 2).

Table 2. Composition of the high-fructose diet and green tea extract used in the study

High-fructose diet (SAFE, France)	Green tea extract (Unilever France)
65% (w/w) fructose	12.75% (w/w) EGCG (epigallocatechin-3-gallate)
20% casein	9.21% EGC (epigallocatechin)
5% corn oil	3.73% ECG (epicatechin gallate)
5% alphacel	2.4% EC (epicatechin)
3.5% mineral mix	5.94% caffeine
1% vitamin mix	0.195% L-theanine
0.3% DL-methionine	
0.2% choline bitartrate	

Experimental Design

Rats were fed a standard Purina chow for one week before being randomly divided into three groups (10 rats/group). The first group was given the high-fructose diet that has been shown to induce insulin resistance, oxidative stress, and inflammation (diet control). The second group was given the high-fructose diet plus 1 g of GTE/kg diet (1 g tea) and the third group was given the high-fructose diet plus 2 g of GTE/kg diet (2 g tea). Rats were sacrificed after 6 weeks on the diet. Food intake and the body weight for rats fed the diet control, 1 g tea, and 2 g tea for 6 weeks were not significantly different. These data are in agreement with a previous report in which the same diet was used and a similar evolution in body weight was reported (Busserolles *et al.*, 2003). The liver and skeletal muscle were removed from the rats, frozen in liquid nitrogen, and stored at –80°C.

Gene Expression Analysis

Total RNA was isolated from rat liver and muscle using TRI_{ZOL} reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA integrity and concentrations were determined using RNA 6000 Nano Assay Kit and the Bioanalyzer 2100 according to the manufacturer's instructions (Agilent Technologies, Palo Alto, CA, USA). Total cDNA synthesis was performed using the ImProm-II Reverse Transcription System (Promega, Madison, WI). The primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). The mRNA names, GenBank accession numbers, and amplicon sizes are described (Table 3). The sequences (5′ to 3′) of the forward primers, TaqMan probes (TET - BHQ1), and reverse primers have been described (Cao *et al.*, 2007a; Cao *et al.*, 2007b). Quantitative real-time PCR assays were performed using an ABI Prism 7700 real-time PCR instrument (Applied Biosystems) (Dawson *et al.*, 2005). The double C_T method of relative quantification was used to determine the fold changes in expression (Livak & Schmittgen, 2001). This was done by first normalizing the resulting threshold cycle (C_T)

values of the target mRNAs to the C_T values of the internal control RPL32 in the same samples. It was further normalized with the diet control (samples with only the high-fructose diet but without tea supplement). The fold change in expression was then obtained. Data were analyzed by SigmaStat 3.1 software (Systat Software, Inc., Point Richmond, CA) using One Way Analysis of Variance (ANOVA). Multiple comparisons were performed with Duncan's Multiple Range Test. Values with different lower case and upper case letters displayed above the columns of the figures are significantly different at $p<0.05$ and $p<0.01$, respectively.

Table 3. The mRNA names, GenBank accession numbers and amplicon sizes of gene targets investigated

mRNA	Accession No	Amplicon size	mRNA	Accession No	Amplicon size
TTP/TIS11/ZFP36	NM_011756	70 bp	AKT1	NM_033230	90 bp
ZFP36L1/TIS11B	NM_007564	60 bp	GRB2	NM_030846	119 bp
ZFP36L2/TIS11D	NM_001001806	77 bp	GSK3B	NM_032080	106 bp
ZFP36L3	NM_001009549	70 bp	GYS1	XM_229128	119 bp
TNF	NM_013693	66 bp	INS1	NM_008386	89 bp
PTGS2/COX2	NM_011198	106 bp	INS2	NM_008387	100 bp
CSF2/GM-CSF	NM_009969	71 bp	INSR	NM_017071	137 bp
ELAVL1/HUR	NM_010485	69 bp	IGF1	NM_184052	78 bp
VEGFA	NM_001025250	68 bp	IGF1R	NM_010513	62 bp
VEGFB	NM_011697	83 bp	IGF2	NM_010514	78 bp
RPL32	NM_172086	66 bp	IGF2R	NM_010515	91 bp
GLUT1/SLC2A1	M13979	123 bp	IRS1	NM_012969	68 bp
GLUT2/SLC2A2	NM_012879	80 bp	IRS2	AF050159	69 bp
GLUT3/SLC2A3	NM_017102	112 bp	PIK3CB	NM_053481	134 bp
GLUT4/SLC2A4	NM_012751	87 bp	PIK3R1	NM_013005	118 bp
			SHC1	XM_216176	85 bp
			SOS1	D83014	104 bp

BASAL GENE EXPRESSION LEVELS

TTP Family mRNA Levels in Rat Liver and Muscle

Four forms of the anti-inflammatory TTP family proteins exist in rats and mice (TTP/ZFP36, ZFP36L1, ZFP36L2, and ZFP36L3) (Blackshear, 2002; Blackshear *et al.*, 2005). The relative expression levels of these genes are important for the evaluation of GTE effects in the tissues. The relative ratios of TTP family mRNAs in the liver and muscle are shown in Table 4. TTP and ZFP36L1 mRNAs are the major TTP family members in liver and muscle (Cao *et al.*, 2007b). The relative levels of TTP, ZFP36L1, and ZFP36L2 mRNAs in liver are 100%, 148%, and 6%, and those in muscle

are 100%, 70%, and 15%, respectively (Table 4) (Cao *et al.*, 2007b). TTP, ZFP36L1, and ZFP36L2 mRNA levels are more abundant in the liver than those in the muscle (Table 4) (Cao *et al.*, 2007b). ZFP36L3 mRNA is too low to be detected by 50 cycles of PCR in the liver or muscle (Cao *et al.*, 2007b). As a comparison, ZFP36L3 mRNA can be readily detected in cultured mouse-derived 3T3-L1 adipocytes and RAW 264.7 macrophages (Cao *et al.*, 2008a; Cao *et al.*, 2008b).

