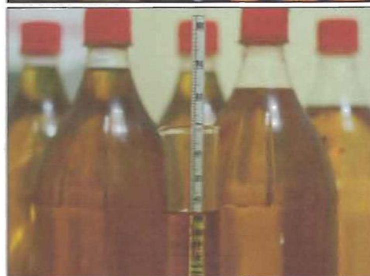
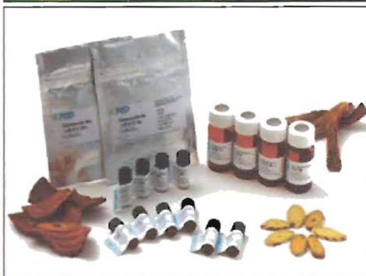
Comprehensive

Bioactive Natural Products

Vol 8

Quality Control & Standardization

V K Gupta
S C Taneja
B D Gupta

Studium Press

Comprehensive Bioactive Natural Products

Volume 8
Quality Control & Standardization

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S.C. TANEJA
B.D. GUPTA

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(Council of Scientific & Industrial Research)
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2010

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Comprehensive Bioactive Natural Products

Vol. 8: Quality Control & Standardization

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Ed. V.K. Gupta

Vol. 3 : ***Efficacy, Safety & Clinical Evaluation II***
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Vol. 4 : ***Antioxidants & Nutraceuticals***
Eds. V.K. Gupta & Anil K. Verma

Vol. 5 : ***Immune-modulation & Vaccine Adjuvants***
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Vol. 7 : ***Structural Modifications & Drug Development***
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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, biotechnology, general biology, and pharmacology to exploit the vast diversity of bioactive natural products.

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

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

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

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

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

Jammu, India

V.K. Gupta
Series Editor

AMITY INSTITUTE FOR HERBAL
AND BIOTECH PRODUCTS DEVELOPMENT

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FNSE, FNESA, FNAASc, FNASc.,
(UN Equator Initiative Laureate)
Director General & Senior Vice President, RBEF
(Former Director, NBRI, Lucknow)

08-06-2009

Foreword to the Series

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

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

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

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

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

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

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

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

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

(P. Pushpangadan)

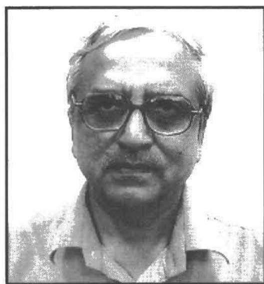
About the Editors

Dr. Vijay Kumar Gupta

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

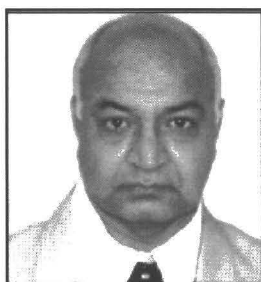
Dr. Subhash Chandra Taneja

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



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

Dr. Bishan Datt Gupta



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

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

Preface

Through out human history people have relied on drugs derived from plants to promote and maintain good health and to fight sickness, pain and disease. Herbal drugs are having prolonged history of frequent use and documentation in texts of established systems of medicine indigenous to a particular country. While modern medicine has, in many parts of the world, replaced traditional medical practices to the benefit of individual and public health, we are becoming increasingly aware of its limitations in dealing with a large number of conditions and diseases, the often unforeseen negative side effects of synthetic drugs, and the ever-rising costs of medical treatment, including pharmaceuticals. As a result, the public and an increasing number of physicians and public health specialists throughout the world are taking a second look at alternative or complementary medicine generally and traditional plant based drugs in particular.

A number of diseases still have no satisfactory cure in modern medicine and certain herbal formulations /traditional medicines are reported to have effective treatment for these ailments. Unfortunately no serious efforts have been made to verify these claims and to develop validated, standardized herbal formulation(s) for the effective cure or management of these diseases. Although there are many formulations available in the market but neither they are scientifically evaluated nor authenticated or quality controlled. Extremely limited knowledge about the ingredients in the herbal drug formulations and their effects in humans, the lack of stringent quality control and the heterogeneous nature of these preparations all necessitate the continuous monitoring of the safety of the herbal products. Assessment of safety and adverse effects of herbal preparations can be much more complex than the modern pharmaceuticals.

Standardization of the herbal drugs by quantitative determination of constituents has been made easy by recent developments in analytical instrumentation. Advances in the isolation, purification, and structure elucidation of naturally occurring metabolites have made it possible to establish appropriate strategies for the determination and analysis of quality and the process of standardization of herbal preparations. TLC, HPLC, GC, quantitative TLC (QTLC), and high-performance TLC (HPTLC) can determine the homogeneity of a plant extract/ formulation. Over-pressured layer chromatography (OPLC), infrared and UV-VIS spectrometry, MS, GC, liquid chromatography (LC) used alone, or in combinations such as GC/MS, LC/MS, and MS/MS, and nuclear magnetic resonance (NMR), are powerful tools, often used for standardization and to control the quality of both the raw material and the finished product.

In the present volume "***Quality Control & Standardization***" of the book series "**Comprehensive Bioactive Natural Products**" an

attempt has been made to cover the recent progress in standardization of the herbal/traditional drugs and strategies adopted for quality assurance of such formulations. Applications of the recent analytical techniques in standardization of these drugs on the bases of marker compounds have also been widely covered. The topics have been contributed by the experts in the fields with relevant and up to date information and studies included in the volume are: Chromatographic techniques applied to the natural products chemistry; Quality control of natural medicines by immunological assay system; Comparison of HPLC and HPTLC methods for the determination of rosmarinic acid; Quality control and standardization of medicinal and aromatic plants; Quantification of podophyllotoxin in *Podophyllum hexandrum*; Standardisation of polyherbal formulations using marker compounds; Variation of lavender oil produced by different techniques; Quality control methods for herbal medicines; Evaluation of biological activity in quality control of herbal medicines; Chemical analysis and quality control of essential oils; Ultraviolet spectrophotometric method for the powdered *Loranthus micranthus*; Exploring pharmacovigilance for traditional herbal medicines; HPLC for evaluation of herbal drugs and formulations; Consumer protection and regulatory requirements for herbal drugs

We believe that the present volume will be a useful compendium of knowledge for chemists, ethnobotanists, pharmacologists, pharmaceutical scientists as well as other researchers in traditional medicines. It will also be of relevance to the Herbal Drug industry.

Jammu, India

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

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1

Chromatographic Techniques Applied to the Natural Products Chemistry

MARIA APARECIDA M. MACIEL[2,*], VALDIR F. VEIGA JR.[2], ANGELO DA CUNHA PINTO[3], FABIANO E.S. GOMES[1] AND TEREZA N.C. DANTAS[1]

ABSTRACT

Medicinal plant users from the entire world keep alive therapeutical informations accumulated for centuries that have been in vogue to research projects involving multidisciplinary fields such as ecology, anthropology, botany, biochemistry, pharmacology and phytochemistry. Both medicinal culture and multidisciplinary investigations lead to the understanding of this inexhaustible natural source: the world flora. In this context, this paper focuses on relevant basic concepts for multidisciplinary researches on medicinal plants, with the primary purpose of stimulate, actualize and instruct anyone that have interest in chemical and pharmacological studies about medicinal plants. Additionally, included general aspects of chromatography and attention will be concentrated to Croton cajucara Benth (Euphorbiaceae) and Copaifera L. (Leguminoseae–Caesalpiniaceae) representing two of the most relevant medicinal plants from Brazil. In order to emphasize basic concepts on classical phytochemistry approaches, this paper shows the importance of such investigation on multidisciplinary studies conducted with the medicinal specie Croton cajucara widely used in the popular medicine of Amazon region of Brazil, which actually represent significant

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importance around the world and Copaifera L. which oil is popularly known as "copaiba oil", will be focused upon, in those studies been representative for HRGC-MS analyzes.

Key words : Botany, chromatography, phytochemistry, medicinal plants, *Croton cajucara* Benth, *Copaifera* L.

INTRODUCTION

Scientific research concerning to natural products has displayed a progressive technological evolution, which could lead to a drastic reduction in the gap observed in the relationship: number of living vegetable organisms versus number of species studied. This evolution can be regarded in terms of new methods for selection and collection of plant materials, new isolation techniques, improvement of spectrometric apparatus and techniques, great amount of biological evaluations, and several studies involving semisynthesis or biosynthesis, which are widely reported in several scientific books and papers. In this context, the development of multidisciplinary researches is encouraged in order to optimize the use of natural bioactive products. Examples of multidisciplinary researches are (among a large amount of other examples) the works of Cechinel Filho and Yunes (1998) who reviewed the strategies for obtaining bioactive natural products from medicinal plants; Maciel *et al.* (2005, 2002a) in a work entitled "Medicinal plants: the need for multidisciplinary scientific studies" emphasizing several botanical, ethnobotanical, phytochemical, and pharmacological approaches in the natural products field; Viegas Jr. *et al.* (2006) with the paper "The natural products and the modern medicinal chemistry", and Albuquerque and Hanazaki (2006), in a work entitled "Ethnodirected research in the discovery of new drugs of medical and pharmaceutical interest: flaws and perspectives" also pointed the importance of natural products as source of new drugs, and the technological advances observed in the pharmaceutical field around the world.

The goal of this article is to emphasize the relevance of medicinal plants investigations involving botany, ethnobotany and phytochemistry. General chromatography aspects will be focused for their applications in obtaining pure biologic compounds aiming pharmacological evaluations. Two of the most relevant medicinal plants from Brazil, *Croton cajucara* Benth and *Copaifera* L. (Leguminoseae-Caesalpiniaceae) will be focused upon. Both of these plants are largely used in the Amazon region of Brazil for centuries and, more recently, since the 1900s, their use has spread nationwide.

Ethnobotany: An Interdisciplinary Science

Ethnobotany is an interdisciplinary science which needs a multidisciplinary team to validate traditional uses of medicinal plants. In this context, it is important to consider as a start point the establishment of a minimum of three qualified professionals working closely together: ethnobotanist, chemist and pharmacologist.

The term ethnobotany was first applied in 1895 by Harshberger, who pointed out how useful it could be in the medicinal plants projects. Since then, several definitions have been found for ethnobotany (Maciel *et al.,* 2005). Among them "scientific botany focusing upon the habitat and use of a specific ethnic group, performed by a scientific botany expert who would eventually associate the eastern scientific classifications with the local one". Ethnobotany applied to the study of medicinal plants work hand in hand with ethnopharmacology, which consists in the interdisciplinary scientific exploration of biologically active agents, traditionally applied or observed by determined human group (Bruhn & Holmstedt, 1982; Bruhn, 1989). One of the pioneers in the study of ethnobotany who was dedicated to the research of flora in Tropical America was Richard Evans Schultes, who may be considered the father of modern ethnobotany. Natural product research involving an ethnobotanical approach to medicinal plants is cited in the literature as an alternative method, which can provide both efficient and successful results contributing to the discovery of new bioactive natural products (Gurib-Fakim, 2006; Gilani, 2005; Maciel, 2005, 2002a; Unander *et al.,* 1995; Cox & Balick, 1994; Cordell *et al.,* 1995, 1991; King & Tempesta, 1994). This field of research focuses on two fundamental factors: plant collection and its medicinal uses. The first factor implies region, time and stage of development favourable to the period of collection. It also involves special proceedings such as the correct identification of the species by a qualified professional, a botanist for example, and a voucher specimen should be deposited in an authorized herbarium.

The selection of the specimen to be analyzed is one of the critical points. An inappropriate choice can result in unsuccessful work. Several approaches to the selection of the specimen have been shown, among them randomized, chemotaxonomical (or philogenetical) and ethnopharmacological collection. The ethnopharmacological selection, however, favours the discovery of bioactive substances, in which the selection of species is in accordance to the therapeutic evidence based on experimental uses by an ethnic group. In this approach, medicinal plants are not simply regarded as raw material. The accounts of a plant's background as a therapeutic resource in the treatment and healing of diseases of a determined ethnic group must

be interpreted as a monetary and time saving procedure, two of the most sought after factors by eastern economies. In the other hand, in the random collection, 10000 different types of plants may represent 50000-100000 different natural products structures, whether bioactive or not (Cordell, 1995; Malone, 1983).

Before starting any experimental work, the specimen must be safely identified. At that point, another specialist is required: a botanist or specialized professional. The lack of scientific identification (or a mistaken identification) may deliberately cancel the whole chemical or even a pharmacological investigation, making it practically useless and impossible to be published. Taking into account this possibility, the collection of the plant must occur in two stages: a previous specimen may be collected for the identification of the species, and then a definitive collection, which contains large amounts of the material destined to the phytochemistry and pharmacological investigations. On the previous stage, small branches with leaves, flowers and fruits at different stages of growth are collected. The samples must represent the general aspect of the plant; branches damaged by insects or damage from handling must be avoided. However, if the representative samples do have such flaws, they should be collected just the same, as any factor is top priority. The collected samples must be sent to a specific herbarium where they are pressed and dried (if such procedures have not been performed at the gathering site). After obtaining scientific identification (voucher specimen), the plant is catalogued containing the followed items: register number, seal of the institution, labelling, plant sample, envelope with flowers and/or fruits; protective catalogued information must be provided. Afterwards, a file must be made including: 1) scientific name of the specimen and its botanic family; 2) name of whom has rated the species; 3) register number of the voucher specimen, 4) location of herbarium in which the register is deposited; 5) location and date of the collection; 6) popular name of the plant; 7) accounts of the part of the plant being used in popular medicine and its therapeutic indications; 8) notes about soil conditions where the plant was collected, as well as type of local vegetation, type of plant (bush or tree), time of the blossom (if it is in flower), time of fruitage (if it bears fruits), colour and smell of several parts of the plant. Finally, latitude and longitude information's, as well as some photographs of the collected plant must be taken in its natural habitat (Maciel *et al.,* 2005, 2002a).

The second stage of the collection destined for the phytochemistry and/or pharmacological approaches consists of only one or several collections. The latter may be done at different times of the year or on different locations. Since different plants might be popularly known

under the same name, the process of plant identification must be taken into account in case the gathering occurs in different regions. Upon the stage which determines the phytochemistry study, the total amount of the material to be collected is defined as well as the part of the plant that will be studied is picked out (roots, stem bark, bark, branches, leaves, flowers, and fruits). In a project that links phytochemistry and pharmacology professionals, the part of the plant used in popular medicine must be chosen. In this case, the least amount of vegetable material to be collected is 2 kg. However, once working conditions in the lab are satisfactory, 3 to 6 kg of collected material must be obtained in order to set apart great amounts of major substances, then enabling pharmacological evaluations of isolated drugs. During the collecting procedure the following steps must be carefully monitored: separation and labelling of the collected material, wrapping in plastic bags, transport of such material, weighing of the dried material, storing, grinding, weighing of the triturated material, obtaining extracts. The drying stage can be achieved in the sun, in a shade or in greenhouse, as long as the temperature is approximately 40°C. If research interest is only in the essential oil of the plant, drying must be avoided. The storing of the dry material must be done in plastic bags packed in cardboard boxes and kept in safe locations of low humidity and temperature. This procedure avoids oxidation and hydrolysis of the plant constituents, and also prevents against attack by microorganisms, among other (Maciel *et al.,* 2005, 2002a).

Chromatography: A General Approach

The first liquid chromatographic separations using the preparative layer technique were carried out in 1930s from vegetable metabolites (pigments, like chlorophyll and carotenes). Chromatography work was initially supported by the pioneering experiments carried out by Tswett in 1906. The origin of chromatography is very well connected to the study of natural products. This association continued to exist during years, and would be inconceivable to imagine the actual advances in the purification of biomolecules without considering the classical chromatography, including the important contribution played by Martin in the 1950s and the introduction of commercially-available high-performance liquid chromatography (HPLC) equipment in the 1970s (Marston, 2007; Hostettmann *et al.,* 2003).

Chromatography is defined as a separation process of components from a mixture containing two or more substances. It depends on the differential distribution of a solute between mobile and stationary phases. Most of the chromatographic techniques work under the same principle observed in solvent extraction. Thus, the separation

results from differences in the distribution constants of the individual sample components between two phases.

Concerning to the main phenomena responsible for the separation of components, chromatographic techniques can be divided in several types, including adsorption, partition, size exclusion, affinity, or ion exchange. Chromatography can be also classified based on mobile phase, in terms of liquid or gas chromatography. In the gas-liquid chromatography, or simply gas chromatography, the mobile phase is an inert carrier gas and the stationary phase is a microscopic layer of liquid or polymer on a inert solid support, while in the liquid chromatography, the mobile phase consist of a liquid, and the stationary phase a packing solid material, usually silica. In general manner, the system contains a stationary, which can be liquid or solid, and a liquid or gas mobile phase, which continuously moves in a definite direction through the stationary phase.

The adsorption of a given substance on solid surfaces is related to the same forces that act in solubility behavior (electrostatic interactions, hydrogen bonds, van der Waals interactions, among other). It is important to emphasize that the acidity and the possibility of formation of hydrogen bonds by silanol group make the silica surface an excellent surface for adsorption. Thus, for determined applications this physicochemical phenomenon may be irreversible, or so strong that would be very difficult to liberate some components from silica surface. The high degree of acidity may also act to promote catalytic degradations, which in certain cases, are aided by the presence of traces of metals in silica. Table 1 shows the increasing order of adsorption in thin layer (TLC) and column chromatography (CC) for compounds that present different polarities, as well as the order of eluting power of some organic solvents in function of their polarity. In general manner, the adsorption order for functionalized compounds is Cl^-, Br^-, $I^- < C{=}C < OCH_3 < COR < CHO < SH < NH_2 < OH < CO_2H$. The type of interaction involved in adsorption phenomena depends on the nature of the sample and stationary phase. Thus, for polar phases, the adsorption of functionalized compounds increases with the degree of polarity of the several classes of organic compounds. The most common solvents used in chromatographic procedures are also listed in Table 1. Toluene and benzene are less polar solvents than chloroform, but they should be avoided due to their high toxicity. Acetone (Me_2CO) is more polar than chloroform ($CHCl_3$), but presents in its structure a reactive carbonyl group which may react with plant metabolites producing artifacts, and should be avoided. Water and organic acids are options for high-polar compounds, but the difficult to remove them from sample, prevent their use in chromatography. Thus, it was established the following order of

Table 1. Adsorption and elution increasing order in TLC analyzes

Adsorption order for organic compounds	Saturated hydrocarbons
	Unsaturated or aromatic hydrocarbons
	Ketones
	Aldehydes
	Esters; amides
	Carboxylic acids
Elution polarity order for solvents	Hexane
	Carbon tetrachloride
	Ethers
	Dichloromethane
	Chloroform
	Adfadf
	Alcohols

increasing polarity: cyclohexane (0.04), hexane (0.1), carbon tetrachloride, toluene, benzene, diethyl ether (2.8), dichloromethane (3.1), tetrahydrofuran (4.0), chloroform (4.1), acetone, ethyl acetate, ethanol (4.3), methanol (5.1), water (10.5), and organic acids. The separation efficiency among solutes depends also on the nature of solvent (eluent or mobile phase), since it may compete with solute molecules for surface adsorption sites (very polar solvents may be easily adsorbed on stationary phase). Two or more solutes that present different adsorption coefficient for a given stationary phase, for example, silica gel, can be separated through liquid-liquid chromatography. However, in order to have an efficient separation, the solvent must be significantly less polar than the components of the mixture and must be able to dissolve them appreciably. Table 2 shows different types of chromatographic techniques based on the distribution of the analyte between mobile and stationary phases (Marston, 2007; Costa-Neto, 2004; Neto & Nunes, 2003; Poole *et al.*, 1990; Zweig & Sherma, 1972).

Generally classical chromatographic separations are carried out in open column using silica gel as stationary phase, and the fractions obtained are monitored by thin layer chromatography. The fractions that showed similar results in TLC analyzes (using several elution systems and revealing reagents) must be pooled and purified. The purity criteria adopted in TLC is characterized by the observation, using several solvents, of a unique and uniform spot in the plate. However, other procedures can be adopted, such as: fraction monitoring by ^{1}H nuclear magnetic resonance or fraction analyzes by gas chromatography.

Table 2. Analyte distribution system

Normal phase	The solid phase is polar and the liquid phase is less polar.
Reversed-phase	The solid phase is non polar and the liquid phase is more polar.
Ion-exchange	The solid phase has attached ions, which in turn, are able to retain counter ions. Ions from sample (present in mobile phase) could be displaced by the original counter ions, and take their place.
Ion-pair	Ion-pair formation, ion interaction.
Size exclusion	The solid phase has pores of different sizes that entrap small molecules and exclude the large ones.
Association to a biological receptor (affinity chromatography)	The solid phase incorporates biological receptors, which are able to interact and retain analytes that shows affinity with them.

Vegetal Material Extraction

The powdered material must be obtained by preparation of the extracts, which could be performed by percolation (cold extraction method), Soxhlet apparatus (hot extraction method) or acid/bases. In the acid bases extraction process, the water-acidic or basic phase as well as organic phase with solvents immiscible in water (Et_2O, $CHCl_3$ or EtOAc) is applied. Percolation extraction, also known as maceration, is the most used procedure to obtain plant extracts, due to the low risks to generate artifacts, since low temperatures is used in this process.

For unique extraction (at room or higher temperatures) it is common to use only one polar solvent as ethanol or methanol. For multiple extractions, it could use three types of solvents in this order: a non polar solvent (hexane or petroleum ether), a moderately polar solvent (chloroform or dichloromethane), and then a polar solvent. However, due to the high toxicity of chlorinated solvents, their use in plant extraction must be avoided been in accordance to the international protocols limiting the production of chlorinated solvents. Thus, the most frequent technique used involves the obtaining of the extract through a unique extraction using ethanol or methanol, which allows to extract both polar and non polar compounds. In this case, subsequent chromatographic fractionations would be utilized in order to obtain up 40 fractions of distinct polarities. For extraction using non polar solvent, only low-polar

compounds will be obtained (Maciel *et al.*, 2002a). The Table 3 shows different extraction approaches that may be used in extraction of natural products from plants.

Supercritical fluid extraction (SFE) is another technique that has become very effective over the years. It is based upon the use of supercritical fluids, usually carbon dioxide (CO_2), sometimes modified by co-solvents in low percentage such as water, methanol, ethanol, propanol, acetonitrile, or dichloromethane. The use of co-solvents allows the work optimization, as increasing the extraction yield and the possibility of extracting high-polar compounds (when using polar solvents). The supercritical fluid used in extraction are gaseous substances, which are usually inert, economic, and present low viscosity and high diffusion rate when compared to liquids. Moreover, this technique has also the advantage of reducing the amount of organic solvents used in extraction when compared to percolation or Soxhlet apparatus. The limitation of the use of SFE consists in the reduced amount of sample that is used in each extraction (usually 200 g). Even if one has extractors to work on a

Table 3. Extraction methods for natural products

Extraction methods	Procedures
Room temperature Extraction (percolation or maceration)	a) Sample + Solvent + Manual stirring; b) Sample + Solvent + Mechanical stirring c) Sample + Solvent (24 h)*.
Hot extraction (Soxhlet)	Sample + Solvent + Heating.
Acid-base extraction	Based upon partition process between acidic or basic aqueous solvents and water-immiscible organic solvents (ether, chloroform, ethyl acetate).
Solid-liquid extraction	Performed at low pressure. The column is filled with dried material and the extraction solvent is pumped through the sample.
Steam extraction	Applied for essential oils. Distillation is performed through a water steam carrier and may occur either for high- or low-volatile compounds.
Supercritical fluid extraction	Performed with the use of supercritical fluids (carbon dioxide, nitrous oxide, among other). This is a viable option for substitution of steam, percolation, or Soxhlet extractions, since with this technique it is obtained high- and low-volatile compounds, as well as moderate polar compounds.

*Also known as static maceration

large scale, the typical volume in the extraction cell varies from 1 to 50 mL, with the usual volume being approximately 10 mL, which limits to the use of small amounts of sample, even if interconnect several cells. A large number of natural products have been actually extracted through SFE such as hop, nicotine, and essential oils with flavouring properties (Zacchi *et al.*, 2006; Castioni *et al.*, 1995; King & Bott, 1993). Souza *et al.* (2006) obtained fixed oils (FO) from the stem bark of *Croton cajucara* Benth through percolation and supercritical fluid extractions (SFE, carried out with CO_2), aiming their phytochemical characterization and evaluation of antifungal properties.

Experimental Chromatography: A General Comments

Column chromatography (CC) represents one of the most efficient and employed method used to separate, and purify natural products. Several types of CC methods can be found: the classical open glass columns, low-, medium-, or high-pressure CC (flash chromatography), and CC equipped with capillary columns and a gas as the mobile phase. In any chromatographic process, the separation efficiency depends directly on the time of contact between mobile and stationary phases, as well onpartition coefficient. In other words: it depends on the amount of silica, the ratio between column diameter and height, and the elution system (solvent polarity).

The classical open glass column containing silica can be employed in preliminary filtrations, chromatographic fractionation, or even in long time chromatographic processes, aiming the immediate isolation of a given compound. For extract filtration, it is recommended to use as stationary phase silica gel having high granulometry (35–70 mesh, for example), and as mobile phase two or three solvents in an ascending polarity gradient, such as:

a) hexane (for obtaining fraction F1), chloroform (F2) and methanol (F3)
b) hexane (F1), chloroform (F2) and ethyl acetate (F3)
c) chloroform (F1), ethyl acetate (F2) and methanol (F3)
d) ethyl acetate (F1), methanol (F2) and methanol: water (7:3; F3)

The filtration procedure is specially used when working with a unique polar extract obtained from methanol, ethanol or methanol-water mixture (hydroalcoholic extract). This procedure is also performed when one wishes to obtaining low polar fractions from a polar extract using the liquid-liquid partition technique (with chloroform or acetyl acetate).

The volume of solvent and the amount of silica to be used in a chromatographic filtration depend on the amount of extract. Usually, for 1 g extract fractions of 200 mL are collected for each eluent. However, the total volume of the eluent should be monitored by TLC, so at the end of the elution process of each solvent, a TLC analyzes is performed in order to verify if any compound remains to be eluted in a given eluent before to move to the next more polar solvent. If the fractioning works adequately, this procedure can be reproduced in large scale for large amount of extracts, using higher amounts of silica and eluent, but remaining the same number of fractions to be collected.

If the distribution of a same compound occurs through several fractions collected, this may be due to the inadequate ratio used between amount of the extract and stationary phase. This ratio may vary a lot, with proportions from 1:10–1:30 (wt extract/wt silica) up to 1:50–1:100 in some cases. The choice of the most appropriate ratio should be conducted in accordance with the chemical nature of the extract (or unpurified fractions), the goal (compound which wants to isolate) and the experimental planning for the next steps. In works where all fractions to be collected will be analyzed in a later chromatographic procedure, a higher ratio of extract/silica can be applied. In this case, the compounds not eluted in a solvent would be presented in the next eluent. On other hand, if the aim of the work is to obtain only a specific fraction, the most non polar, for example, it should use a lower ratio and/or increase significantly the amount of eluent to ensure that all compounds soluble in low polar solvents would be eluted.

For extract fractionation purposes it is recommended the use of silica gel 70–230 mesh (in ratio 1:10–1:30), and pure or purified solvents in an ascending polarity gradient, as shown below:

a) hexane: ethyl acetate (100:0–0:100), for obtaining up to 20 fractions. The increasing of the solvent mixture polarity should obey the follow: F1 and F2 obtained from hexane 100%, F3 to F16 from ethyl acetate 1, 2, 3, and 5%, F17, F18, F19 and F20 from ethyl acetate 10, 20, 50, and 100%, respectively;

b) hexane: ethyl acetate: methanol (100:0:0–0:100:0 and 0:90:10–0:0:100), for obtaining up to 20 fractions, which should be obtained as follow: F1 eluted with hexane 100%, F3 to F10 from mixtures of hexane: ethyl acetate at ratios 99:1, 97:3, and 95:5, F11, F12, F13, and F14 obtained from 90:10, 80:20, 50:50, and 0:100, respectively; remained fractions should be obtained from ethyl acetate: methanol mixtures at proportions

90:10 (F15), 80:20 (F16), 70:30 (F17), 50:50 (F18), 30:70 (F19) and 0:100 (F20);

c) in order to obtaining a small number of fractions: hexane 100% (F1), hexane:dichloro-methane 1:1 (F2), dichloromethane: ethyl acetate 1:1 (F3), and ethyl acetate: methanol 1:1 (F4);

d) the elution process should also occur with a fast increasing of the gradient polarity of the solvent, as in the example: F1 obtained from hexane or ethyl ether, F2 from dichloromethane or chloroform, F3 from ethyl acetate, and F4 from methanol, or in mild conditions by slightly increasing the solvent polarity as in the use of hexane: ethyl ether: chloroform mixtures where F1 would be eluted with 99.5:0.5:0; F2 (99:1:0); F3 (95:5:0); F4 (80:20:0); F5 (70:30:0); F6 (50:50:0) and then, the third eluent more polar is added: F7 [50(hexane):50(ethyl ether):10(chloroform), F8 (50:50:30), F9 (50:50:40), F10 (50:50:60), and finally F11 (0:0:100).

In the extract fractionation procedure the ratio between the amount of extract and the volume of fractions obtained should also be 1 g extract for 100–200 mL of each fraction collected. Similarly for the filtration procedure, the volume of the fractions to be collected may vary a lot, depending upon the nature of the compound that is being eluted and the monitoring of the elution of a given compound, which may be accompanied either through coloration changes in silica or the formation of crystals at the bottom of the column. Thus, the change of solvent should only be performed when no more remained substances are being eluted.

For extract analyzes using a chemosystematic approach, the chromatographic study can be conducted analyzing all fractions obtained from a single chromatographic procedure, or obtaining new fractions from the first ones through a new chromatographic procedure. If the aim of the work is discovering new chemical entities from plants, the analyzes would be carried out only in the fractions that showed the most promising data spectra (NMR analyzes). When the work aims to correlate phytochemical data to biological/ pharmacological properties of the plant, it is recommended the use of a chemopharmacological approach for the analyzes of fractions obtained from filtrations or pooled fractions (after TLC and GC analyzes). For this purpose a new fractionation of the bioactive fractions should be directed to the isolation of bioactive compound(s), in a process known as bioassay-guided fractionation. Scheme 1 shows a general procedure for isolation of terpenoid compounds and non polar flavonoids (Maciel *et al.*, 2005, 2002a, 2000). Scheme 1 integrates both phytochemical and pharmacological approach, characterizing an

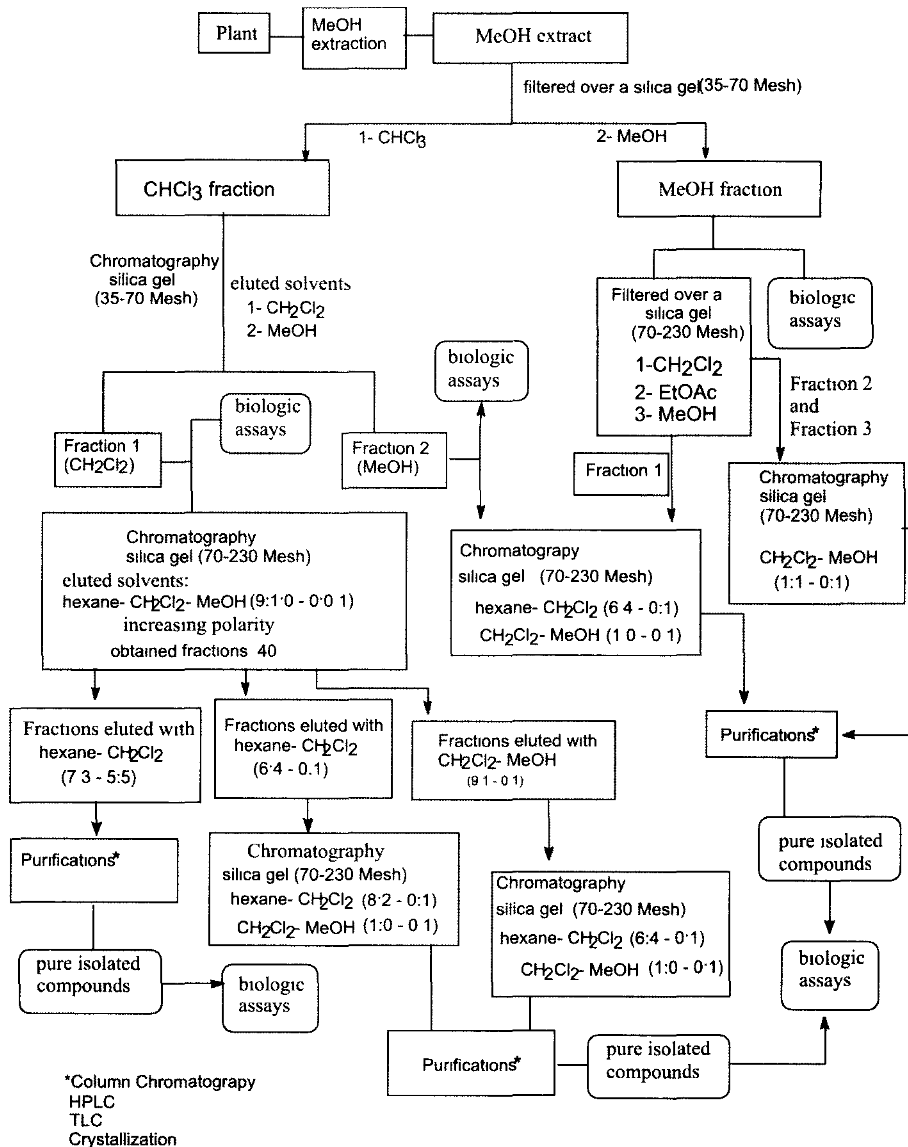
interdisciplinary relationship. The pharmacological assessment of crude extract, crude fractions, and isolated substances must rigorously be oriented by the therapeutic benefits previously divulged for the specimen. Adequate selection of the specific biological screen will allow a general evaluation of the therapeutically potential of the specimen in focus, as well as provide information about the toxicity of the plant.