Table 4. Relative mRNA levels of TTP and GLUT family genes in rat liver and muscle [modified from (Cao *et al.*, 2007a; Cao *et al.*, 2007b)]

Tissue	mRNA	Expression ratio (relative to TTP or GLUT1) (Fold)	Expression ratio (relative to liver) (Fold)
Liver	TTP/TIS11/ZFP36	1.00	1.00
	ZFP36L1/TIS11B	1.48	1.00
	ZFP36L2/TIS11D	0.06	1.00
	ZFP36L3	0.00	
Muscle	TTP/TIS11/ZFP36	1.00	0.21
	ZFP36L1/TIS11B	0.70	0.07
	ZFP36L2/TIS11D	0.15	0.50
	ZFP36L3	0.00	
Liver	GLUT1/SLC2A1	1.0	1.0
	GLUT2/SLC2A2	32.0	1.0
	GLUT3/SLC2A3	0.02	1.0
	GLUT4/SLC2A4	0.01	1.0
Muscle	GLUT1/SLC2A1	1.0	0.1
	GLUT2/SLC2A2	0.003	0.00001
	GLUT3/SLC2A3	1.6	9
	GLUT4/SLC2A4	294	4705

TTP Family-related mRNA Levels in Rat Liver and Muscle

TNF and cyclooxgenase-2 (COX2) mRNA levels are very low in both liver and muscle (Cao *et al.*, 2007b), and TNF mRNA levels in the liver are about 2-fold those in the muscle (Cao *et al.*, 2007b). COX2 mRNA levels in the liver are about 40% of those in the muscle (Cao *et al.*, 2007b). The expression levels of Hu antigen R (HuR), vascular endothelial growth factor a (VEGFA) and VEGFB in the liver are about 2.5-fold, 2-fold, and 25% of those in the muscle, respectively (Cao *et al.*, 2007b). Granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA is undetectable by 50 cycles of PCR in the liver or muscle (Cao *et al.*, 2007b). As a positive control for the assay, GM-CSF mRNA is detected in cultured RAW264.7 cells (Cao *et al.*, 2008a).

Glucose Transporter Family mRNA Levels in Rat Liver and Muscle

The predominant glucose transporters in mammals are GLUT1, GLUT2, GLUT3, GLUT4, and GLUT5 (Shepherd & Kahn, 1999). The relative ratio of GLUT family mRNAs in the liver and muscle are shown in Table 4. GLUT2 is the major GLUT mRNA in the liver and GLUT4 is the major one in the muscle (Cao *et al.*, 2007a). The relative mRNA levels of GLUT1, GLUT2, GLUT3, and GLUT4 in liver are 1-, 32-, 0.02-, and 0.01-fold, and those in muscle are 1-, 0.003-, 1.6-, and 294-fold, respectively (Table 4) (Cao *et al.*, 2007a). GLUT1, GLUT2, GLUT3, and GLUT4 mRNAs in the muscle are 0.1-, 0.00001-, 9-, and 4705-fold of those in the liver, respectively (Table 4) (Cao *et al.*, 2007a).

Insulin Signaling Pathway mRNA Levels in Rat Liver and Muscle

Numerous components exist in the insulin signal transduction pathway (Taha & Klip, 1999). We have analyzed the mRNA levels of the following genes (refer to abbreviation list for gene/mRNA abbreviations) coding for insulin signaling pathway components (INS1, INS2, INSR, IRS1, IRS2, AKT1, GRB2, IGF1, IGF2, IGF1R, IGF2R, GSK3B, GYS1, PIK3CB, PIK3R1, SHC1, SOS1) in liver and muscle of rats fed a high-fructose diet (Cao *et al.*, 2007a). AKT1, GRB2, GSK3B, INSR, IGF2R, IRS1, IRS2, PIK3CB, PIK3R1, and SHC1 mRNAs are more abundant in the liver than those in the muscle, whereas GYS1, INS2, IGF2, and IGF1R mRNAs are more abundant in the muscle than those in the liver (Cao *et al.*, 2007a). INS1, INS2, IGF1, or IGF2 mRNAs is either undetectable or expressed at very low levels (Cao *et al.*, 2007a).

REGULATION OF GENE EXPRESSION INVOLVED IN INFLAMMATION

Green Tea Increases Anti-inflammatory TTP mRNA Levels in Rat Liver and Muscle

Green tea extract (1 g solid/kg diet) increases TTP mRNA levels by 50% and 140% in the liver and skeletal muscle, respectively, but does not have significant effects on TTP homologues ZFP36L1 or ZFP36L2 mRNA levels in the liver (Fig 1A) (Cao *et al.*, 2007b). However, GTE (2 g solid/kg diet) does not have significant effects on TTP, ZFP36L1 or ZFP36L2 mRNA levels in either tissue (Cao *et al.*, 2007b). The percentage increases of TTP mRNA in the muscle (Fig 1B) are greater than those in the liver (Fig 1A). Because TTP mRNA levels in the liver are about 5-fold higher than those in the muscle (Table 4), the net increases of TTP mRNA levels in the liver are more than those in the muscle (Cao *et al.*, 2007b).

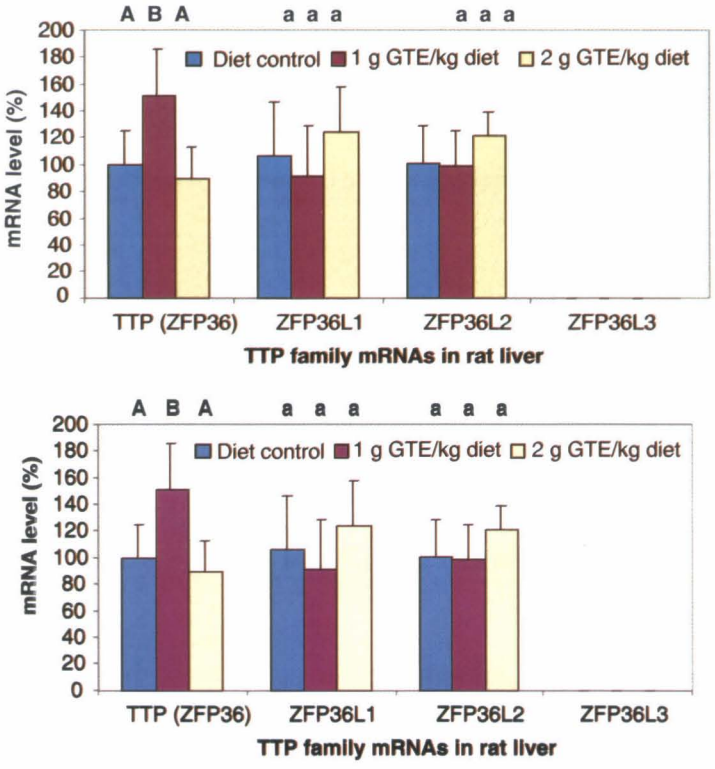

Fig 1. Green tea extract effects on TTP family mRNA levels in rat liver and muscle. RNAs were isolated from livers and muscles of rats with metabolic syndrome induced by a high-fructose diet and transcribed into cDNAs. RNA-derived cDNAs (25 ng) were used for quantitative RT-PCR assays. The results represent the percentage means and the standard deviations from 5-8 samples. Values with different upper case and lower case letters displayed above the columns of the figure are significantly different at $p<0.01$ and $p<0.05$, respectively [modified from (Cao *et al.*, 2007b)]

Green Tea Decreases Pro-inflammatory TNF mRNA Levels in Rat Liver and Decreases TNF and COX2 mRNA Levels in Rat Muscle

The increases of the anti-inflammatory TTP mRNA levels with GTE suggest that green tea may have destabilizing effects on pro-inflammatory AU-rich (ARE)-containing mRNAs such as TNF, GM-CSF, and COX2 mRNAs, whose stability are known to be destabilized by TTP (Carballo *et al.*, 1998; Carballo *et al.*, 2000; Sawaoka *et al.*, 2003). PCR assays demonstrate that GTE at 1 g decreases TNF mRNA levels by 30% but does not have significant effects on COX2 mRNA levels in the liver (Fig 2A) (Cao *et al.*, 2007b). PCR analyses also show that GTE at 1 g decreases both TNF and COX2 mRNA levels in the skeletal muscle by 30% and 40%, respectively (Fig 2B) (Cao *et al.*, 2007b). However, GTE at 2 g does not have significant effects on TNF

or COX2 mRNA levels in the liver or muscle (Cao *et al.*, 2007b). GM-CSF mRNA was undetectable by the PCR assays in the liver or muscle of rats treated with or without the tea supplement.