The chromatographic separations suggested in Scheme 1 is performed on a column of silica gel and the similar fractions detected by thin layer chromatography (TLC) analyzes must be brought together and purified according to specific separation techniques. The adopted criteria for purification are the one in which varying the eluted solvent system, only one uniform dot is revealed in TLC by specific reagents. Since the choice of methods for isolation of natural products must be correlated with the polarity of the target substances, other methods may be applied. Most of them involve high performance liquid chromatography (HPLC) for isolation and purification of substances, in addition to solid-phase extraction or solid-phase purification procedures (Li *et al.,* 2007; Tsai *et al.,* 2007; Chafer *et al.,* 2005; Smith, 2003; Adlof & List, 2003; Daood *et al.,* 2002; Poole *et al.,* 1990).

Finally, the identification of organic constituents must be based on the following spectrum analyzes: 1) infrared spectroscopy analyzes (IR), in which the spectrum is interpreted based on the presence or absence of functional groups; 2) ultraviolet spectroscopy (UV), which informs the presence of unsaturated compounds. In practice, it is for the most part limited to conjugated systems; 3) spectroscopy of nuclear magnetic resonance of hydrogen (H^1 NMR) and carbon (C^{13} NMR) which determines the nature and chemical environment of hydrogen and carbon, respectively, and has been the most relevant technique to detailed molecular structures, 4) mass spectrometry (MS), which offers data on the molecular weight and the molecular formula as well as the identification of fragments characteristic to the molecule.

Maciel *et al.* (2003, 2002a, b, 2000, 1998a, b) show how this methodology works for isolation of bioactive clerodane-type diterpenes from *Croton cajucara* Benth (discussed herein later), as well as for the separation of flavonoids compounds. The eluent system could be changed to a mixtures of hexane: EtOAc in gradient of polarity giving the same efficient results. Another example of a bioassay-guided methodology in multidisciplinary works was applied by Veiga Jr. *et al.* (2005a, b, 2001, 1997), in the studies of copaiba oils (discussed herein later). In this late example, the aim of the work is to purify specific fractions with low impurities or obtaining fractions to be

analyzed in gas chromatography-mass spectrometry (GC-MS) techniques. In this case, the proportion 1 g of sample per 50 g of silica can be applied in order to obtain fractions of 50 mL each, using several mixtures of solvents as eluent as shown in Table 4. The application of this methodology gives fractions with distinct polarities, easing the analyzes of the chemical structure, functional groups and



Scheme 1. General phytochemical approach for isolation of bioactive compounds

Table 4. An example of elution systems for column chromatography*

Eluent	Volume eluted (mL)
Hexane 100%	100
Hexane: EtOAc 5%	100
Hexane: EtOAc 10%	100
Hexane: EtOAc 20%	100
Hexane: EtOAc 40%	50
hexane: EtOAc 50%	50
EtOAc 100%	50
EtOAc: MeOH 5%	50
EtOAc: MeOH 50%	50
MeOH 100%	50

*For 1 g of extract or fraction obtained through filtrations or chromatographic fractionation

classes of natural compounds that would be obtained in each fraction. For chromatographic resolution of polar extracts, it is common to find in the two first fractions aliphatic, saturated and unsaturated hydrocarbons, and fatty acids and alcohols, as well as phytol, chlorophyll and steroids. Between the third and fifth fractions, diterpenes and triterpenes less oxygenated may be found, if present in the extract. These fractions may be analyzed by GC-MS, HRGC (High Resolution Gas Chromatography), or HRGC-MS (High Resolution Gas Chromatography-Mass Spectrometry). Additionally, they may also be analyzed by Nuclear Magnetic Resonance (NMR) in order to confirm the GC analyzes and point for the presence of another molecule already reported in literature or not.

After performing a given chromatographic procedure, the semi-purified fractions obtained should be re-fractionated through more refined processes, using silica gel with low granulometry, usually 230-400 mesh in isolation or purification procedures. As this silica possesses small diameter, it produces a very compact stationary phase when packed, difficulting or slowing the flow of the eluent in the column. The use of air pressure or pumps in open chromatographic columns was initially idealized by Still *et al.* (1978) and has been named "flash chromatography", and constitutes a good technique to speed up the chromatographic process and reducing its duration (usually the time spent is about 2 h, including the time spent for column preparation). Similarly to the classical open column chromatography, it should perform initially a preliminary TLC

Table 5. Parameters for flash column chromatography

D Inner diameter column (cm)	V Volume of eluent (mL)	v Volume of each fraction (mL)	m Sample mass (mg)	
			$\Delta R_f > 0.2$	$\Delta R_f > 0.1$
1	100	5	100	40
2	200	10	400	160
3	400	20	900	360
4	600	30	1600	600
5	1000	50	2500	1000

analyzes in order to optimize the separation of the components present in the sample. This separation observed in TLC (ΔR_f higher than 0.1 or higher than 0.2) as well as the amount of sample are used as parameters for the choice of the column size (inner diameter) and the total volume of solvent to be eluted in each fraction (in a total of 20 fractions).

The use of silica gel 230–400 mesh in resolution procedures through flash chromatography may reproduce the separation obtained through TLC. Thus, it is possible to achieve a good separation using the same elution systems if it is in accordance with a proportion between the amounts of sample, the difference observed in R_f of the components in TLC analyzes, and the column diameter, which is directly proportional to the amount of the used silica gel. This relation is shown in Table 5 (Pinto *et al.,* 2000; Still *et al.,* 1978). Another important property of this technique is related to the dry-pack method used to fill the column with silica. In this case, the pack of the column is performed without solvent, which is added only after the silica is settled. The pressure is another important parameter that must be controlled because too much pressure may mix the components eluted, and low pressure may be insufficient to elute the components at an acceptable rate. In Table 6 are presented some suggestions of flow rate commonly applied in function of the column size and the amount of silica used (Pinto *et al.,* 2000; Still *et al.,* 1978). Recently, Martins *et al.,* (2006) employed

Table 6. Flow rates adopted in flash column chromatography

d Inner column diameter (cm)	Flow rate (cm/min)
2.0	4-10
5.0	20-40

this methodology (silica gel packed column using vacuum), where the mobile phase was hexane, dichloromethane, ethyl acetate, methanol, and water in polarity gradient, with analyzes of obtained fractions through TLC using the Godin's reagent as revelator. This work resulted in the isolation of the flavonoids quercetin and chrysosplenol, among other chemical constituents from *Acanthospermum australe*.

In a wide context, other chromatographic techniques can be used aiming the isolation and purification of natural products. Several types of stationary phases can be used as well as those impregnated with a specific reagent or solution. A common procedure for isomer resolution (when the difference is due to the position of the unsaturation) frequently used chromatography involves the use of silica (70–230 mesh) containing 5% to 20% of silver nitrate, which is heated up to 110°C during 3 h for dryness and activation (Adlof & List, 2003; Williams & Mander, 2001; Pinto *et al.,* 1997). Once dried and activated, the silica impregnated with silver nitrate can be used in a resolution chromatographic procedure in darkness or in low-light ambiances to prevent the oxidation of silver. Despite the darkening of the silver added to the silica affect little the chromatographic resolution, is not affected. Silica impregnated with silver nitrate can be also used in a similar manner in preparative TLC (Pinto *et al.,* 1997).

Another impregnation technique aims the resolution of specific classes of compounds, like carboxylic acids, in a process similar to ion-exchange chromatography. In this technique, the silica (70-230 mesh) is impregnated with a potassium hydroxide solution 10% in an amount sufficient to humidify the silica. As observed previously in the impregnation with silver nitrate, the silica impregnated with potassium hydroxide must be heated up to 110°C during 3 h prior to be used in chromatography, whose process is similar to filtration, where hexane, chloroform, and methanol can be used as eluents. The methanol fraction contains the carboxylic acid salts of potassium presented in the mixture, which should be neutralized, as well as some acids that are also eluted with chloroform. The hexane and chloroform fractions would contain hydrocarbons and alcohols, respectively, presented in the sample (Pinto *et al.,* 2000).

Modern Techniques of Chromatography

The progress of the natural product chemistry has always been linked to technological advancement of the analytical chemistry. The characterization of complex mixtures of compounds often requires the use of modern techniques which can improve significantly the

sensitivity and selectivity of the structural analyzes of the compounds studied. Thus, it is possible to detect accurately compounds of interest and directing their isolation and purification for further characterization. Exhaustive and preliminary chromatographic procedure is avoided in modern approaches for isolation and purification of natural products, which involves the use of high performance liquid chromatography (HPLC) in conjunction with MS, UV, IR and NMR (hyphenated techniques).

HPLC-based techniques require large volumes of solvents, and in HPLC-NMR these solvents must be deuterated, further increasing the costs. A single HPLC chromatograph coupled to all spectroscopy techniques (HPLC/MS, HPLC/IR, HPLC/UV, HPLC/NMR) exists only in a few research labs around world (Marston, 2007; Stoll *et al.*, 2007; Theunits *et al.*, 2007; Henry & Yonker, 2006; Petrovic *et al.*, 2005; Wolfender *et al.*, 2003; Dallüge & Brinkman, 2003; Queiroz *et al.*, 2002a, b; Grob, 2000; Poole, 2000; Albert, 1999; Smith, 1999). Recently, Morston (2007) reported an extensive revision concerning to the major chromatographic methods used in phytochemistry with concentration to HPLC chromatographic application.

With the growing interest in the discovery of new metabolites from marine or land microorganisms and superior organisms, the need to separate mixtures in small or large scale, in an economic, fast and efficacious manner is claimed. Because of these three requisites it is very important to know and judge the best choice of chromatographic technique to be applied in a given problem. When aiming economic and time issues (avoiding the long and exhaustive procedures of the open column techniques), the coupled techniques like HPLC-UV, HPLC-IR, HPLC-MS, and HPLC-NMR are the best options to be used in extracts and unpurified or semi-purified fractions.

In the last decade, HPLC was one of the most employed techniques for isolation and analyzes of natural products from complex matrices, like plant extracts. This technique was originally denominated as High Pressure Liquid Chromatography, but this terminology was swept away when it was noted that its differential in comparison with other techniques lay in chromatographic performance and not in the use of high pressure. Currently, HPLC is widely applied in cosmetics, foods, pharmacy, chemistry, and biotechnology.

Concerning to gas chromatography (GC), we can say that it is a physical method of separation of compounds from a mixture through the passage of a mobile gas phase through a adsorbent phase. This technique is employed for volatile compounds, so the compounds found in the mixture should present a reasonable vapor pressure at the separation temperature. One of the main disadvantages of GC

lay in the fact that as the ionic nature of the compound increases (and therefore its volatility reduces) the performance of resolution is reduced. In counterpart, this is not observed in HPLC, which is able to separate both polar and no polar compounds without any volatility restrictions. Besides compound volatility, the GC technique is also limited by the density of the pack column; so columns with more than 3 meters length should be avoided due to the high pressure existing in the injection chamber. Despite these limiting factors, the conventional GC technique reigned absolute until the early of the 1980 decade, when it was replaced by HRGC, which provides best results and sharp peaks. The HRGC technique must be operated only with high resolution capillary columns (range from 30 to 50 cm length) for samples that contain volatile compounds. In this technique, the separation of the sample components is achieved in a chromatographic column, where the tube material (metal, glass or quartz), the adsorbent, the type and amount of liquid phase, the coating procedures, the length and diameter of the column, and the temperature are all important factors to be considered in order to achieve a good resolution. Recent papers have examined some problems and suggested modern solutions for sample preparation and methods of purifications, concentration and extraction of solid matrices (Smith, 2003).

Over the past twenty years, many types of columns have been developed for GC, and according Neto and Nunes (2003) they can be classified in two main groups: capillary and open tubular columns. The tube materials are usually copper, stainless steel, aluminum, or glass. Several types of porous polymers has been manufactured as coating agents for capillary columns, as for example the Porapak series (Waters Associates, USA) and the Chromosorb series (Johns Manville, USA), which are modified styrene copolymers. The actual capillary columns are made of glass or fused silica, with inner diameter less than 0.3 mm and film thickness less than 0.5 mm, which allows a good interaction between this film and the tube surface.

For the analyzes of specific compounds, like essential oils, sterols, or triterpenes, there are several temperature programmings that match some characteristics of the sample and allow to evaluate the degree of oxidation of the components. In this context, the following works are representatives:

a) Patitucci *et al.* (1995) reported the use of HRGC for detection of terpene compounds in crude plant extracts, where it was possible to observe the existence of a common chromatographic profile for species belongs to each genus or family studied.

b) Veiga Jr. *et al.* (2005a, b, 2001, 1997) performed several phytochemical studies with specific oven temperature programming in HRGC aiming the identification and separation of compounds present in several species of the genus *Copaifera*, as well as in hundreds of commercial copaiba oils.

Generally it has been proved that the identification of molecules may depend roughly on the analyzes of NMR spectra, since a large number of isolated compounds from natural sources have molecular weight above 200 g/mol, high population of isomers and several chiral centers, which demand a lot of effort to identify accurately any substance. Rigorously, to be proceeded to the identification of compounds through HRGC techniques it should be necessary that a substance has the same retention time in three different stationary phases, in order to ensure the identification of this substance with high reliability. Such analyzes finds little use in practice (Khoo *et al.,* 1973). In contrast, this procedure is very useful in HRGC coupled to mass spectrometry (HRGC-MS) (Veiga Jr. *et al.,* 2005a, b, 2001, 1997; Patitucci *et al.,* 1995). However, the match of two mass spectra solely is not sufficient to ensure accurately the identification of one substance, even if the spectra is compared automatically through a spectra library, like observed in many papers. A routine methodology for HRGC-MS analyzes frequently used by researchers for identification of compounds consists in the combined analyzes of the retention time and mass spectra. Another approach is use is a co-injection method or another (a second) capillary column containing a different stationary phase. In complex mixtures, the combined use of a co-injection method, at least two columns with different stationary phases, and several mass spectrometry techniques, like ion trap mass spectrometry, are useful tools for studying and identification of compounds with high accuracy. The HRGC-MS is usually applied for identification of compounds present in mixtures in which separation is difficult or impracticable, like in cases that separation leads to isomerization of compounds as observed in some essential oil samples. For these samples, HRGC-MS is the major and the most used technique, as evidenced by the main periodic title of this field, the Journal of the Essential Oil Research (Veiga Jr. *et al.,* 1997; Patitucci *et al.,* 1995).

Chromatographic resolution leads to efficient separation of different classes of compounds. Therefore, only the adjusting of the retention time is not sufficient to determine if two distributions of molecules was effectively separated. It is also necessary to introduce a second effect: the enlargement of the peak along the column. The peak width keeps a direct relationship with the separation efficiency of the column, and this relationship result of the intrinsic characteristics

of the chromatographic process. This peak enlargement is due to scattering of the molecules by diffusion in the fluid media, either in mobile or in stationary phase when in liquid state (Poole *et al.*, 1990). The sample (analyte) is carried by the carrier gas along the column, and should have been instantly volatilized in column inlet prior to enter in the column. So as it moves through the column, it shall enter in successive equilibrium distribution between the two phases (similar to plates of a typical distillation tower) represented by the partition coefficient between the two phases (Poole *et al.*, 1990). The rate in which the components progress in the column depends on the interactions between them and the stationary phase, and on the mobile phase flow rate, which in turn are function of the partition and distribution coefficient, which may be obtained using the component concentration in mobile and stationary phases. Besides the inherent aspects of the chromatographic system there are two other important factors related to separation efficiency (generally attributed, wrongly, only to the column efficiency) that should be considered: the amount of sample and the nature of the stationary phase. Mass overload may impair the performance of the column, leading to peak broadening, with distortions in the peak shape at the front of the peak.

Isolations of Terpenoids from *Croton cajucara*

Croton cajucara Benth (Euphorbiaceae) occurs widely in the Amazonian region, where it is popularly known as 'sacaca' and has a history of safe use in folk medicine. In this region, both stem bark and leaves are marketed and indicated for health care and treatment of several diseases, such as: diabetes, diarrhoea, malaria, fever, stomachache, liver, kidney and vesicle disorders, and to lower blood cholesterol. With the aiming to poll both phytochemistry and pharmacological approaches, Maciel *et al.* (2003, 2002a, b, 2000, 1998a, b) developed a methodology that could meet the needs of pharmacological studies for this plant in order to confirm its empirical therapeutic data. Therefore stem barks and leaves from trees aging from 1½ to 6 years were investigated, aiming at the isolation of major compounds for pharmacological assays.

The classical phytochemical study of *Croton cajucara* carried out with 6 kg of stem bark from plants with 4–6 years old, showed that this part of the plant is rich in clerodane-type diterpenes (Maciel *et al.*, 2003, 1998a, b). From the stem bark the isolated and characterized clerodanes are: *trans*-dehydrocrotonin (DCTN), *trans*-crotonin (CTN) *cis*-cajucarin B, *trans*-cajucarin B, cajucarin A, cajucarinolide, and isosacacarin (Fig 1, 2). The triterpene acetyl aleuritolic acid (AAA) was also obtained (Fig 1). The 19-*nor*-clerodane-type diterpene DCTN

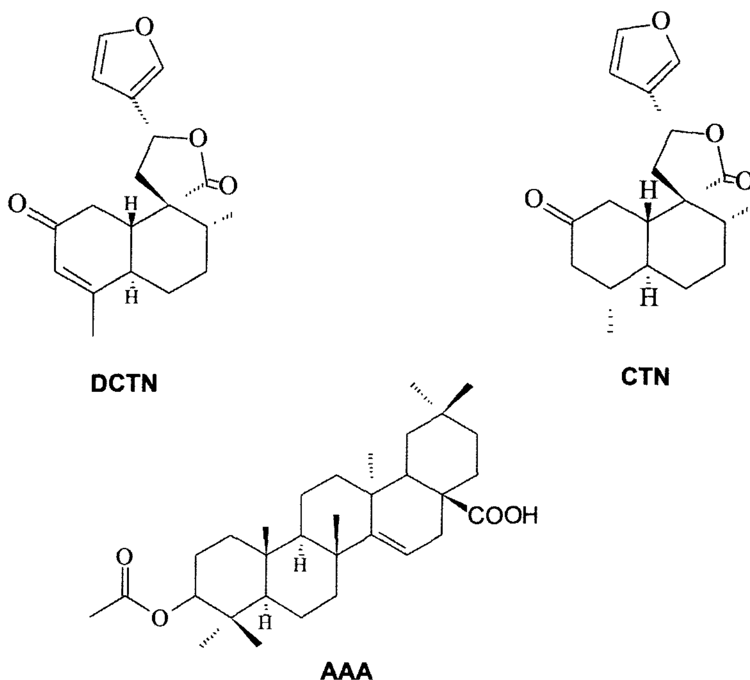


Fig 1. Chemical structures of the bioactive terpenoides *trans*-dehydrocrotonin (DCTN), *trans*-crotonin (CTN) and acetyl aleuritolic acid (AAA)

was the major component isolated from stem bark of trees with ages ranging from 3 to 6 years-old, while in trees with 1½ years old this compound was absent. Meanwhile, the triterpene AAA was found to be the major component isolated from trees aging 1½ years-old (Maciel *et al.,* 2003, 2002a, b, 2000, 1998a, b).

The pharmacological properties of the terpenoids DCTN, CTN, and AAA showed a striking correlation with the folk traditional therapeutic use of *Croton cajucara*, in which among them we can cite: anti-inflammatory and antinociceptive actions (for DCTN, CTN, AAA – related to the use for inflammations in general); anti-tumor activity (DCTN); hypoglycemic effect (DCTN – treatment of diabetes); anti-spasmodic activity (DCTN, AAA – treatment of diarrhea); anti-ulcer properties (DCTN, CTN – treatment of stomach disorders). The cardiovascular benefit of *trans*-dehydrocrotonin was also evidenced. The 19-*nor*-clerodane cajucarinolide and its diastereoisomer isocajucarinolide also presented anti-inflammatory properties (Khan *et al.*, 2009; Perazzo *et al.*, 2007; Maciel *et al.,* 2007a, b, 2006a, b, 2002a, b, 2000; Silva *et al.,* 2005, 2001a, b; Hiruma-Lima *et al.,* 2002, 1999; Agner *et al.,* 2001; Grynberg *et al.,* 1999; Ichihara *et al.,* 1992).

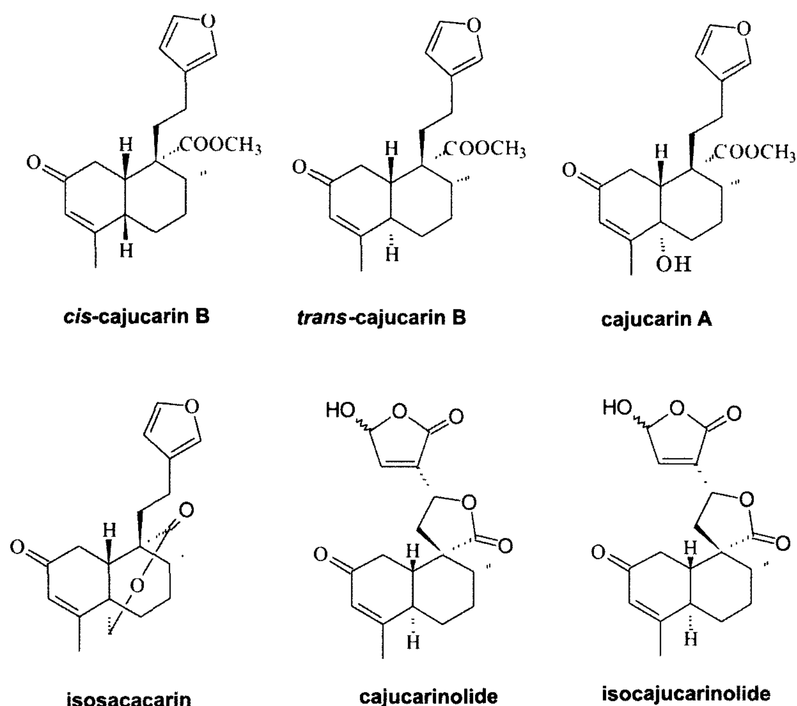


Fig 2. Chemical structures of the clerodane-type diterpenes obtained from *Croton cajucara*

Hexane and methanolic extracts obtained through Soxhlet extraction of *Croton cajucara* (6 kg of stem bark of plants with age ranging from 4 to 6 years-old) were submitted to chromatographic fractionation using silica gel (35–70 and/or 70–230 mesh) as adsorbent. TLC was carried out using silica gel 60 H and revelation were employed upon with sulfuric acid:methanol (1:1) and Dragendorff 's reagent. TLC also was revealed by UV radiation at wavelength of 254 and 360 nm. (Maciel *et al.*, 2000, 1998a, b). Phytochemical investigation performed with the hexane extract (471.8 g) gave, after filtration in open column containing silica gel (35–70 mesh), the Fractions A (eluted in hexane), B (eluted in dichloromethane) and C (eluted in methanol).

Fraction B was submitted to a new filtration procedure in a open column containing silica gel (35–70 mesh), eluted with mixtures of hexane:dichloromethane:methanol in gradient of polarity, from which 53 fractions (500 mL each) were obtained. After evaluation by TLC analyzes, 6 major fractions groups (7–21; 22–25; 26–29; 30–33; 34–41 and 42–53) were obtained. Schemes 2 and 3 show the purification procedure for each group of fraction, leading to the isolation of the

terpenoids DCTN, CTN, and AAA. The mother liquids derived from crystallization of Fraction B (Scheme 1) were submitted to new chromatographic fractionation described in Scheme 3, affording additional amounts of AAA, CTN and DCTN, as well as the 19-*nor*-clerodanes *trans* and *cis*-cajucarins B. These last two compounds are diastereoisomers and were purified only using preparative chromatography (silica gel PF 254). Therefore, each plate was eluted three times with a mixture of hexane:acetyl acetate (8:2), and it was observed upon UV radiation the presence of a spot with a minimal color differentiation at the extremities. The resolution of the cajucarins B was performed as follows: first, both extremes were separated from the center of the spot. After extraction with solvent and subsequent filtration, it was confirmed that the central region of the spot corresponded to the stereoisomeric mixture of cajucarins B, and the extremities of the spot corresponded separately to the diastereoisomers *cis*- and *trans*-cajucarins B. Their purity was assessed by high-resolution mass spectrometry (HRMS), their chemical structures were determined by spectroscopic techniques, and their stereochemistry was elucidated by ^{1}H high-resolution NMR (Maciel *et al.,* 2003, 1998a).

Even using conventional chromatography procedure it was possible to isolate and purify the diastereoisomeric *cis*- and *trans*-cajucarins B (Scheme 4). Meanwhile, the isolation and purification of the diastereoisomeric pair cajucarinolide and isocajucarinolide was only possible with the use of modern chromatographic techniques (Ichihara *et al.,* 1992). Despite this evidence the diastereoisomeric *cis*- and *trans*-cajucarins B, additionally to the minor constituents isosacacarin, cajucarin A and cajucarinolide were isolated by a conventional chromatograph methodology, as shown in the Schemes 4 and 5 (Maciel *et al.,* 2003, 2000, 1998a). The clerodane isosacacarin was target of a total synthesis (Grossman & Rasne, 2001).

The Fraction C, after new filtration using open column containing silica gel (70–230 mesh), gave 32 fractions (500 mL each fraction), which after TLC analyzes afforded 5 groups of fractions (2–3; 4–10; 11–18; 19–24 and 25–30). Scheme 4 show isolation procedures for the obtained clerodanes isosacacarin, cajucarinolide, and cajucarin A, in addition to the terpenoids AAA and DCTN.

From the methanolic extract (202.0 g, Scheme 2) were obtained AAA (0.3 g), DCTN (26.3 g) and isosacacarin (0.07 g) as shown in the (Scheme 5).

The total amount of DCTN isolated from the hexane (Schemes 2–4) and methanolic extract (Scheme 5) was 85.9 g (1.4%) and 4.9 g (0.08%) for AAA. NMR spectroscopy data of these terpenoids were

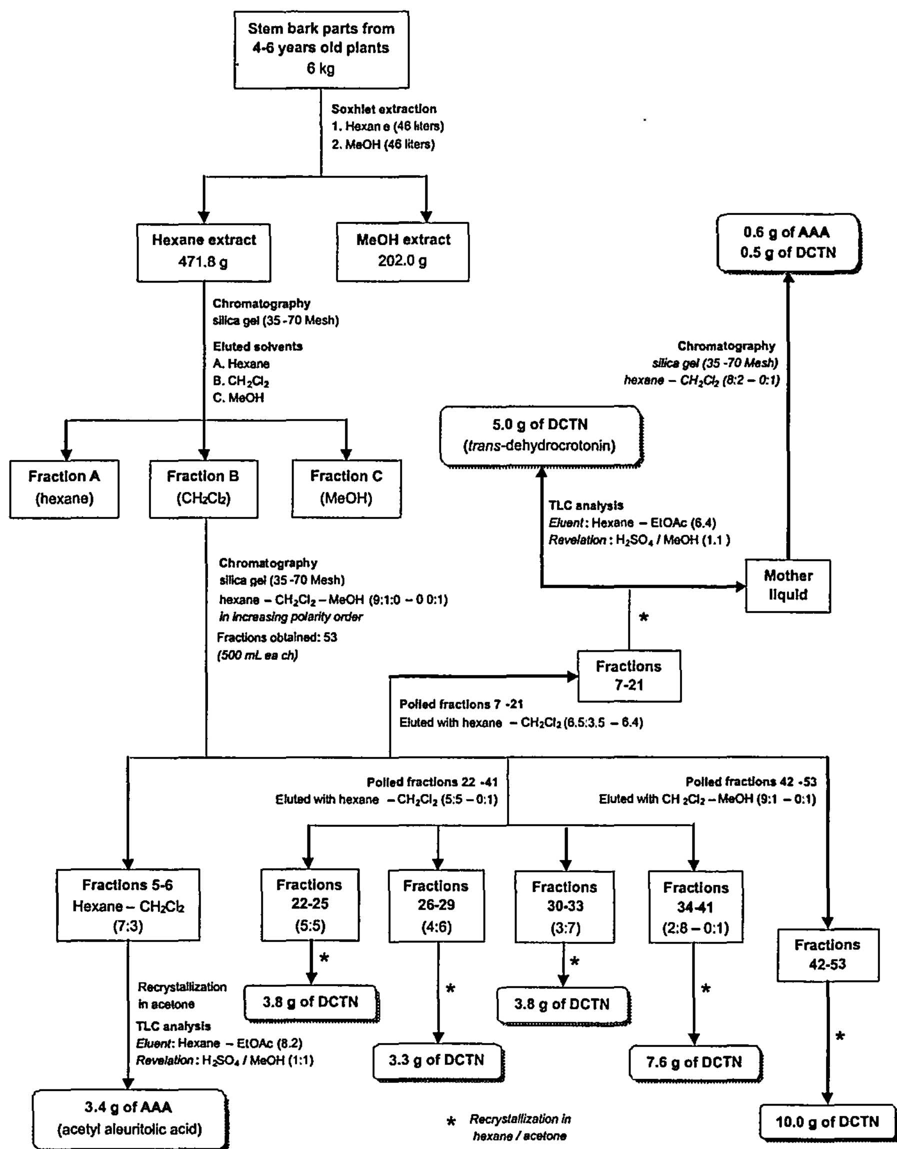
obtained in low- and high-resolution (600 Mhz) equipments (San Gil *et al.*, 2008; Maciel *et al.,* 2003, 1998a).

Ion-exchange chromatography was successfully applied in the separation of the triterpene acetyl aleuritolic acid (AAA), with high selectivity for acid fractions (Barreto Jr. *et al.,* 2005). This study opens new perspectives for the utilization of this technique aiming not only to obtain acid fractions present in low percentage in bioactive extracts, but also to the isolation of acid compounds present in extracts and oils.

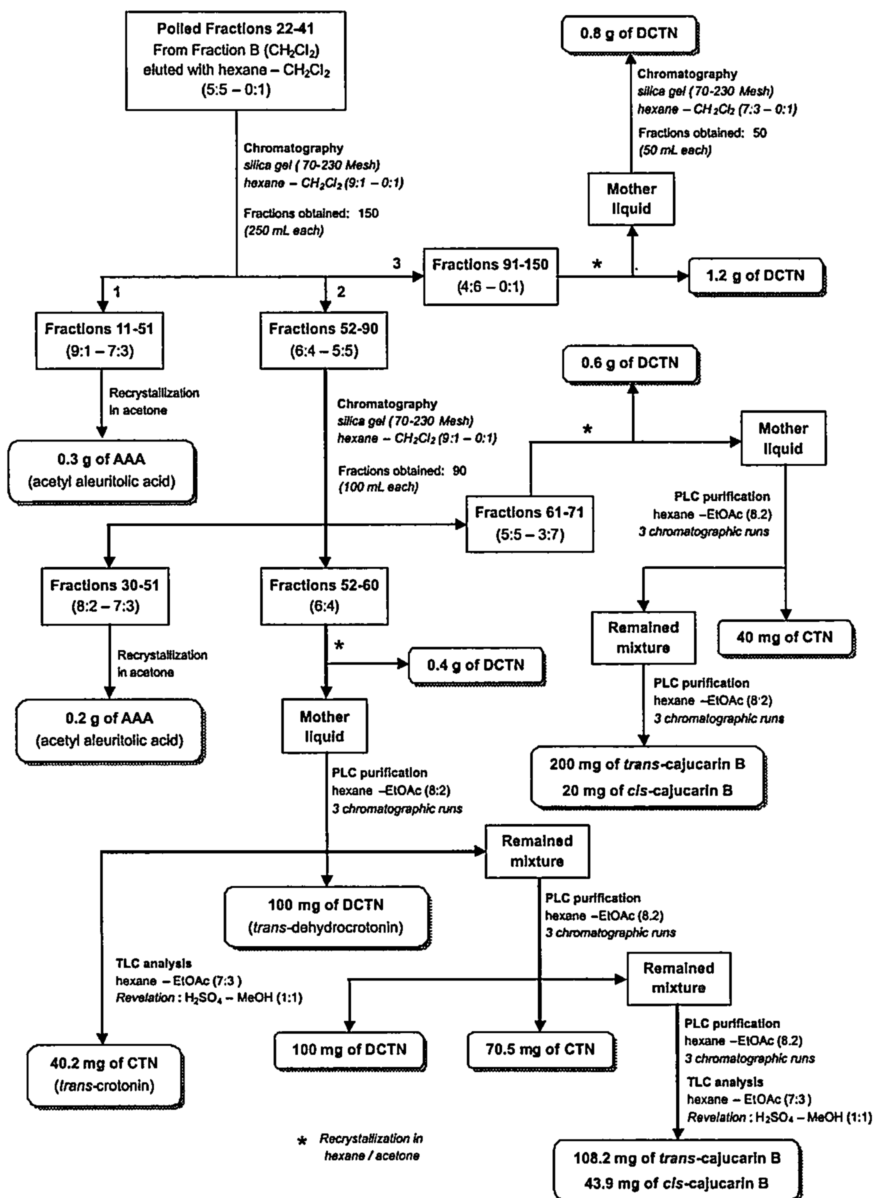
For the characterization of non polar constituents present in oils obtained from *Croton cajucara*, the use of GC-FID and GC-MS techniques was sufficient to characterize chemically a fixed oil from the methanolic extract (unique extraction) of the stem bark of this *Croton*. In this study, the presence of the bioactive clerodane CTN and the diastereoisomeric *cis*- and *trans*-cajucarins B, as well as the presence of the major sesquiterpenes α-copaene (20.1%) and ciperene (21.8%) was confirmed (Souza *et al.*, 2006). The anti-ulcer activity of the oil essential oil obtained from the stem bark of *Croton cajucara* was proved by Hiruma-Lima *et al.* (2000), and the anti-inflammatory and antinociceptive properties by Bighetti *et al.* (1999).

Copaifera L.