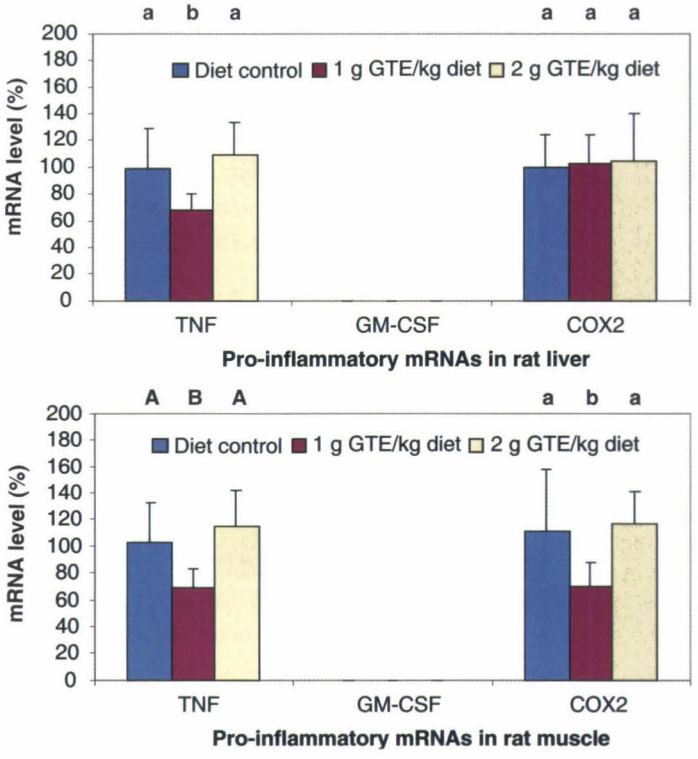

Fig 2. Green tea extract effects on TNF, GM-CSF, and COX2 mRNA levels in rat liver and muscle. RNA isolation, cDNA synthesis, and real-time PCR assays are described in Fig 1 legend [modified from (Cao *et al.*, 2007b)]

Green Tea Effects on HuR and VEGF mRNA Levels in Rat Liver and Muscle

In contrast to TTP, HuR is known to be a stabilizing protein for some ARE-containing mRNAs (Atasoy *et al.*, 1998; Raghavan *et al.*, 2001; Katsanou *et al.*, 2005). GTE at 1 g does not significantly affect HuR mRNA levels in the liver or muscle (Cao *et al.*, 2007b). However, GTE at 2 g increases the mRNA levels by 40% in the liver but not in the muscle (Cao *et al.*, 2007b). Similar to TNF and COX2 mRNAs, VEGF mRNA also contains ARE in the mRNA, which are destabilized by TTP family proteins (Ciais *et al.*, 2004; Essafi-Benkhadir *et al.*, 2007; Suswam *et al.*, 2008). PCR assays demonstrate that GTE at 1 or 2 g does not have significant effects on VEGFA or VEGFB mRNA levels in the liver or muscle of rats fed the high-fructose diet (Cao *et al.*, 2007b).

REGULATION OF GENE EXPRESSION INVOLVED IN GLUCOSE UPTAKE AND INSULIN SIGNALING PATHWAY

Green Tea Regulates the Expression of Genes Involved in Glucose Uptake

Green tea extract (1 g GTE/kg diet) increases GLUT1 and GLUT4 mRNA levels by 111% and 165%, respectively, but does not have significant effects on GLUT2 or GLUT3 mRNA levels in the liver (Fig 3A) (Cao *et al.*, 2007a). GTE (2 g GTE/kg diet) increases GLUT4 mRNA levels by 92%, but does not have significant effects on GLUT1, GLUT2, or GLUT3 mRNA levels in the liver (Fig 3A) (Cao *et al.*, 2007a). However, GTE (1 g GTE/kg diet) does not alter the mRNA levels of GLUT1, GLUT2, GLUT3, or GLUT4 in the muscle (Fig 3B) (Cao *et al.*, 2007a). GTE (2 g GTE/kg diet) increases GLUT2 and GLUT4 mRNA levels by 81% and 38% over the diet control, respectively, but does not have significant effects on GLUT1 or GLUT3 mRNA levels in the muscle (Fig 3B) (Cao *et al.*, 2007a).

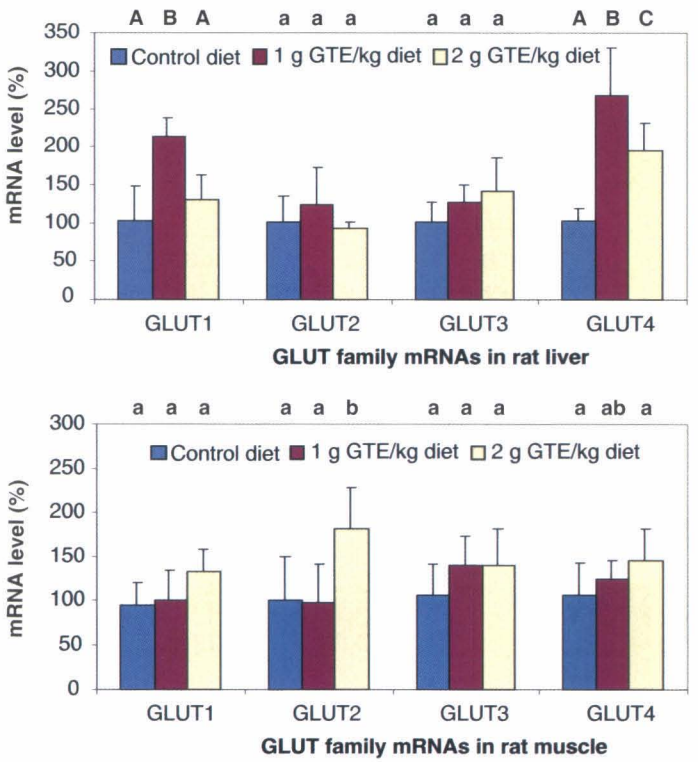

Fig 3. Green tea extract effects on GLUT family mRNA levels in rat liver and muscle. RNA isolation, cDNA synthesis, and real-time PCR assays are described in Fig 1 legend [modified from (Cao *et al.*, 2007a)]