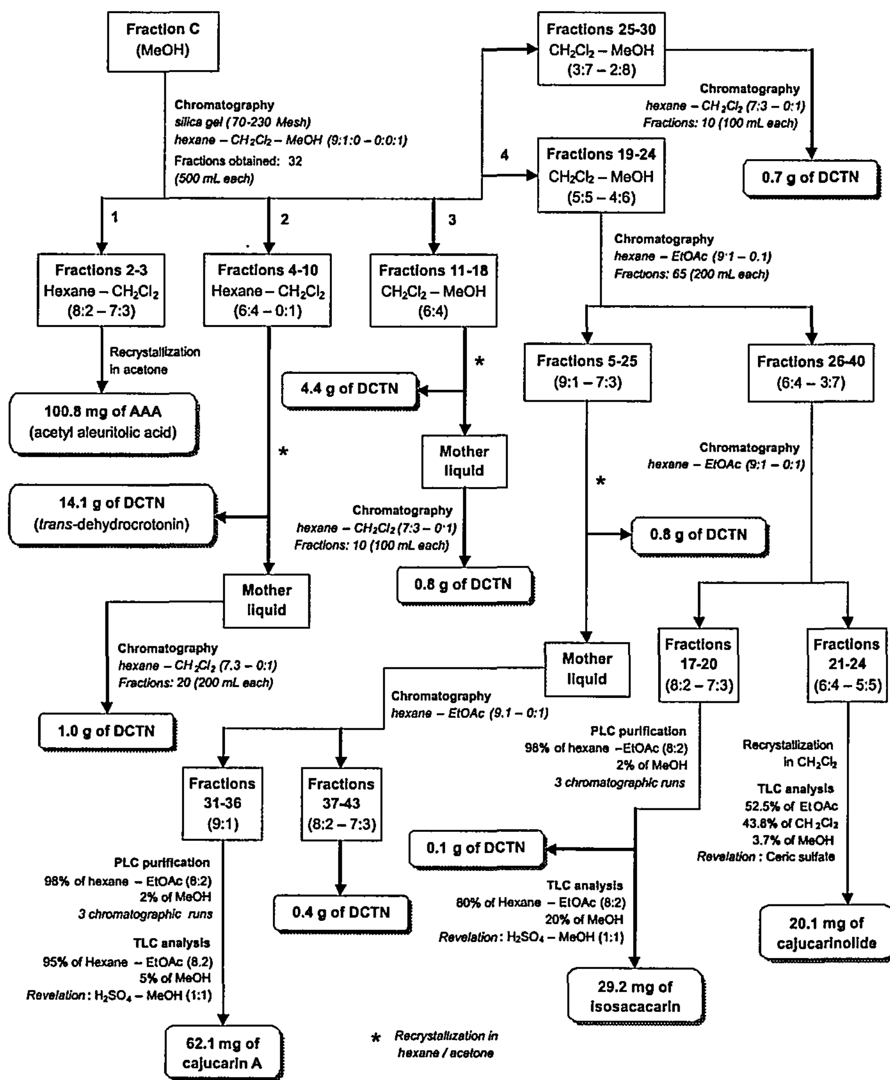
The Copaiba trees of the *Copaifera* L. type (Leguminosae – Caesalpinoideae) exude a resinous oil called Copaiba oil from its trunk. This species is found in the northern South America, mainly in both the Brazilian States Pará and Amazonas. Since the 16th century, its use as an anti-inflammatory and healing agent was reported when the first settlers of the Americas divulged that female Indians rubbed it on navels of newborn babies and the wounds of warriors suffered after battles (Salvador, 1975). The knowledge of the medicinal characteristics of the copaiba oil derives from an Indian legend, telling the tale of wounded animals rubbing their wounds against the copaiba trees to heal their injuries. Nowadays, the medicinal use of the oil of copaiba has spread to all regions of Brazil. It is taken orally, topically application and also as a cream. In the northern States of Brazil, the practice of topically administration to treat sore throats is pretty common. The copaiba oils have been widely used as a relevant phytotherapic in traditional medicine being indicated as a stimulant, diuretic, purgative, expectorant, healing, antitetanic, antihemorrhagic, anti-inflammatory, antiulcerogenic, antiseptic of the urinary system, treatment of bronchitis, syphilitic illness, skin disorders, leucorrhoea, psoriasis, diarrhoea, urticaria, dysentery, infections of the pulmonary and urinary systems and it



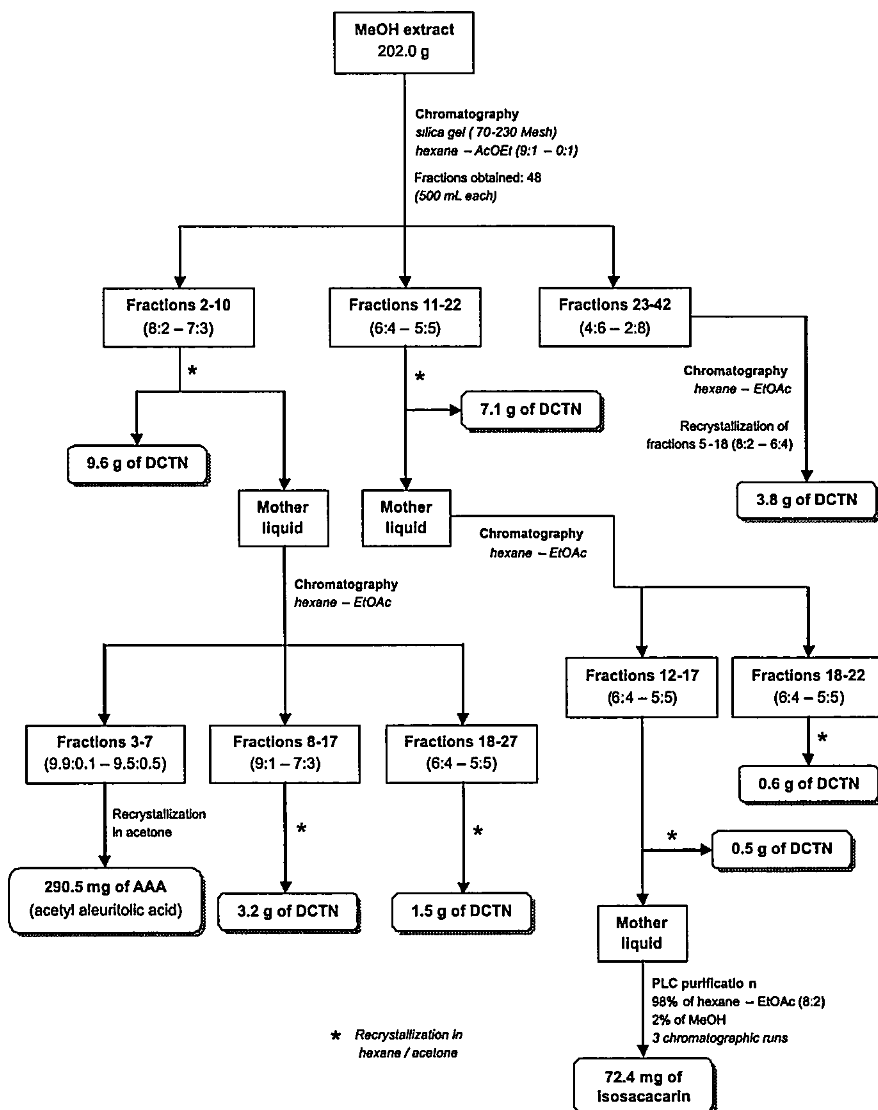
Scheme 2. Chromatographic fractioning of Fraction B obtained from hexane extract of *Croton cajucara* with isolation procedures for DCTN and AAA. TLC = Thin-Layer Chromatography



Scheme 3. Chromatographic fractioning of the polled fractions 22–41 from Fraction B (Scheme 2) with isolation procedures for DCTN, CTN, AAA, and *trans*-cajucarin B and *cis*-cajucarin B. TLC = Thin-Layer Chromatography; PLC = Preparative Layer Chromatography



Scheme 4. Chromatographic fractioning of Fraction C obtained from hexane extract of *Croton cajucara* with isolation procedures for DCTN, AAA, isosacacarin, cajucarinolide and cajucarin A. TLC = Thin-Layer Chromatography; PLC = Preparative Layer Chromatography



Scheme 5. Chromatographic fractioning of methanolic (MeOH) extract of *Croton cajucara* with isolation procedures for DCTN, AAA, and isosacacarin. PLC = Preparative Layer Chromatography

even combats different types of cancer. Despite side-effects, gastrointestinal irritation, diarrhoea, sialorrhoea, depression of the central nervous system caused by high dosages of the oil, its popular use has been intensified (Basile *et al.,* 1988; Alencar, 1982; Salvador, 1975).

Several techniques have been proposed to analyze the chemical composition of the copaiba oils. High Resolution Gas Chromatography is one of the most successful techniques, since it is cheap, sensitive and able to inform the type of adulteration was performed. Coupled with mass spectrometry detectors, gas chromatography becomes a even more powerful way to analyze copaiba oils. Two examples can be reported: the use of a "windows" system that delimit retention times to detect classes of terpenoids; and the use of the selective ion monitoring at mass spectrometry, capable to detect other classes of compounds. At the first case, the retention time definition to sesquiterpenes and diterpenes enable the quality control of the copaiba oils, usually mixed with *Eperua* and *Burseraceae* oleoresins, species that contain not only sesquiterpenes and diterpenes, but triterpenes and monoterpenes too. The second methodology uses a selection of some ions that are not usually observed at the sesquiterpenes and diterpenes mass spectra of copaiba oils, but are very common (and with high abundance) at some metabolites from the adulterated material, *e.g.* fatty acids, from seed oils, and hydrocarbons, from diesel oil.

Column open chromatography using silica impregnated with potassium hydroxide is another chromatographic method used to analyze copaiba oils. This modified silica works not only as a normal phase but as a cation exchange process too. This silica enables the elution of all the metabolites that are not carboxylic acids, which could be eluted with using methanol and mobile phase. Since copaiba oils have large amounts of diterpenic carboxilic acids, this kind of pre-fractionation is very useful and helps the isolation of the two classes of metabolites: sesquiterpenes and diterpenes.

Although numerous therapeutic uses of the copaiba oil are mentioned, the pharmacological studies done on such oils are minimum. Majority of these studies neither indicate the source of the oil nor the time and location of the gathering. The lack of such information opens gateways to doubts concerning the authenticity of such oils, once studies on the authenticity of 16 different types of commercial copaiba oils by means of high resolution gas chromatography have proven that different copaiba oils are often mixed. Falsifications with possible blends using soybean oils have also been proved. In addition, ethnobotanic research related to copaiba

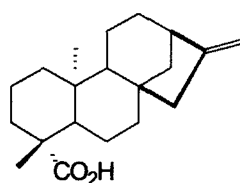
oils mention that not all trees exude appropriate copaiba oils to medicinal use (Basile *et al.,* 1988). The chemical constituents of the copaiba oil are not provided in much of printed research. The botanic species have not even been identified for many. There is just a simple mention on the commercial copaiba oil. Pharmacological studies done with the *Copaifera multijuga* oil indicated that the inhibition of oedema of the rat's paw varies with the chemical compound of the oils of this species gathered at different times (Veiga Jr. *et al.,* 2001, 1997).

Among the many medicinal virtues attributed to the copaiba oils by most of the researched are anti-inflammatory and antitumoral. Zanini and co-workers proved the anti-inflammatory efficiency of a commercial oil of copaiba without any botanic identification (Basile *et al.,* 1988). Low toxicity was observed (LD_{50} 3.79 mL/kg) in experiments done on mice. Recent studies with diverse commercial copaiba (Fernandes *et al.,* 1992; Gilbert *et al.,* 1972) and *Copaifera multijuga* oils (Veiga Jr. *et al.,* 2001), the fragment rich in hydrocarbons shows better anti-inflammatory activity than that of the sesquiterpenic alcohols and diterpenic acids. Fernandes and co-workers (Fernandes *et al.,* 1992) researched on the anti-inflammatory and analgesic activities of the *Copaifera cearensis* oil. They also assessed the copalic acid, bisabolol and solidago acid methyl ester; all present in copaiba oils. Results showed that the anti-inflammatory and analgesic actions of the oil are higher than those pure terpenoids. Different types of commercial oils proved protective against penetrations of *Schistosoma mansoni* cercarie (Gilbert *et al.,* 1972), insect repellents (Lacey, 1981; Jones, 1983) and antibacterial activities (Opdyke, 1976).

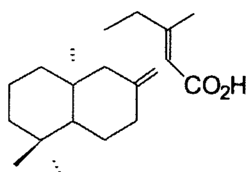
The chromatographic fractionating of the *Copaifera langsdorfii* oils guided by bioassay against MIC carcinoma in mice, proved that the diterpenes colavenol and hardwickiic acid are potent antitumoral agents (Ohsaki, 1994).

Antioxidant characteristics are described in the methanolic extract of rinds of *C. reticulata.,* which proved to be active when tested according to reductions of free radicals to induce DNA damage. It showed IC_{50} 3 µg/mL, less than the standard used, catechin (IC_{50} 5 µg/mL) (Desmarchelier *et al.,* 1997a). The total reactive antioxidant potential of this extract was also analyzed based on reduction of free radicals in quimioluminescence analyzes showing an activity of 7500 mm in relative values to the standard, Trolox (Desmarchelier *et al.,* 1997b). Extracts of seeds of *Copaifera multijuga* were analyzed for haemolytic and agglutinating activities, while only the haemolytic actions have been confirmed (Alves *et al.,* 1995).

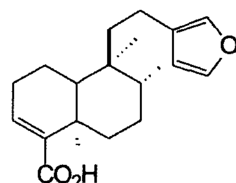
As seen in the summarized description of the pharmacological research cited above, except for the diterpenes kolavenol and hardwickiic acid (Fig 3) there is no relationship between pharmacological characteristics and isolated chemical substances of copaiba oils (Ohsaki *et al.,* 1994). However, many of the constituents already isolated or detected in copaiba oils have already had pharmacological characteristics described in literature. Among the diterpenes, the kaurenoic acid is the most researched, having been described as a trypanosomicide agent (Alves *et al.,* 1995; Batista, 1999), bactericide, larvicide against *Aedes aegypti,* stimulant to uterine contraction, antinociceptive and proved to be weakly active against the biotype *Bacillus subtilis* (Velikova *et al.,* 2000; Block *et al.,* 1998; Slimestad *et al.,* 1995; Page *et al.,* 1992; Oguntimein *et al.,* 1987; Lwande *et al.,* 1985).



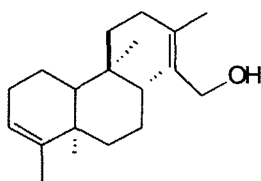
kaurenoic acid



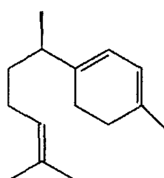
copalic acid



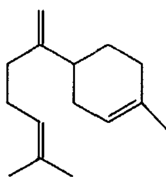
hardwickiic acid



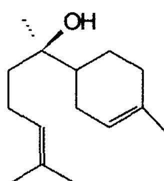
kolavenol



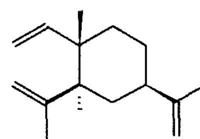
curcumene



bisabolene



bisabolol



elemene

Fig 3. Chemical structures of the chemical constituents obtained from *Copaifera* L.

Some sesquiterpenes, such as α-curcumene and β-bisabolene (Fig 3), have the antiulcerongenic and antiviral activities reported (Yamahara *et al.*, 1992; Denyer *et al.*, 1992). β-bisabolene is also reported as abortive (Pei-Gen & Nai-Gong, 1991). Bisabolol is known as responsible for anti-inflammatory and analgesic characteristics of the chamomile (*Matricaria chamomilla*) (Zekovic *et al.*, 1994). Sesquiterpenes β-elemen, caryophyllene and δ-cadinene (Fig 3) are anticarcinogenics agents (Leewenberg, 1987; Kubo & Muroi, 1993). Caryophyllene showed the following characteristics: antiedemic, fagorrepelent, anti-inflammatory, antitumoral, bactericide, insecticide and spasmolitic. Some of those activities were based on the oxide caryophyllene (Bettarini & Borgonovi, 1993; Kang *et al.*, 1992; Zheng *et al.*, 1992; Keeler & Tu, 1991; Shimizu, 1990).

CONCLUSIONS

The scientific progress of researches concerning medicinal plants is evidenced by several works aiming the pharmacological validation of a given specimen through chemical and pharmacological assays. An example is the genus *Croton* (Euphorbiaceae; widely distributed around the globe, with high prevalence in Africa, Brazil and Mexico), which is internationally represented by the species *Croton zambesicus* Muell Arg. (Africa), *Croton lechleri* Muell. Arg. (USA) and *Croton tiglium* Klotzsch (Asia), which lead the documentation record of this genus with 57 indexed-periodic papers. Among the Brazilian native species *Croton cajucara* Benth, *Croton zehntneri* Pax e Hoffm. and *Croton sonderianus* Müll Arg. are the most represented with 86 papers, but *Croton cajucara* Benth solely contributes with 60 work inserted in a multidisciplinary approach (Maciel *et al.*, 2006a). Additionally, the study of the specie *Croton cajucara* projected the bioactive compound *trans*-dehydrocrotonin (DCTN-isolated from the stem bark of this plant), which represents actually the most studied clerodane-type diterpene around the world. Recently, Costa *et al.* 2007 described in a review the extensive results of pharmacological studies of DCTN, as well as its semi-synthetic derivatives, and also presented insights into the use of DCTN as a therapeutic agent and some potential advantages of its incorporation in drug delivery systems.