Green Tea Regulates the Expression of Genes Involved in Insulin Signaling

Green tea extract (1 g GTE/kg diet) increases glycogen synthase kinase 3 beta (GSK3B) and insulin receptor substrate 2 (IRS2) mRNA levels by 34% and 55%, respectively (Fig 4A) (Cao *et al.*, 2007a), but does not have significant effects on AKT1, GRB2, GYS1, INS1, INS2, INSR, IGF1R, IGF2R, IRS1, PIK3R1, SHC1, or SOS1 mRNA levels in the liver (Cao *et al.*, 2007a). GTE (2 g GTE/kg diet) increases GSK3B and phosphatidylinositol 3-kinase, catalytic, beta (PIK3CB) mRNA levels by 30% and decreases Src homology 2 domain-containing transforming protein 1 (SHC1) mRNA levels by 63% (Fig 4A) (Cao *et al.*, 2007a), but does not have significant effects on AKT1, GRB2, GYS1, INS1, INS2, INSR, IGF1R, IGF2R, IRS1, IRS2, PIK3R1, or SOS1 mRNA levels in the liver (Cao *et al.*, 2007a). In the muscle, GTE (1

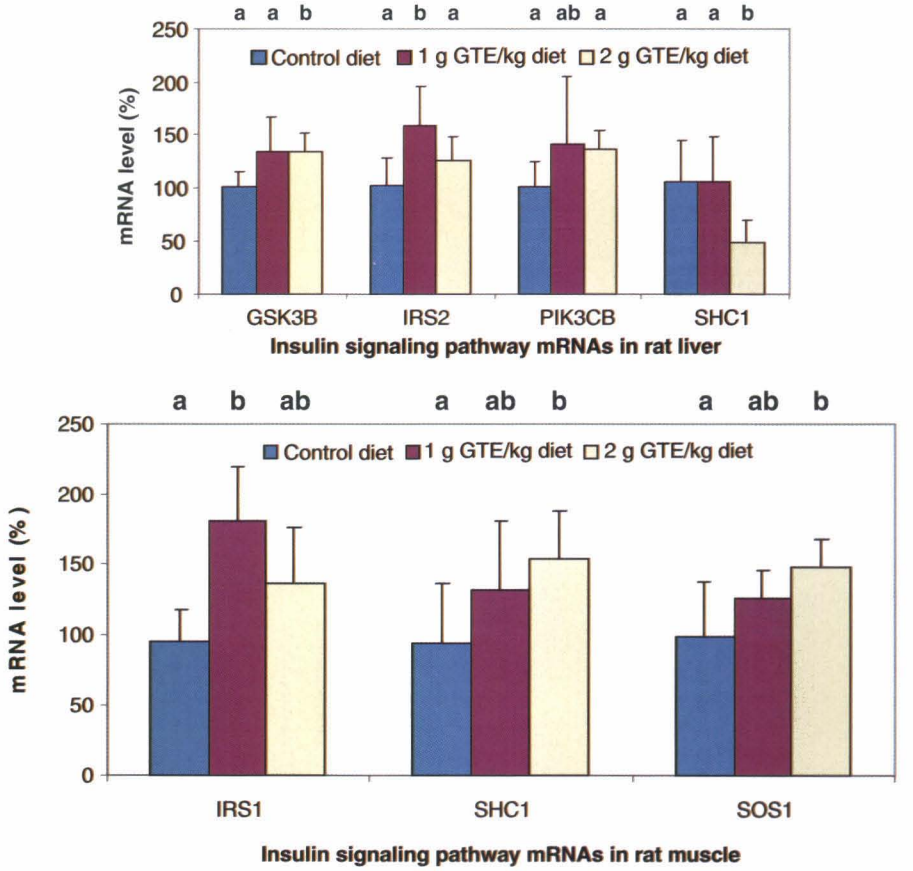

Fig 4. Green tea extract effects on insulin signaling pathway mRNA levels in rat liver and muscle. RNA isolation, cDNA synthesis, and real-time PCR assays are described in Fig 1 legend [modified from (Cao *et al.*, 2007a)]

g GTE/kg diet) increases IRS1 mRNA levels by 84% (Fig 4B) (Cao *et al.*, 2007a), but does not have significant effects on AKT1, GRB2, GSK3B, GYS1, INS1, INS2, INSR, IGF1R, IGF2R, IRS2, PIK3CB, PIK3R1, SHC1 or SOS1 mRNA levels (Cao *et al.*, 2007a). GTE (2 g GTE/kg diet) increases SHC1 and Son of sevenless 1 (SOS1) mRNA levels by 60% and 49%, respectively (Fig 4B) (Cao *et al.*, 2007a), but does not have significant effects on AKT1, GRB2, GSK3B, GYS1, INS1, INS2, INSR, IGF1R, IGF2R, IRS1, IRS2, PIK3CB, or PIK3R1 mRNA levels in the muscle (Cao *et al.*, 2007a).

DISCUSSION

Plants have been used for the treatment of diabetes for thousands of years (Gray & Flatt, 1997), however, no investigation at the molecular level was substantially performed to support this practice. Several studies indicate that tea extract and its major polyphenol, EGCG, has insulin-potentiating activity in *in vitro* and in animal models (Broadhurst *et al.*, 2000; Anderson & Polansky, 2002; Wu *et al.*, 2004b; Anderson *et al.*, 2005; Li *et al.*, 2006b; Anton *et al.*, 2007) and that green tea has anti-inflammatory properties (Dona *et al.*, 2003). We recently analyzed the effects of a green tea polyphenolic extract on the mRNA levels of glucose transporter family, insulin signal transduction pathway family, anti- and pro-inflammatory family genes in liver and muscle of rats fed a high-fructose diet known to induce insulin resistance, oxidative stress (Faure *et al.*, 1999), and inflammation in rats (Kelley *et al.*, 2004).

One of the major findings is that GTE increases anti-inflammatory TTP and decreases pro-inflammatory TNF gene expression in liver and muscle of rats fed a high-fructose diet (Cao *et al.*, 2007b). Recent investigations have established a mechanism for the regulation of inflammatory responses at the post-transcriptional level by TTP family proteins (Blackshear, 2002; Anderson *et al.*, 2004). TTP binds ARE in some mRNAs and destabilizes those transcripts encoding proteins such as TNF-α (Carballo *et al.*, 1998; Lai *et al.*, 1999; Cao *et al.*, 2003; Cao, 2004), GM-CSF (Carballo *et al.*, 2000; Carballo *et al.*, 2001), and COX2 (Sawaoka *et al.*, 2003). The mRNAs encoding TNF-α and GM-CSF are stabilized in TTP knockout mice and in cells derived from them (Carballo *et al.*, 1998; Carballo *et al.*, 2000). Excessive secretion of these cytokines in TTP knockout mice results in a severe systemic inflammatory response including arthritis, autoimmunity, and myeloid hyperplasia (Taylor *et al.*, 1996; Phillips *et al.*, 2004). Up-regulation of TTP reduces inflammatory responses in macrophages (Sauer *et al.*, 2006). These lines of evidence support the conclusion that TTP is an anti-inflammatory protein. Therefore, agents that induce TTP gene expression may have potential therapeutic value for the prevention and/or treatment of inflammation-related diseases. Our results show that GTE increases TTP and decreases TNF gene expression in rats fed a high-fructose diet known to induce inflammation, suggesting

that part of the mechanism of tea's anti-inflammatory effects may involve TTP at the post-transcriptional level.

The other major finding is that GTE increases GLUT4 mRNA levels in rat liver and muscle of rats fed a high-fructose diet (Cao *et al.*, 2007a). The increased GLUT4 mRNA level in the muscle may be physiologically significant for the following reasons: 1) GLUT4 is the major GLUT family mRNAs in the muscle (Table 4), 2) GLUT4 protein is the insulin-responsive glucose transporter in the muscle and adipose tissue (Shepherd & Kahn, 1999), 3) the amount of GLUT4 protein is decreased in obese subjects (Garvey *et al.*, 1991), 4) insulin increases GLUT4 mRNA and protein levels in fetal rat brown adipocytes (Valverde *et al.*, 1999; Hernandez *et al.*, 2003) and increases GLUT4 protein levels in 3T3-F442A adipocytes (Yu *et al.*, 2001), and 5) the amount of GLUT4 protein is increased in fructose-fed rats with green tea powder supplementation resulting in amelioration of fructose-induced insulin resistance (Wu *et al.*, 2004b). The increased GLUT4 mRNA levels with GTE suggest a positive effect of these polyphenolic compounds on the long-term regulation of glucose transport. The significance of the increased levels of GLUT1 and GLUT4 mRNAs in the liver and GLUT2 mRNA in the muscle is not clear because these GLUT mRNAs are the minor forms in their respective rat tissues (Table 4).

Our results also show that GTE consumption affects the expression of other genes coding for key proteins in the insulin-signaling pathway. GTE increases GSK3B, IRS2 and PIK3CB, but decreases SHC1 mRNA levels in the liver, and GTE increases IRS1, SHC1, and SOS1 mRNA levels in the muscle (Cao *et al.*, 2007a). However, GTE consumption exhibits none or minimal effects on the other mRNA levels in the liver or muscle, including INS1, INS2, INSR, AKT1, GRB2, IGF1, IGF2, IGF1R, IGF2R, GYS1, and PIK3R1 mRNAs (Cao *et al.*, 2007a). The small effects of GTE consumption on mRNA levels in the insulin signaling pathway in our studies are in agreement with previous observations from other studies showing that the effects of insulin on the expression of a number of genes in the insulin signaling pathway are minimal in mouse 3T3-L1 adipocytes (Wang *et al.*, 2006; Cao *et al.*, 2008b). The plausible reason for the small effects of GTE and insulin on mRNA levels in the insulin signal transduction pathway in rat liver and muscle, and cultured mouse-derived 3T3-L1 adipocytes, is probably due to the functional effects of insulin signal transduction mainly through protein phosphorylation. It is also possible that the effectiveness of GTE is dependent upon factors such as the timing and dosages, and the physiological status of the target cells. Additional studies are needed to determine a useful role for GTE and its components in improving the negative health effects of glucose intolerance, insulin resistance, diabetes, and obesity.

Food intake for rats fed 1 g GTE/kg diet is 20.5 g/d. On a per kg basis, the rats consuming 1 g of GTE per day consume approximately 60 mg/kg body weight per day. Humans consuming 1 cup of tea made from a tea bag containing 2 g of solids consume roughly 900 mg of tea solids or approximately 12.8 mg/kg. On a per kilogram basis, 1 g GTE/kg diet used in our study is equivalent to humans drinking approximately 5 cups of tea per day. The reason(s) why GTE at 2 g/kg of diet does not produce more TTP mRNA levels than 1 g of GTE/kg diet in the liver is not clear. The effects of GTE on insulin levels in the plasma showed a similar pattern (Anderson *et al.*, 2005). Additional dose response studies of GTE and its components are needed.

CONCLUSIONS

We have investigated the effects of GTE on the expression of multiple genes coding for inflammatory factors, GLUT family and components in the insulin signal transduction pathway (Fig 5). Quantitative real-time PCR demonstrate that anti-inflammatory TTP mRNA levels are increased significantly and pro-inflammatory TNF mRNA levels are reduced in the liver and skeletal muscle of rats fed a high-fructose diet and given 1 g GTE/kg in the diet.

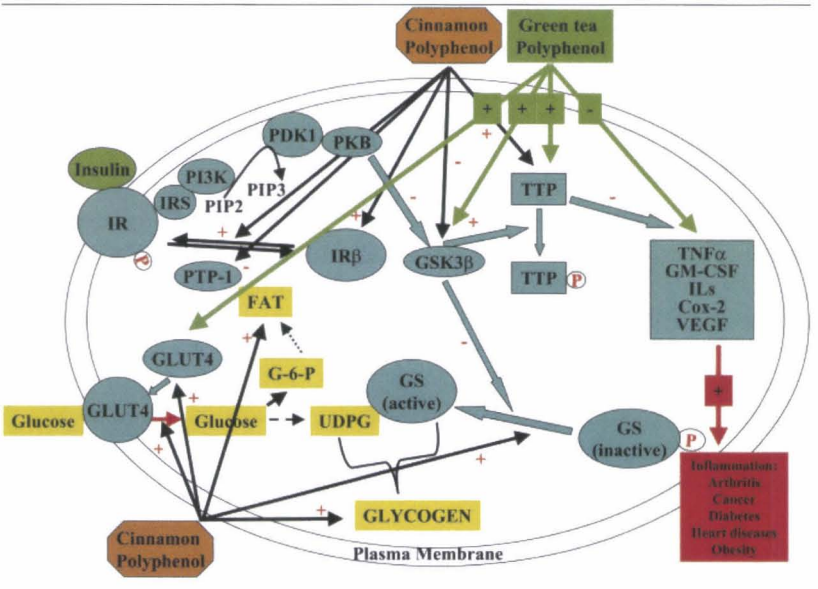

Fig 5. A working model for green tea extract in inflammation, glucose uptake, and insulin signaling pathway leading to potential beneficial effects. This model is modified from a model for cinnamon polyphenol effects (Cao *et al.*, 2007c). The effects of green tea polyphenol extract on TTP and TNF gene expression are described in Figs 1, 2 (Cao *et al.*, 2007b), and those on GLUT4 and GSK3B gene expression are described in Figs 3, 4 (Cao *et al.*, 2007a) ("+" and "-"represent positive and negative effects, respectively) [from (Cao *et al.*, 2007a)]

Additional results suggest that GTE consumption regulates gene expression in glucose uptake and insulin signaling pathways in the same rats. These findings support plausible reasons for drinking adequate amounts of green tea for improved health consequences.