It is interesting to reinforce the importance of other Brazilian medicinal plant *Copaifera* L. Copaiba oils at the brazilian traditional medicine have been reported since the discovery of the country, at the XV Century. The oils that receive this name are oleoresins exuded from the trunk of the trees of some *Copaifera* species (Leguminoseae – Caesalpiniaceae). Copaiba oils are composed of sesquiterpenes and diterpenes that can differ qualitatively and quantitatively with several biotic and abiotic aspects, as type of soil, *Copaifera* specie, season,

among other. Their pharmacological activities have been shown to change with this chemical variation. Since this oil has a great commercial value, it is not uncommon to find it adulterated with cheaper oils, soy seed oil, for example.

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2

Quality Control of Natural Medicines by Immunological Assay System

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ABSTRACT

We demonstrated the immunoblotting of glycyrrhizin (GC) on the positive charged polyethersulphone (PES) membrane instead of a polyvinylidene difluoride (PVDF) membrane. The membrane was treated by $NaIO_4$ solution to give cleavage of the sugar moiety in GC and visualized using the eastern blotting technique. GC and the extract of licorice roots could be developed by acetonitrile-water-formic acid solvent system. We applied it to the quantitative immunoassay using graphic analysis of NIH Image software. The immunochromatographic strip test was developed based on a competitive immunoassay in which the detector reagent consisted of colloidal gold particle coated with the respective anti-GC monoclonal antibody (MAb). Detection limit for the strip test was 250 ng/mL. The assay system can be used for the analysis of GC in licorice roots and the traditional Chinese medicines (TCM). Immunoaffinity column conjugated with anti-ginsenoside Rb1 MAb can it possible to isolate the antigen molecule, ginsenoside Rb1 from the ginseng crude extract by one-step purification. On the other hand, the washing fraction contained all other components except ginsenoside Rb1. We named its fraction as the knockout extract which is important for the determination of pharmacologically active compound in the natural medicines.

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Key words : Ginseng, immunochromatographic strip test, knockout extract, licorice, monoclonal antibody, natural medicine, new eastern blotting

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INTRODUCTION

Licorice (*Glycyrrhiza* spp.) is one of the most important natural medicines and has been used for medicinal purposes for at least 4,000 years. The major constituent of licorice is the triterpene saponin, glycyrrhizin (GC) which have been widely applied in pharmaceutical area and the food industry as flavoring and sweetening additives (Tomoda *et al.*, 1990). GC has many pharmacological properties such as anti-viral (Sasaki *et al.*, 2002), anti-mutagenic (Zani *et al.*, 1993), anti-tussive (Kamei *et al.*, 2003), anti-inflammatory (Kai *et al.*, 2003), anti-microbial (Tanaka *et al.*, 2001), and hepatoprotective activities (Nose *et al.*, 1994). Therefore, GC is used as marker for a quality control of *Glycyrrhiza* species.

Ginseng, the natural medicine of *Panax ginseng* root is also the most important component in traditional Chinese medicine (TCM). Major active components are the ginsenosides, which consist of protopanaxatriol and/or protopanaxadiol possessing a dammarane skeleton in their molecules. It is well known that the concentration of GC and ginsenosides are varying depending on the method of extraction, subsequent treatment (Kitagawa *et al.,* 1987), or even the season of its collection (Shan *et al.,* 2001).

Quality control and standardization of natural medicines are necessary since as factors affecting the difference of chemical constituent composition and concentration, many reasons such as genetic distinction, botanical sources, production area, harvest time, processing method, etc are considered. Qualitative and/or quantitative analysis of natural medicines still mainly owe to HPLC analysis until today. HPLC method is useful for the analysis of natural medicines because it can survey different kind of components once. However, since HPLC analysis often needs much time, pre-treatment and organic solvent for good separation of components contained in natural medicines. Recently organic solvents used for HPLC become troublesome related to protection of environment. From these reasons we are ongoing to open new assay systems using monoclonal antibodies (MAbs) against naturally occurring bioactive compounds. The first trial is setting up enzyme-linked immunosorbent assay (ELISA) instead of HPLC (Shan *et al.,* 2001; Sakata *et al.,* 1994; Shoyama *et al.,* 1996; Tanaka *et al.,* 1999; Fukuda *et al.,* 2000a; Zhu *et al.,* 2004; Morinaga *et al.,* 2000; Morinaga *et al.,* 2001; Kim *et al.,* 2004; Xuan *et al.,* 1999; Tanaka *et al.,* 1996;

Lu *et al.*, 2003). The sensitivity is nearly many hundred times rather than HPLC, and rapid, good reproducible without organic solvent system.

A PVDF membrane is most widely used for the analysis of proteins in western blotting experiments (Granger *et al.*, 1988; Reig & Klein, 1988; Towbin *et al.*, 1979). Regarding small molecule compounds, Towbin *et al.* (1984) reported immunostaining of glycosphingolipids transferred from a thin-layer chromatography (TLC) plate to a nitrocellulose membrane, however the transfer efficiency and the reproducibility were low. The direct immunostainings of glycosphingolipids on TLC plate were limitedly succeeded because the other small molecule compounds are easily washed out by buffer solution without fixing (Uemura *et al.*, 1983; Kundu *et al.*, 1983; Suetake *et al.*, 2003; Meisen *et al.*, 2004). Recently we also succeeded to detect gangliosides by TLC immunostaining (Miyamoto *et al.*, 2006).

We have reached to a new methodology called eastern blotting (Shan *et al.*, 2001) which is a kind of immunostaining using blotting system to PVDF membrane. Eastern blotting method clearly stained an antigen component in the crude extract of natural medicine like glucoalkaroids (Tanaka *et al.*, 1997), ginsenoside Rb1 (G-Rb1), G-Rc, G-Rd, G-Re and G-Rg1 (Fukuda *et al.*, 1999; Fukuda *et al.*, 2000b; Fukuda *et al.*, 2001), glycyrrhizin (GC), (Shan *et al.*, 2001) and saikosaponin (Zhu *et al.*, 2007). In order to develop a new eastern blotting system available for quantitative and qualitative analysis of natural medicines, we succeeded it by using PES membrane instead of PVDF membrane (Morinaga *et al.*, 2005). Therefore, the assay system will be reviewed here.

Other application is carried out by a strip chromatographic assay system. In order to screen large number of plant samples for the presence of GC, a rapid sample assay system is required to be applied to small quantities of test materials. Previously, we produced MAb against GC and developed ELISA method for determination of GC in licorice (Tanaka *et al.*, 1998; Shan *et al.*, 2001). Therefore, the immunochromatographic assay using antibody conjugate with gold particle for determination of antigen provides an accurate, easy to use and rapid tool for detection of active compound in natural medicines. The immunochromatographic strip test has been developed based on a competitive immunoassay in which the detector reagent consisted of colloidal gold particle coated with the respective anti-GC MAb.

Immunoaffinity column conjugated with anti-ginsenoside MAb can make it possible to isolate antigen molecule by single column. On

the other hand we noticed the washing fraction contained all other components except only antigen molecule which is resemble to a knockout gene. Therefore, we named it a knockout extract (Fukuda *et al.*, 2000c; Wang & Shoyama, 2006; Fukuda *et al.*, 2007). The knockout extract can be used for the confirmation of real pharmacologically active component in crude extract. These three applications will be reviewed here.

MATERIALS AND METHODS

New Eastern Blotting Technique for GC

GC and the extracts of licorice roots and TCM were applied to a PES membrane and developed by acetonitrile-water-formic acid (45:55:2, by volume). The developed PES membrane was dried and dipped into water containing $NaIO_4$ and stirred at room temperature for 1 h. After washing with water, 50 mM carbonate buffer solution containing BSA was added, and stirred at room temperature for 3 h. After washing the PES membrane with phosphate buffered solution (PBS), the membrane was treated with PBS containing skim-milk for 2 h to reduce nonspecific adsorption. The PES membrane was immersed in anti-GC MAb and stirred at room temperature for 3 h. After washing the PES membrane twice with PBS containing Tween 20 (T-PBS) and water, a 1:1000 dilution of peroxidase-labeled goat anti-mouse IgG in PBS containing gelatin was added, and the mixture was stirred at room temperature for 1 h. The PES membrane was washed twice with T-PBS and water and then exposed to dimethylaminoazobenzene/4-chloro-1-naphthol mixture solution for 15 min at room temperature. The reaction was stopped by washing with water, and the immunostained PES membrane was allowed to dry.

Immunochromatographic Strip Test

Preparation of Antibody-colloidal Gold

The colloidal gold solution was adjusted to pH 9.0 with 0.2 M potassium carbonate solution. The MAb was added to the colloidal solution and stirred gently at 4°C for 10 min. The conjugate was stabilized with BSA in tris-HCl and adjusted to a final concentration of 1%. The mixture was incubated for 1 h at room temperature and centrifuged. The supernatant was discarded and the pellet was resuspended in 1% BSA. This step was repeated twice. The concentrated conjugate in 1% BSA was stored at 4°C until use. Each conjugate pad was loaded with detector reagent mixture and dried for 2 h at room temperature. The pads were assembled onto the strip test.

Preparation of Chromatographic Strip

GC-HSA conjugates used as the test capture reagent were synthesized as previously reported (Shan *et al.*, 2001). Anti-mouse IgG was used as the control capture reagent. Control and test capture reagents were applied onto the nitrocellulose membrane. After drying the membrane at room temperature for 1 h, the membrane was immersed in PBS containing BSA and incubated with stirring at room temperature for 2 h. The membrane was washed twice with PBS containing Tween 20 (T-PBS) for 10 min. After drying, the membrane was cut into single test strips (450 x 6 mm). Each test strip consisted of the nitrocellulose membrane, absorbent pad, detector reagent in conjugate pad and sample pad. The sample solution was transferred to a tube into which the lower edge of the test strip was dipped. The sample was migrated upwards and the results of test were read after 10 min.

Preparation of Knockout Extract

Preparation of an Immunoaffinity Column for Ginsenoside Rb1 Using an Anti-ginsenoside Rb1 Monoclonal Antibody

Purified IgG in Bio-Rad Affi-Gel Hz coupling buffer diluted was dialyzed against the coupling buffer two times. $NaIO_4$ solution was added to the IgG solution and stirred gently at room temperature in the dark for 1 h. After the reaction, glycerol was added to the reaction mixture and stirred for 10 min for the inactivation of $NaIO_4$, then dialyzed against the coupling buffer. After the Affi-Gel Hz Hydrazied gel was washed with the coupling buffer, the buffer was removed. IgG, dissolved in the coupling buffer, was added to these gels and stirred gently at room temperature for 24 h. The immunoaffinity gel was packed into a plastic mini-column. Columns were washed with phosphate buffer containing NaCl. The column was stored at 4°C in PBS containing sodium aside.

Purification of Ginsenoside Rb1 from a Crude Extract of the Roots of P. ginseng by Immunoaffinity Column Chromatography

An extract of ginseng roots was redissolved in PBS and then filtered to remove insoluble portions. The filtrate was loaded on the immunoaffinity column and allowed to stand overnight at 4°C. The column was washed with the washing buffer solution, and then eluted with HOAc buffer containing KSCN and MeOH. The ginsenoside Rb1-containing fraction was concentrated and surveyed by TLC, developed with *n*-BuOH-EtOA-H_2O(15:1:4), followed by western blotting.

Washing fraction was freeze dried and redissolved in MeOH. MeOH solution was analyzed by TLC.

RESULTS AND DISCUSSION

New Eastern Blotting

Glycosides like GC and ginsenoside having sugar linkage in a molecule are complicated and the structure elucidation of glycosides needs much efforts. However, recently various kinds of plant glycoside have been isolated due the developments of technology for separation and analytical methodology for the structure elucidation. Previously we succeeded in the eastern blotting of GC on a PVDF membrane using anti-GC MAb (Tanaka *et al.,* 1997, Shan *et al.,* 2001). In this methodology we separated the GC molecule into two functional parts, the epitope part (mainly aglycone) and the sugar part. The sugar part in GC was oxidatively cleavaged to release aldehyde group which was conjugated with protein to fix on a PVDF membrane. The aglycone part of GC was bound by the anti-GC MAb as shown diagrammatically in Fig 1. However, since separation of GC and staining on a TLC plate were impossible because GC is easily washed out by buffer solution without fixing, we first separated GC by TLC plate and transferred to PVDF membrane by blotting procedure. After that, the membrane was treated by $NaIO_4$ and protein, and finally stained by anti-GC MAb. However, since the transfer efficiency at blotting step was difficult to control, this method could not be applied for the quantitative analysis system. Therefore, we improved a new eastern blotting technique for GC in licorice roots and TCM (Shan *et al.,* 2001) applying a chromatographic separation system utilizing a PES membrane, and evolve to the quantitative analysis using NIH Imaging software for the estimation of TCM containing licorice root.

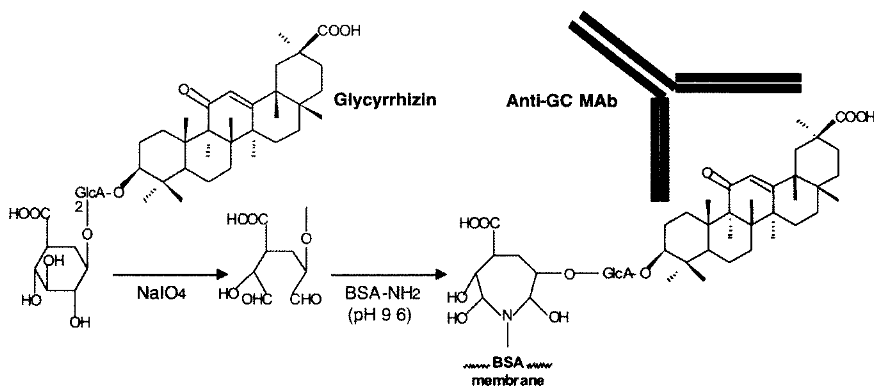

Fig 1. Schematic diagram illustrating the eastern blotting of GC onto the membrane

In the case of new eastern blotting technique, we demonstrated the immunoblotting of GC on the positive charged PES membrane instead of a PVDF membrane. PES membranes are widely used for the ultrafiltration system (Duarte *et al.*, 2003) and enzyme immobilization unit (Gomes *et al.*, 2004). However, no success with immunoblotting using a PES membrane has been reported yet. Fortunately, we found a new fact that the positive charged PES membrane was suitable for the immunoblotting of GC. We noticed that its intrinsic hydrophilicity and strong physical property against organic solvents may make it possible to separate GC chromatographically on this membrane. GC and the crude extracts of licorice roots were applied to a PES membrane and developed by various ratio of methanol-water or acetonitrile-water containing various ratio of acetic acid or formic acid. After drying, the membrane was treated by $NaIO_4$ solution to give cleavage of the sugar moiety in GC and visualized using the eastern blotting technique. As shown in Fig 2, GC and that of licorice roots could be developed by acetonitrile-water-formic acid (45:55:2, by volume). From these results we applied it to the quantitative immunoassay using graphic analysis of NIH Image software calculating the areas of coloring spots on membrane. Standard curve of GC was prepared by plotting area (y) against the logarithm of GC concentrations (x). The regression equation of the standard curve of GC was y = 1549.7Log (x) + 573.0 (y^2 = 0.988) and the full linear range of the assay was extended from 1.0 to 8.0 μg. The detection limit of GC was 0.5 μg of direct applied amount.

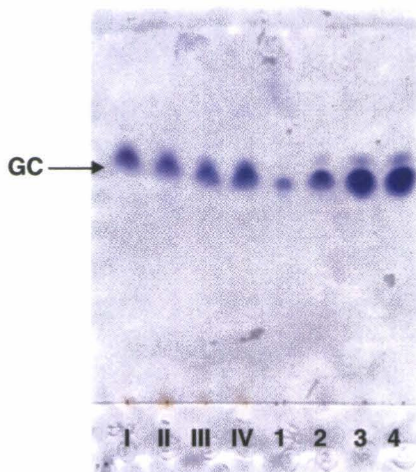

Fig 2. New eastern blotting technique for GC utilizing a PES membrane. Lanes I-IV indicated Seihoku licorice, *Glycyrrhiza glabra*, *G. inflate* and *G. uralensis*, respectively. Lanes 1–4 indicated GC standard with the loaded amount being 1.0, 2.0, 4.0 and 8.0 mg, respectively

Fig 3 shows the immunodetection of GC in licorice roots and TCM with and without licorice by using a new eastern blotting technique. Lane 3 was Daisaiko-to extract, which does not contain licorice, indicating no band by eastern blotting. On the other hand, the band of GC in Syakuyakukanzou-to and Shousaiko-to extracts (Fig 3, lanes 1 and 2), which contain licorice, and various licorice root extracts clearly appeared (Fig 3, lanes 4–11). These areas of coloring spots on this membrane were calculated using NIH Image software and the GC concentrations were determined as indicated in Table 1. These results were in a good agreement with those from the HPLC analysis (Table 1). The advantages of the new eastern blotting technique over the HPLC method are mainly its saving cost-performance (*e.g.* organic solvents and analytical equipments), speed and ease of use, which are useful if large numbers of smaller samples are to be analyzed.