ABBREVIATIONS

ANOVA: one way analysis of variance; ARE: AU-rich element; AKT1: thymoma viral proto-oncogene 1; COX-2/PTGS2: cyclooxgenase-2/ prostaglandin-endoperoxide synthase 2; EGCG: Epigallocatechin-3-gallate; GLUT: glucose transporter; GM-CSF/CSF2: granulocyte-macrophage colony-stimulating factor; GRB2: growth factor receptor bound protein 2; GSK3B: glycogen synthase kinase 3 beta; GTE: green tea extract; GYS1: glycogen synthase 1; HuR/ELAVL1: Hu antigen R/embryonic lethal, abnormal vision-like 1; IGF1: insulin-like growth factor 1; IGF1R: insulin-like growth factor I receptor; IGF2: insulin-like growth factor 2; IGF2R: insulin-like growth factor 2 receptor; INS1: insulin I; INS2: insulin II; INSR: insulin receptor; IRS1: insulin receptor substrate 1; IRS2: insulin receptor substrate 2; PIK3CB: phosphatidylinositol 3-kinase, catalytic, beta; PIK3R1: phosphatidylinositol 3-kinase, regulatory subunit 1; SHC1: Src homology 2 domain-containing transforming protein 1; SOS1: Son of sevenless 1; RPL32: ribosomal protein L32; TNF: tumor necrosis factor; TTP: tristetraprolin; VEGF: vascular endothelial growth factor; ZFP36: zinc finger protein 36; ZFP36L: ZFP36-like.

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Euphorbious Plants as Molluscicides and Piscicides: A Review

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ABSTRACT

Many aquatic snails act as vectors for the larvae of trematode and thereby cause a number of diseases. Two diseases carried by aquatic snails, fascioloiasis and schistosomiasis cause immense harm to man and his domestic animals. The WHO has tested thousands of synthetic compounds for the eradication of freshwater target snails. Though effective, these pesticides have so for not proved themselves to be entirely satisfactory. With a growing awareness of environmental pollution, which such compounds can cause, efforts are being made to find out molluscicidal and piscicidal products of plant origin. Presence of predatory fishes in fish culture pond is also a serious problem due to their growth and better utilization of cultured carp habitats and food. Due to their carnivorous nature they engulf the fingerlings of cultured carps and adversely effect the aquaculture production. A number of compounds, such as saponins, tannins, miscellaneous alkaloids, alkenyl phenols, glycoalkaloids, flavonoids, sesquiterpenes lactones, terpenoids and phorbol esters present in various plants have been found to be toxic to target freshwater snails and predatory fishes.

Key words : Euphorbiales, molluscicides, piscicides, snail

INTRODUCTION

Although the exact number of existing molluscan species is still a matter of speculation, Abbott (1954) has estimated a total of about 1,10,000 living

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species, 80,000 amongst which are gastropods, 10,000 bivalves, and 5,000 belonging to the other three classes of mollusca. (Godan, 1983) on the other hand believe the number of living species is about 1,20,000.

Terrestrial snails and slugs cause considerable damage to both cultivated and useful non-cultivated plants. The animals can make their appearance in any damp area, but damage can occur also during relatively dry weather. Along with slugs, terrestrial snails also cause considerable damage to vegetable gardens, agricultural crops and fruits orchards. Singh and Agarwal (1981), reported that *Pila globosa* an amphibious snails causes damage to paddy crops in northern part of India.

In freshwater, the larvae of parasite trematodes also pass part of their life. Many aquatic snails act as vectors for the larvae of trematodes and there by, cause a number of diseases. Two diseases carried by aquatic snails, schistosomiasis and fascioliasis cause immense harm to man and his domestic animals (Bali *et al.,* 1986; Yadav & Singh 2001, 2002, 2003; Yadav *et al.,* 2004, 2005). *Fasciola hepatica*, the large liver fluke, common in sheep, cattle, goat and other herbivorous animals throughout the World. Froyed (1975), reported that about 21% cattle and 7% sheep were infected with liver fluke in Great Britain. In India, the freshwater snails *Lymnaea acuminata* and *Indoplanorbis exustus* are the intermediate host of *Fasciola hepatica* and *Fasciola gigantica* (Hyman, 1970; Singh & Agarwal, 1988a; Singh & Agarwal, 1992b; Yadav, 2000; Yadav & Singh, 2001, 2003), which causes immense harm to domestic animals of this country. Schistosomiasis is caused by *Schistosoma*, it is a devastating disease of mankind second only to malaria in its deleterious effect (Jobin, 1973).

The control of harmful freshwater snails through synthetic pesticides has been review in detail by various workers (Ritchie, 1973; Godan, 1983; Agarwal & Singh, 1988; Singh & Agarwal, 1990; Singh *et al.,* 1996).

SYNTHETIC MOLLUSCICIDES

Metaldehyde

The molluscicidal activity of metaldehyde is generally influenced by temperature and humidity. Its treatment affects the gastropods by two ways; first its irritant effect which causes gastropods to secretes large amount of mucus resulting in desiccation and secondary its neurotoxicity at high concentration (Stringer, 1946).

Niclosamide

This is the standard molluscicides, active against all the stages of snails at 24h exposure up to 0.03 ppm. The niclosamide is not an irritant, as the snails treated with this compound, did not exhibit any symptoms like crawling, excessive production of mucus or quick contraction in to the shell (Khajuria & Bali, 1988).

Carbamate and Organophosphate Compounds

Carbamate and organophosphate (OP) compounds are esterase inhibiting neurotoxicants, with acute cholinergic effects preceded by inhibition of acetylcholinesterase (AChE) (Matsumura, 1985).

Carbamate

Pesticides such as carbaryl (Barry, 1969; Brar & Simwat, 1973; Singh & Agarwal, 1982), Aldicarb (Judge, 1969; Singh & Agarwal, 1981), Isolan (Daxl, 1971), Methiocarb (Rayner, 1975) and Mesurol (Smith & Boswell, 1970) have been used against various molluscan pests.

Organophosphate

Pesticides which currently being used for the control of various species of gastropods pests are Zinophos (Barry, 1969), Phorate (Barry, 1969; Singh & Agarwal, 1983), Nuvan (Tripathi & Agarwal, 1998), Dimeton, Trichlorofon (Singh & Agarwal, 1981) and Karathane (Oteifa *et al.,* 1975).

Synthetic Pyrethroids

The pyrethroids act primarily on nerve membrane by changing it to Na^+ and K^+ permeability. This causes repetitive discharges of nerve at the synapse and neuromuscular junction (Narahashi, 1983). Adlung and Kauth (1956), however, found that a 5% oil emulsion of pyrethrum was quite effective against the freshwater snails, *Radix auricularia, Lymnaea stagnalis* and *Physa fontinalis.*

PLANT ORIGIN MOLLUSCICIDES

With growing awareness of environmental pollution caused by synthetic molluscicides (Ritchie, 1973; Srivastava & Singh, 2001; Saravanan *et al.,* 2003; Selvarani & Rajamanickam, 2003, Park *et al.,* 2004), efforts are being made to find out molluscicides of plant origin. Being the product of biosynthesis, they are highly toxic and easily biodegradable in environment (Marston & Hostettman, 1987; Singh & Agarwal, 1992a; Singh *et al.,* 1996).

A continuous search for new classes of molluscicidal natural products is essential, so that problems of selectivity and low activity can be overcome. In addition, as wide a range as possible of structurally related compounds should be isolated (or synthesized) for structure-activity and mode of action studies.

This paper review all the relevant information on Euphorbious plants, which is considered as one of the most promising known molluscicidal and piscicidal plants.

Euphorbiales as Molluscicides (Table 1)

Snails exposed to latex of *Euphorbia royleana* exhibited typical symptoms of nerve poisoning and death took place within 24 h. It was shown that the latex was an acetylcholinesterase inhibitor and its anti-AChE activity in the snail *Lymnaea acuminata* was very high in comparison to any synthetic organic pesticides (Singh & Agarwal, 1984a). In another study, Singh & Agarwal (1984b) also observed that the latex of *Euphorbia royleana* reduced the level of 5-hydrodxy-tryptamine (5-HT) and dopamine in the nervous tissues of *Lymnaea acuminata*. Singh and Agarwal (1992 a) reported that, the latices of several euphorbious plants significantly reduced the alkaline and acid phosphatase activity in nerve tissue of *Lymnaea acuminata*. Cheng (1971) and Amin (1972) have recorded the molluscicidal properties of *Thea olesosa, Croton tiglium, Sehima argenta* and *Jatropha* spp.

Pharmacological action of *Croton tiglium* is due to the presence of alkaloids (Rizk, 1987). The alkaloids are naturally occurring organic bases which contain at least one nitrogen atom either in the heterocyclic ring or linked to an aliphatic skeleton. They are found in vascular plants and rarely occur in gymnosperms cryptogams and monocotyledons. Okunji and Iwu (1988), screened several plants of different families for molluscicidal properties and suggested that the toxic properties of these plants may be due to the presence of alkaloids. Toxicity in *Codiaeum* spp. is due to the presence of tanin in the latex (Wealth of India 1985). Tanin are complex phenolic compounds, divided in to two groups (i) The hydrolysable tanins, which one esters of gallic acid and also glycosides of these esters and (ii) The condensed tanins, which are polymers derived from various flavonoids. The molluscicidal activity was found to be related to the free phenolic groups of the tanins.

Euphorbiales as Piscicides (Table 2)

The method is simple, the poisonous ingredients are pounded and thrown in to a pool or dammed up sections of a small river. After a short time the fish begin to rise to the surface and can then readily be taken by hand. The fish can be eaten without health problems (Singh, 2001). According to Neuwinger (1994), 258 fish poisonous plants are present in Africa, based on 25 years of field research by the author in tropical Africa and evaluation of herbarium notes. 10-20 percent fishing poisonous are probably still unknown. They are spread among 167 plant general and 60 families. The evaluation shows a clear dominance of the leguminose Caesalpiniaceae, Mimosaceae, Papopmaceae in the hierarchy of fish poisoning plants. It also remarkable that a great proportion is in euphorbiales.

Table 1. A list of Euphorbious plants having molluscicidal activity

Plant	Plant part Tested	Class of active moiety	Extracts	Species	Reference(s)
Acalypha ornata	Leaf, Root	Unknown	Methanol	*B. globosus*	Adewunmi & Sofowora, 1980
Alchornea cordifolia	Fruit	Alkaloids, sterols	Water	-	Okunji & Iwu, 1988
Bridelia atroviridis	Stem bark	Unknown	Methanol	*B. globosus*	Adewunmi & Sofowora, 1980
B. ferruginea	Leaf	Sterols, saponins	Water	*B. glabrata, B. pfeifferi*	Okunji & Iwu, 1988
Cryptogonone argentea	Root	Unknown	Water	*B. globosus*	Adewunmi & Sofowora, 1980
Euphorbia antisyphlitica	Latex	Unknown	Water	*L. acuminata*	Singh & Agarwal, 1987
E. lactea criststa	Latex	Unknown	Water	*L. acuminata*	Singh & Agarwal, 1988a
E. neutra	Latex	Unknown	Water	*B. globosus*	Adewunmi & Sofowora, 1980
E. pulcherima	Root	Unknown	Water	*B. globosus*	Adewunmi & Sofowora, 1980
E. royleana	Latex	Unknown	Water	*L. acuminata*	Singh & Agarwal, 1984a.
Jatropha gossypifolia	Latex	Jatrophane	Water	*B. globosus*	Adewunmi & Sofowora, 1980
Manihot glaziovii	Stem bark	Unknown	Water	*B. globosus*	Adewunmi & Sofowora, 1980
Schima argenta	-	-	Water	*B. globosus*	Cheng, 1971
Thea olesosa	-	-	Water	*B. globosus*	Amin, 1972.
Uapaca guinensis	Stem bark	Sterols, saponins	Methanol	*B. glabrata, B. pfeifferi*	Okunji & Iwu, 1988
Croton tiglium	Latex, stem bark	Saponins, tanins	Water	*L. acuminata, I. exustus*	Yadav & Singh, 2001
Codiaeum variegatum	Latex, stem bark	Saponins, tannis	Water	*L. cuminata, I. exustus*	Yadav & Singh, 2001
Euphorbia pulcherima	Latex, stem bark	Ellagic acids	Water *I. exustus*	*L. cuminata,*	Singh *et al.,* 2004
Euphorbia hirta	Latex, stem bark	Ellagic acids	Water *I. exustus*	*L. cuminata,*	Singh *et al.,* 2004

Table 2. A list of Euphorbious plants having piscicidal activity

Plant	Plant part Tested	Class of active moiety	Extract	Species	Reference(s)
Euphorbia royleana	Stem bark	Unknown	Water	*Channa punctatus*	Singh & Singh, 2000
Euphorbia tirucalli	Stem bark	4-deoxy-phorbol	Water	*C. punctatus*	Tiwari *et al.*, 2001
Euphorbia tirucalli	Latex	4-deoxy-phorbol	Water	*C. punctatus*	Tiwari *et al.*, 2001
Glochidion velutinum	Stem bark	Unknown	Water	*O. punctatus*	Kulakkattolickal, 1989
Mallotus philippensis	Stem bark	Unknown	Water	*O. punctatus*	Kulakkattolickal, 1989
Manihot esculenta	Root	Unknown	Water	*O. punctatus*	Kulakkattolickal, 1989
Euphorbia royleana	Latex	Unknown	Water	*Channa marulias*	Singh & Singh, 2005
Jatropha gossypifolia	Latex	Unknown	Water	*Channa marulias*	Singh & Singh, 2005
Croton tiglium	Latex	Unknown	water	*Channa punctatus*	Yadav & Singh, 2002
Codiaeum variegatum	Stem bark	Taraxerol	Petroleum ether	*Channa punctatus*	Yadav *et al.*, 2005