Immunochromatographic Strip Test

We previously reported that anti-GC MAb had high specificity with GC (Shan *et al.*, 2001). Furthermore, in the former section we developed a new eastern blotting system available for quick, reproducible, highly sensitive, quantitative and/or qualitative analysis of natural medicines. Therefore, the other immunochromatographic strip test based on an immunoassay system with MAb has been introduced for GC analysis possessing high sensitivity and specificity.

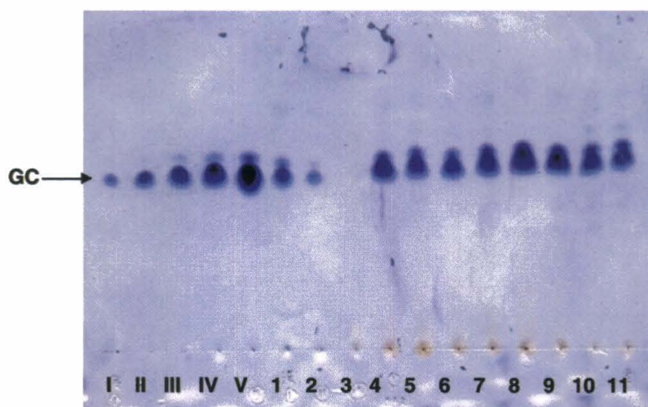

Fig 3. Immunodetection of GC in licorice roots and traditional Chinese medicines by using a new eastern blotting technique. Lanes I-V indicated GC standard with the loaded amount being 0.5, 1.0, 2.0, 4.0 and 8.0 mg, respectively. Lanes 1–3 were Syakuyakukanzou-to, Shousaiko-to and Daisaiko-to, respectively. Lanes 4–11 were Seihoku, stir-baked Seihoku, stir-baked Seihoku, Tohoku, Seihoku licorice, *Glycyrrhiza glabra*, *G. inflata* and *G. uralensis*, respectively

Table 1. GC concentration in licorice roots and traditional Chinese medicines determined by NIH Image and HPLC analyzes

Sample	Concentration (mg/g dry weight powder)	
	NIH Image	**HPLC**
Syakuyakukanzou-to	6.0 ± 0.2	6.2 ± 0.1
Shousaiko-to	2.5 ± 0.4	1.9 ± 0.2
Daisaiko-to	ND	ND
Seihoku licorice	25.3 ± 4.7	25.1 ± 0.2
Stir-baked Seihoku licorice	24.5 ± 2.7	26.3 ± 0.2
Stir-baked Seihoku licorice	20.1 ± 1.0	21.8 ± 0.1
Tohoku licorice	26.1 ± 2.3	23.3 ± 0.4
Seihoku licorice	48.7 ± 5.8	42.2 ± 0.4
Glycyrrhiza glabra	36.2 ± 3.4	37.4 ± 0.1
G. inflata	25.9 ± 2.3	22.0 ± 0.7
G. uralensis	28.0 ± 3.1	38.6 ± 0.4

The data are the means of triplicate assays ± the SD.
ND = not detectable.

The immunochromatographic strip test (Fig 4) was developed base on a competitive immunoassay methodology using anti-GC MAb as a detector antibody. A sample solution was applied to the sample pad, and GC in the sample was bound by the detector reagent in the conjugate pad. This pad contained anti-GC MAb detector reagents. The GC was bound to the detector reagents, and free GC and detector reagent migrated up the strip with the sample.

When the sample solution passed over the capture reagent (GC-HSA), the detector reagent that was free of analyte bound the capture reagent at capture spots, while the control capture reagent (anti-mouse IgG) bound to the anti-GC MAb held to the detector reagent at the control spot. When GC was contained in the sample, they competed with the immobilized GC conjugate with HSA on the membrane for the limited amount of antibody of the detector reagent. Therefore, the immobilized capture reagent was prevented from binding with detector reagent on the membrane when adequate amount of GC was present in the sample. Thus, a positive sample produced no visible test spot in the test capture zone and the control test spot was always visible. Color appeared at both capture and control spots if the sample contained no GC (negative sample, Fig 5 lane 1). On the other hand, no color developed on the capture

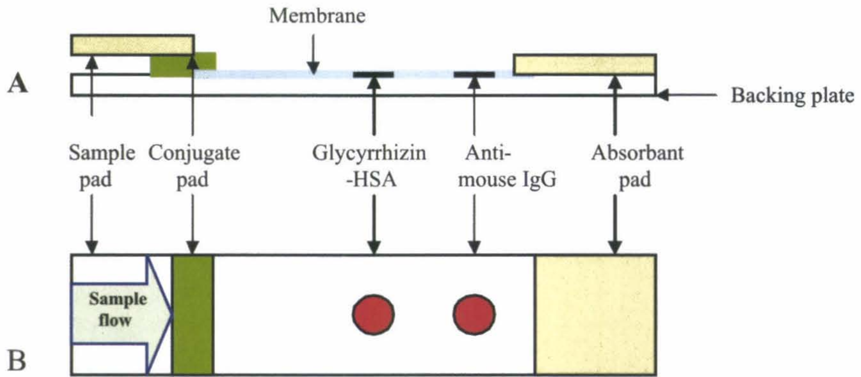

Fig 4. Schematic of an immunochromatographic test strip. (A) Cross-section; (B) Top view

spot when the sample contained GC (positive sample as shown in Fig 5 lane 2). Detection limit for GC using the strip test was 250 ng/mL. The appropriate sample volume size was 200 µl, and the assay can be performed in about 10 min. GC was detected by the immunochromatographic strip test (Fig 6) when a sample were analyzed. Fig 6 shows that *G. glaba*, *G. uralensis*, *G. inflata*, and Licorice root no. 1–5 contained GC (lanes 1–8). On the other hand, GC was below the detection limits in snack food and sauce (lanes 9-10). These results were confirmed by competitive ELISA using anti-GC MAb (Shan *et al*., 2001). *Glycyrrhiza* species contained high levels of GC, 1.91 ± 0.6 to 3.72 ± 0.10% dry wt. (Table 2). Our

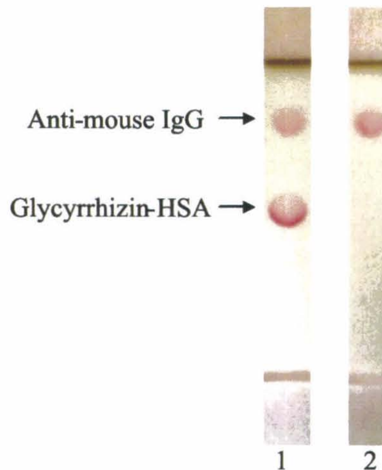

Fig 5. Immunochromatographic strip test of glycyrrhizin. (1) Negative sample; (2) Glycyrrhizin positive sample

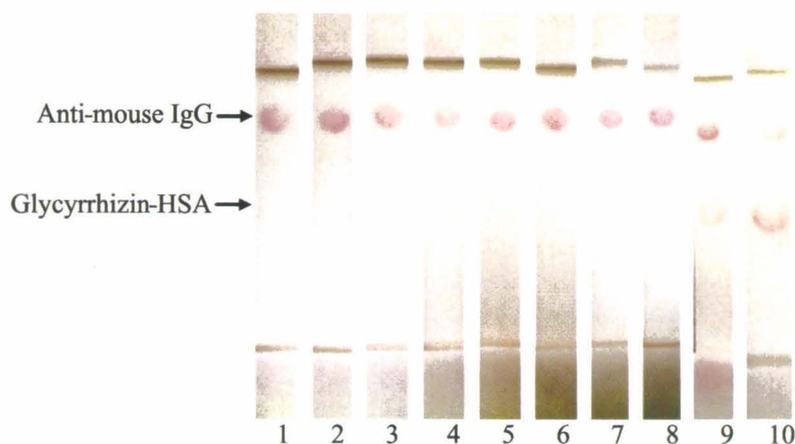

Fig 6. Immunochromatographic strip test of glycyrrhizin from *Glycyrrhiza* species. (1) *Glycyrrhiza glabra* (2) *G. uralensis* (3) *G. inflata* (4) Licorice root no. 1 (5) Licorice root no. 2 (6) Licorice root no. 3 (7) Licorice root no. 4 (8) Licorice root no. 5 (9) Snack food (10) Sauce

results indicated that immunochromatographic assay accelerated the analytical procedure and did not require handling reagents. Furthermore, the assay can be available when we need to analyze in the field study. Therefore, the immunochromatographic strip assay was suitable as a rapid and simple procedure for screening GC concentrations in plants, biological fluid and food samples. From above results the combination of immunochromatographic assay and

Table 2. Glycyrrhizin contents in *Glycyrrhiza* spp. and food samples determined by ELISA and immunochromatographic strip test

	Glycyrrhizin content	
Sample	**Strip test**	**ELISA (% dry wt)**
Glycyrrhiza glaba	+	3.49 ± 0.34
G. uralensis	+	2.75 ± 0.13
G. inflata	+	3.24 ± 0.28
Licorice root no. 1	+	3.64 ± 0.09
Licorice root no. 2	+	1.91 ± 0.16
Licorice root no. 3	+	2.39 ± 0.21
Licorice root no. 4	+	3.49 ± 0.19
Licorice root no. 5	+	3.72 ± 0.10
Snack food	-	$(3.20 \pm 0.10) \times 10^{-4}$
Sauce	-	$(11.20 \pm 0.10) \times 10^{-4}$

ELISA was useful methods for the qualitative and quantitative analysis of GC.

Knockout Extract

A crude extract of *P. ginseng* roots was loaded onto the immunoaffinity column and washed with the washing solvent. Fig 7 shows the fractions 1–8 containing overcharged ginsenoside Rb1, which was determined by ELISA. Ginsenosides Rc, Rd, Re and Rg1 were also detected in these fractions by the eastern blotting procedure. A sharp peak appeared around fractions 20–24, which contained ginsenoside Rb1. Overcharged ginsenoside Rb1, eluted with washing solution, was repeatedly loaded and finally isolated in pure form. The antibody was stable when exposed to the eluent, and the immunoaffinity column showed almost no decrease in capacity after repeated use more than 10 times under the same conditions, as was reported for a single-step separation of forskolin from a crude extract of *Coleus forskohlii* root (Yanagihara *et al.,* 1996).

After washing fractions were deionized the solvent was lyophilized. Fig 8 indicated the TLC profile of purification step. Line 1, 2 and 3 were the crude extract, the washing fraction and the eluted fraction, respectively. Interestingly the washing fraction contained all of compounds in the ginseng crude extract except ginsenoside-Rb1. It becomes evident that ginsenoside-Rb1 molecule can be eliminated by

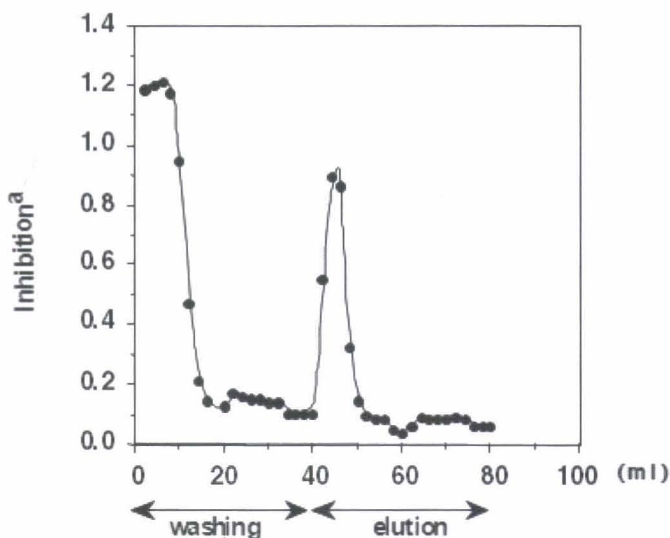

Fig 7. Elution profile of *Panax ginseng* crude extract used immunoaffinity column monitoring by ELISA using anti-G-Rb1 MAb

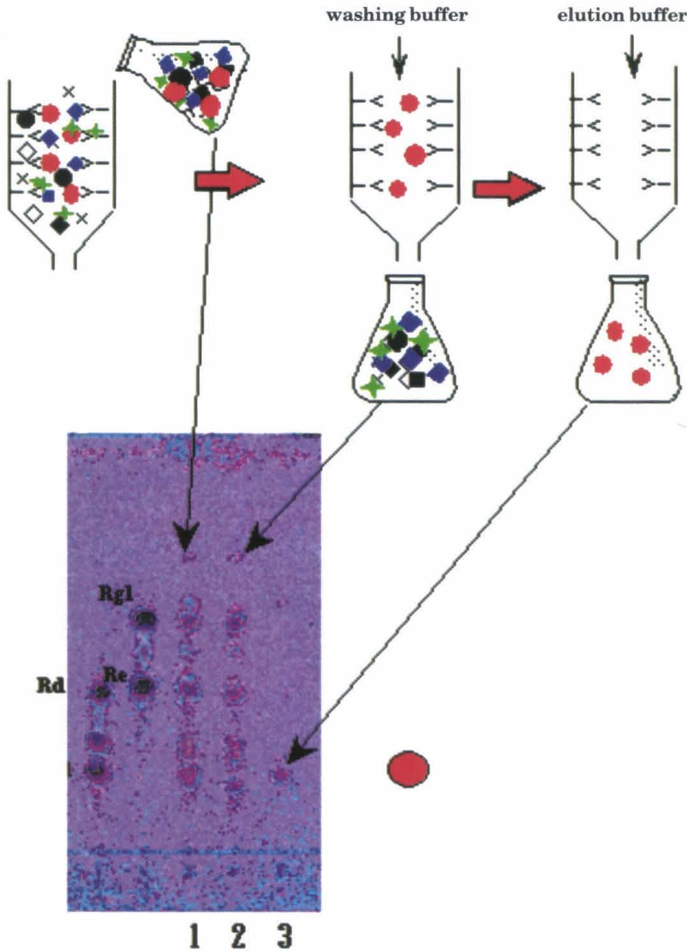

Fig 8. Preparation of knock-out extract eliminated G-Rb1 from *Panax ginseng* crude extract using immunoaffinity column conjugated with anti-G-Rb1 MAb. (Lines 1,2 and 3 indicate crude extract, knock-out extract and purified G-Rb1, respectively. Red spot shows G-Rb1)

an immunoaffinity column conjugated with anti- ginsenoside-Rb1 MAb and the washing fraction was knocked out only antigen molecule. Therefore, we named this washing fraction as a knockout extract. This knockout extract is important for the determination of real pharmacologically active component in natural medicines. Furthermore, when we need the related total saponins like ginsenosides, saikosaponins and solasodine glycosides, a wide cross-reactive MAb can be used for separation of total saponins and a knockout extract as reported previously in the case of solasodine glycosides (Putalun *et al.,* 1999).

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3

Comparison of HPLC and HPTLC Methods for the Determination of Rosmarinic Acid from *Orthosiphon stamineus*

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ABSTRACT

A validated HPTLC and HPLC method with UV detection were developed to determine the content of rosmarinic acid (RA) in Orthosiphon stamineus leaf extract. Similar fingerprints and quantitative data were obtained for leaf samples collected from different locations. The methods give good accuracy, reproducibility and selectivity for the quantitative analysis of rosmarinic acid.

Key words : *Orthosiphon stamineus*, rosmarinic acid, HPTLC, HPLC

INTRODUCTION

Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxyphenylacetic acid (Fig 1), which are important natural bioactive substances occurring widely in food plants (Ho, Lee & Huang, 1992). It is a well known natural product extracted from rosemary plant (*Rosmarinus officinalis*), and other members of Labiatae, Boraginaceae, Lamiaceae, Zosteraceae families and in lower plants such as the hornworts (Litvinenko *et al.,* 2001). 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).

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Orthosiphon stamineus Benth. (Lamiaceae) is taken as beverage to improve health and for treatment of kidney, bladder inflammation, gout and diabetes in Southeast Asia (Hegnauer, 1966; Wagner, 1982). The therapeutic effects of *O. stamineus* leaf extracts are ascribed mainly to its polyphenol content. Our previous report described HPTLC and HPLC profiling of *O. stamineus* extracts using RA as marker (Akowuah & Zhari, 2006). The present report describes a validated HPTLC and HPLC method for quantification of RA from *O. stamineus* leaf extract.

MATERIALS AND METHODS

Chemical and Reagents

Rosmarinic acid was purchased from Sigma Chemical Company (St. Louis MO, USA). Acetic acid, dicloromethane, methanol, and water 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 analytical or HPLC grade.

Plant Samples

Plants were grown from cuttings using standard agronomic practices at experimental farms in 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

One gram of dry leaf sample of *O. stamineus* was extracted with 10 mL of methanol for 4 h with continuous stirring with magnetic stirrer at 40°C. The extracts were filtered (Whatman No. 1) with a Buchner filter and concentrated under vacuum. The methanol extract was redissolved in methanol to a total volume of 100 mL for HPTLC and HPLC analyses.