The latices of several genera of the Euphorbiaceae and in particular of different species of *Euphorbia* have been used extensively by fisherman in different countries as fish poison of high biological activity (Watt & Breyer – Brandijk, 1976; Novock *et al.*, 1980). The rhizome of *Euphorbia biglandulosa* are pounded in order to release the latex and then thrown in stagnant waters of rivers. The rapidly dissolving poison first paralyses and then kills the fish. The sap though causes in irritation in the human skin has no intoxicating effect on the people who handle the latex or eat the fish. The toxicological action of the latex can be attributed to a new class of diterpenes such as esters of phorbol, 12-deoxyphorbol, 12-deoxy-12-hydroxy-phorbol, ingenal, 5-deoxyingenol, 2-deoxyingenol, resiniferotoxin and tinyatoxin (Kinghorn & Evans, 1975). It has been reported that phorbol esters interact with and activate the recently discovered protein kinase-C (Takai *et al.*, 1977). Tadpoles were found to be most sensitive to aqueous extracts of oil cake of this plant to the presence of sterol glycoside, saponins, flavonol glycosides. A terpene, 4-deoxyphorbal the active component of plant *Euphorbia tirucalli* is highly poisonous to fish (Kamat & Muthe, 1995).

These pesticidal compounds come from more than 50 families of angiosperms. The toxic effect of ripe fruit pulp of hingan, *Balanites roxyburghii*, containing saponin on fishes *Glossogobius giurius*, *Chanda*

nama, *Sarotherodon mossambica* and *Channa marulius* have been evaluated in the laboratory. *Channa marulius* was found more resistant to the toxic effect of hingman fruit than other three fishes. The hingman fruit pulp used for eradication of weed and unwanted fishes from the culture ponds. Same result was also found in case of karanj, *Pongamia pinnata* seed on different fishes (*G. giuris*, *Chanda nama*, *Oreochromis mossambicus*) and these extracts are also effects the non-target aquatic organism (Mohapatra & Nayak, 1998; Mohapatra & Sovan, 2000).

ACTIVE COMPOUND PRESENT IN EUPHORBIOUS PLANTS

Saponins

Saponins are naturally occurring plant glycosides which form a soapy lather with water they consist of a sugar moiety and an aglycone unit. The compounds responsible for the molluscicidal activity were found to be triterpenoid saponins, with LC_{100} values as low as 2 ppm (Hostettmann *et al.*, 1982).

Alkaloids

Several classes of alkaloids occur in certain genera of the family Euphorbiaceae and in particular *Croton* and *Phyllanthus* species. Imidazole alkaloids have been detected in only the genus *Glochidion.* Croton spp. contain several types of alkaloids, *viz.* isoquinolines (aporphines, *e.g.* sparsiflorine; proaporptines, *e.g.* crotonsparine and dihydroproaporphines, *e.g* crotosparinine), morphinandienones (*e.g.* crotonosine).

Flavonoids

The family Euphorbiaceae is rich in flavonoids, particularly flavones and flavonoles, which have been identified from several genera. They occur both as O- and C-glycosides and as methyl ethers. The flavonoids were detected in different parts of the plant other than roots.

Diterpeneoids

Numerous species of Euhporbiaceae, which contain diterpenes, show molluscicidal properties. Those with known molluscicidal properties are *Euphorbia royleana, E. antisyphilitica, E. lacteal cristata, E. pulcherima, Jatropha gossypifolia, E. hirta, E. neutra, Croton tiglium* and *Codiaeum variegatum* (Singh & Agarwal, 1984a, 1984b, 1987b, 1988a, 1990b, 1991, 1992a, 1992b; Yadav & Singh, 2001, 2002). These plants have been used as fish poisons, insecticides and molluscicides.

Monoterpenoids

α-phellandrene, myrcene, sabinene, α-terpinoline, borned, camphor, geranial, linalool, ascaridol and isoscaridol.

Sesquiterpenes Lactones

Aromadenderene, α-bergamoptene, β-elemene, α-humulene etc.

Iridoids

Iridoids are those monocyclic monoterpenoids which possess a lactone ring instead of having the ρ-menthane skeleton. The iridoid glycosides, ligstroside and oleuropein, have been claimed to possess activity against *Biomphalaria glabrata* at 100 ppm at 250 ppm respectively.

Naphthaquinones

Simple naphthaquinone have considerable activity whereas prenylated and dimmer naphthaquinones are comparatively inactive against snails. The prothrombogenic drug menadione has significant molluscicidal activity.

Alkenyl Phenols

The anacardiac acid component, at high concentrations, LC_{100}, induced immediate distress behaviour expressed by twitching of the tentacles (Sullivan *et al.*, 1982). With anacardic acid, the highest mortality took place only within the first 24 h of exposure as during the subsequent 48h there were rarely any deaths.

Chalcones

Chalcones are phenyl styryl ketones. 2, 4-dihydroxy-3′, 6′-dimethoxy-chalcone showed molluscicidal activity at 40 ppm within 6 h against *Biomphlaria pfeifferi* and *B. sudanica*.

Furanocoumarins

Molluscicidal activities of five such compounds (Xanthotoxin, Bergapten, Isopimpinellin and Chalepnsin) have been reported. These compounds possess strong toxicity against snails through their practical use is limited because they are phytotoxic.

Isobutylamides

Three isobutylamides (Affinin, N-isobutyl-2E-octadienamide and fagaramide), at relatively high concentrations, have been found to be toxic to snails. Compound 102 was tested against *Physa occidentalis* while compounds 103 and 104 were tested against *Biomphalaria glabrata* and found to be lethal (Marston & Hostettmann, 1985).

CONCLUSIONS

Despite all the data available for plant sources, very little is known about the active principles themselves; only about 70 natural products with recognized molluscicidal activity have been isolated. When it is considered that more than 20,000 compounds were screened in order to discover

the synthetic molluscicide Bayluscide® the immensity of the effort required in the search for natural, highly active molluscicides can be appreciated. However, the problem is somewhat simplified by the information already available from the testing of plant material, where the activity is known but the structures of the active components remain to be elucidated.

There are a very large number of plants, which contain compounds lethal to target as well as non-target organism at doses, which are much below for synthetic compounds (Shall *et al.,* 1988; Singh *et al.,* 1996; Amusan *et al.,* 1997; Singh & Singh, 2005; Yadav *et al.,* 2004). Use of such products has the additional advantage that these contain biodegradable compounds, which are less likely to cause environmental contamination. We strongly feel that if the herbaceous products are used as molluscicides or piscicides they would not only control the harmful snails and weed or predatory fish populations but would also have easy availability, easy biodegradability and greater acceptance by the users.

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