HPTLC Analysis

Chromatography was performed on pre-activated (100°C) silica gel $60F_{254}$ HPTLC plates (10 × 10 cm; 0.25 mm layer thickness; Merck). The CAMAG densitometry (Camag Model-3 TLC scanner equipped with Camag CATS 4 software), a reflectance spectrometer of monitoring range 190–700 nm was employed for the analysis. The slit was set to 8 × 0.4 mm and data acquisition and processing were

performed using the software winCATS. Samples (10 µl) were applied to the layers at 8 mm wide bands, positioned 10 mm from the bottom of the plate, using a Camag (Mutten, Swizterland) Linomat IV automated TLC applicator with nitrogen flow providing delivery from the string at a speed of 10 µL/s was maintained for all analyses. TLC plate development was performed using a Camag twin-trough glass tank, which had been pre-saturated with mobile phase for 2 h. Solvent was allowed to run up the plate to a height of 8 cm. TLC analyses were made under room temperature. A mixture of acetic acid: methanol: dichloromethane (5:15:35; v/v/v) was used as mobile phase. After development, the layers were dried and the components were visualized by UV light at 365 nm. The quantitative determination was performed by winCATS software program.

The method was validated for linearity, limits of detection (LOD), precision and accuracy. A stock solution of RA was prepared in methanol at 1.0 mg/mL. Standard solutions were prepared by dilution of the stock solution with methanol to give solutions containing the RA in the concentration range of 1-100 µg/mL. The system suitability of the HPTLC method were evaluated by the intra-day and inter-day precision and accuracy of replicates. The precision and recovery experiments were performed at three concentrations of standard solutions of RA. The validated HPTLC method was used for determination of RA from methanol extracts of *O. stamineus* leaf samples collected from our experimental farms in Malaysia. The concentration of RA was determined by external standard method.

HPLC Analysis

HPLC analysis was performed using an Agilent Technologies Series 1100 system equipped with an automatic injector, a column oven, and UV detector. A LiChrosorb RP-18 (250 mm × 4.6 *i.d.* mm, 10 µm particle size) (Merck Darmstadt, Germany) was used. The temperature was maintained at 25°C, with injection volume of 20 µL and flow rate of 1.3 mL/min. RA was separated by using reverse-phase LiChrosorb C-18 column and detected at 340 nm. The separation was achieved by applying a linear gradient from 100% A (water + 0.1% acetic acid) to 100% B (methanol + 0.1% acetic acid) in 40 min followed by 20 min isocratic at 100% B.

The HPLC method was validated for linearity, limits of detection (LOD), precision and accuracy. A stock solution of RA was prepared in methanol at 0.1 mg/mL. Standard solutions were prepared by dilution of the stock solution with methanol to give solutions containing RA in the concentration range of 0.1–100 µg/mL. The precision and recovery experiments were performed at three

concentrations (0.1, 5, and 100 µg/mL) of standard solutions of RA. The validated HPLC method was used for determination of RA from methanol extracts of *O. stamineus* leaf samples collected from our experimental farms in Malaysia. The concentration of RA was determined by external standard method.

Statistical Analysis

Results were analyzed by SPSS 11.5 (SPSS, USA). Correlations were obtained by Pearson correlation coefficient in bivariate correlations. P-values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

HPTLC Analysis

A mixture of acetic acid: methanol: dichloromethane (5:15:35) used as mobile phase gave a good resolution of the RA together with symmetrical and reproducible peaks at R_f 0.58. Fig 2A shows the 3-dimensional HPTLC profiles of reference standards of RA and methanol extracts of samples from the experimental farms. The calibration curve for the RA was linear over the range of 1–100 µg/mL with correlation coefficient greater than 0.9995 in all standard curves. The limit of detection (LOD) of RA by HPTLC was determined by repeated scanning of the lowest detectable standard prepared (1 µg/mL) six times, multiplying the standard deviation of the peak area by three and converting from area to concentration (Merken & Beecher, 2000). The limit of detection (LOD) of RA by HPTLC was 0.4 µg/mL.

Table 1 shows the precision and recovery at three concentrations of standard solutions of RA. The precision of the HPTLC instrumentation was checked by repeated scanning of the same spot of RA seven times each on a same day (intra-day precision) and on five consecutive days (inter-day precision) and the relative standard

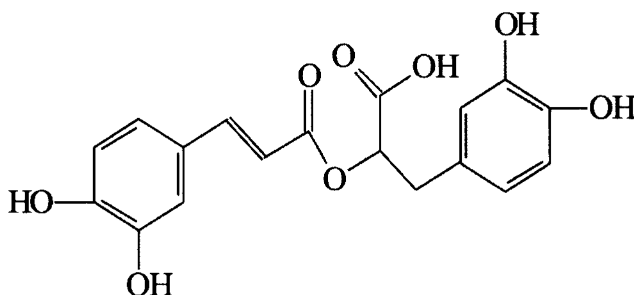

Fig 1. Structure of rosmarinic acid

Table 1. Accuracy and analytical precision of rosmarinic acid by HPTLC and HPLC

Concentration added (µg/mL)	HPTLC Assay			
	Precision *(n=6)*		Accuracy *(n=3)*	
	Intra-day (RSD, %)	Inter-day (RSD, %)	Recovery[a] (%)	RSD[b] (%)
1	1.81	1.86	96.47	1.85
10	1.25	1.26	96.12	1.27
100	1.08	1.15	99.63	1.12
Concentration added (µg/mL)	**HPLC Assay**			
	Precision *(n=6)*		**Accuracy *(n=3)***	
	Intra-day (RSD, %)	**Inter-day (RSD, %)**	**Recovery[a] (%)**	**RSD[b] (%)**
0.1	0.94	0.96	98.44	1.15
5	0.87	0.89	99.25	0.94
100	0.83	0.82	100.16	0.72

RSD: relative standard deviation in percentage

[a] Recovery = (calculated conc/spike conc) × 100

[b] RSD = (SD/conc) × 100

deviations values were calculated. The results showed acceptable precision with the method as revealed by relative standard deviation data. The RSD of the inter- and intra-day precisions of standard solutions of RA were less than 2%. The recovery was determined by spiking a sample of the extract with three different standard solutions of RA and analyzed quantitatively in triplicate. The average recovery of RA by HPTLC assay was found to be 97.41%. This indicates good accuracy of the method.

The HPTLC method was used to determine the content of RA in *O. stamineus* leaf samples collected from our experimental farms in Malaysia. Qualitatively, similar HPTLC fingerprints were obtained for all the extracts giving reliable indication of the same identity (Fig 2A). RA was well separated in the extracts by the HPTLC method and detected in all the samples at R_f 0.58. The UV-Vis absorption spectra recorded on the CAMAG TLC scanner at the start, middle and end position of the RA band were superimposable indicating the purity of the peak (Fig 2B). Using the techniques of the HPTLC and the UV-Vis spectra the amount of RA in the methanol extract of the leaf samples were found to range from 0.61 to 1.90% dry weight

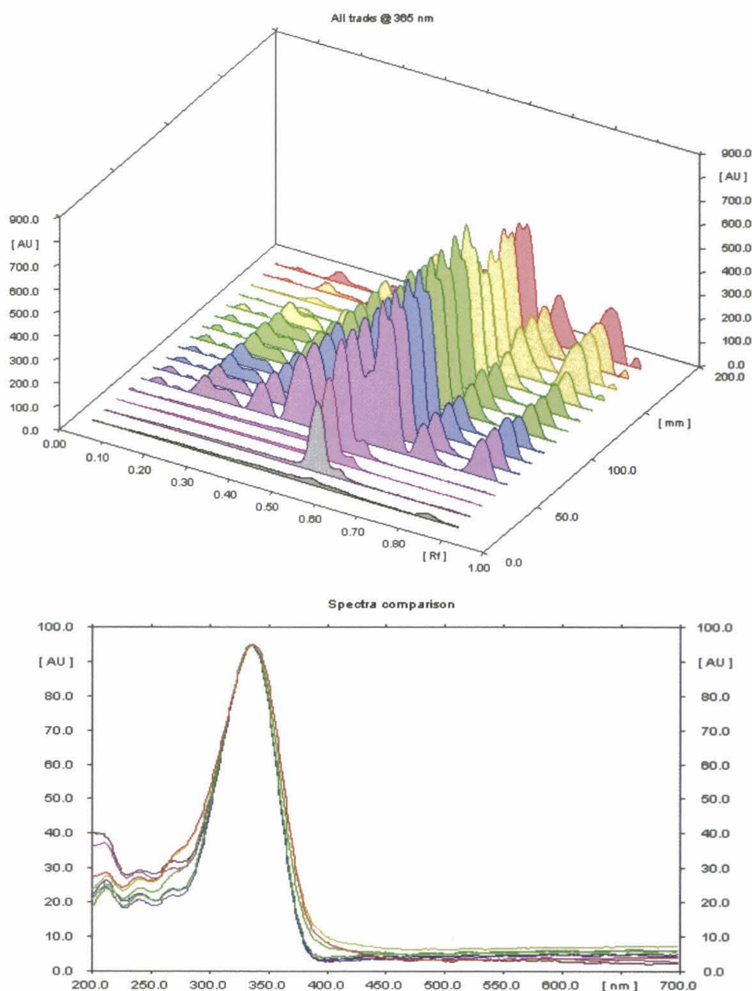

Fig 2. HPTLC profile of *Orthosiphon stamineus* leaf extracts. (A) Three-dimensional chromatogram and; (B) Peak purity spectra for RA recorded at the start, middle and end position of the spot (See the experimental section for the analytical protocol)

(Fig 4). The HPTLC procedure can be used as fast screening method for *O. stamineus* leaf samples and herbal formulations.

HPLC Analysis

Fig 3 shows the chromatogram obtained for HPLC analysis of the methanol extracts of *O. stamineus*. RA was determined by using the chromatographic conditions described in the experimental section and eluted at 15.23 min. The peak of RA was identified by comparison

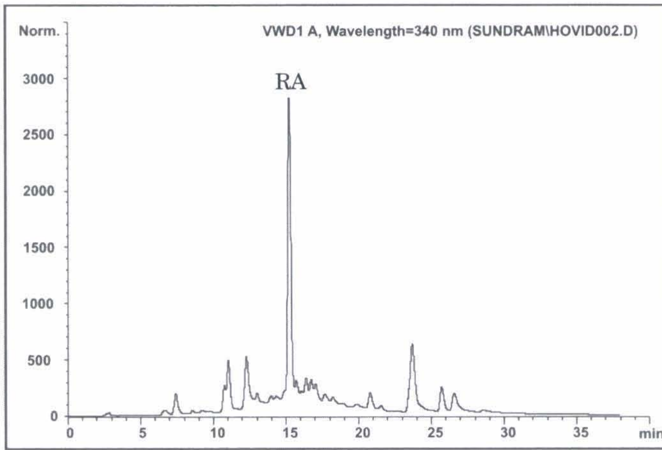

Fig 3. HPLC profile of *Orthosiphon stamineus* leaf extract (See the experimental section for the analytical protocol)

of the retention time of reference standard of RA and extract in the same chromatographic conditions. RA exhibited good linearity in the range from 0.1–100 µg/mL with the square of correlation coefficient (R^2) greater than 0.9999 in all calibration curves. The limit of detection was determined by injecting the lowest detectable standard prepared (0.1 µg/mL) six times, multiplying the standard deviation of the peak area by three and converting from area to concentration (Merken & Beecher, 2000). The limit of detection (LOD) of RA by HPLC assay was 0.05 µg/mL.

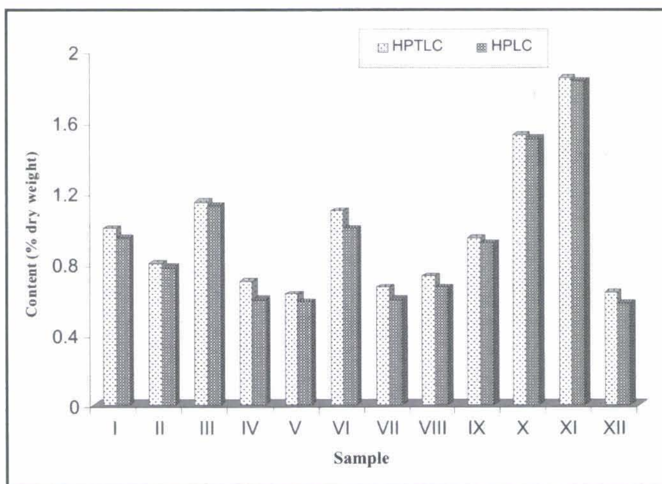

Fig 4. The content of rosmarinic acid in extracts of twelve *Orthosiphon stamineus*. Leaf samples determined by HPTLC and HPLC method (n=3)

Table 1 shows the precision and recovery at three concentrations of standard solutions of RA. To assess the precision of the method, standard solutions of RA were determined six times on the same day and one time for five consecutive days using calibration curves obtained daily. The precision of the method at three concentrations of the standard solutions of RA was expressed as the relative standard deviations (RSD) values by calculating the standard deviation (SD) as percentage of the mean calculated concentration. The results showed acceptable precision with the HPLC method as revealed by relative standard deviation data shown in Table 1. The RSD of the intra-day and inter-day variation studies were less than 1.0% for standard solution of RA. The recovery was determined by spiking a sample of the extract with three different standard solutions of RA and analyzed quantitatively in triplicate. The average recovery of RA by HPLC assay was found to be 99.28%, which indicates good accuracy of the method.

The HPLC procedure was applied to determine the content of RA in extracts for twelve batches of *O. stamineus* leave samples collected from our experimental farms in Malaysia. The quantitative analysis was performed under the described chromatographic conditions using the external standard technique. Typical HPLC profile of the methanol extracts of the leaf is shown in Fig 3. The HPLC assay gave a separation sufficient for peak identification of RA which was eluted at the retention time (R_{t-}) of 15.23 min. The amount of RA ranged from 0.58 to 1.51% dry weight in the methanol extracts. The HPLC method can be used to obtain quantitative data of herbal medicinal products containing *O. stamineus.*

HPTLC method showed slightly higher values for the twelve samples of *O. stamineus* leaf from our experimental farms but there was no statistically significant difference ($P>0.5$) between the mean values all the samples. When the content of the RA in the methanol extracts of the twelve sample was analyzed by linear regression a good relationship between HPTLC and HPLC methods was observed for RA ($r_{xy} = 0.9992$). This shows that the HPTLC method is reliable for good estimation of RA, therefore the method could be used for initial screening or semi-quantitative analyses since the method is less time consuming, simple, and accurate.

CONCLUSIONS

RA content of methanolic leaf extract of *O. stamineus* was determined by HPTLC and HPLC method. Qualitatively, similar HPTLC and HPLC fingerprints were obtained for all the extracts giving reliable indication of the same identity. The HPTLC method for the

determination gave accurate results when compared to the HPLC method. The methods may be of value in standardization of preparations containing *Orthosiphon stamineus* leaves.

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4

Quality Control and Standardization of Medicinal and Aromatic Plants

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ABSTRACT

In India, medicinal and aromatic plants have always been the principal form of medicines and presently they are becoming popular throughout the world. Medicinal and aromatic plants have curative properties due to the presence of various active chemical constituents, viz., alkaloids, steroids, terpenoids, essential oil, glycosides, flavonoids etc occurring in different parts of those plants. The different chemical composition of active chemicals may also found as secondary plant metabolites in one or more parts of these plants. Quantification of active chemicals is the first step of standardization and the presence and concentration of the same can be followed to decide the genuiness of the drug or formulation. Even though quite a good number of plant species under the group of medicinal and aromatic plants are grown in our country under different agro-climatic condition, no systematic investigations have so far been carried out for their active chemical constituents and their variations under different condition. Lack of standardization constitutes a major constraint to the development and use of medicinal and aromatic plants. Under these perspective quality control and standardization of important medicinal and aromatic plants like Kalmegh (Andrographis paniculata), Brahmi (Bacopa monnieri),Senna(Cassia anguistifolia), Ashwagandha (Withania somnifera), Ghritakumari (Aloe vera), Haldhi (Curcuma longa), Vasak (Adhatoda vasica), Lemon grass (Cymbopogan winterianus Jowitt), Mentah (Menthae arvensis aeth) and Thymol

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(Ajowani fructus aeth) were carried out by sophisticated instrumental techniques and also quantified and identified their active principles.

Key words : Quality control, standardization, medicinal and aromatic plants, commercial formulation, analytical technique, active chemicals

INTRODUCTION

India has a rich heritage of medicinal plant wealth. It has been identified as one of the top twelve mega bio-diversity center of the world with immensely rich in medicinal and aromatic plants with diverse eco-systems. From the ancient times these plant resources have been used as pharmaceutical and therapeutic agents. More over risk of side effects of modern drugs, expensive treatments and development of resistance in microbes to antibiotics and chemotherapeutic agents are compelling people to adopt plant based medicine, crude herbal drugs and other holistic health care management approaches.

Medicinal plants are finding diverse use as raw materials not only for medicine but also for articles of daily uses, cosmetics, nutrients and pesticides. As a result, the demands for plant based raw materials have increased enormously in both the national and international markets. The effectiveness of these systems, in turn, mainly depends upon the proper use and sustained availability of genuine raw materials. In the present time people are more quality conscious and demand stringent quality control to consumer products. The aspect of assuring consistency and quality in herbal medicines has proved a bottle-neck in expanding business opportunities and delivering drugs from the developing countries to the world market. The inherent problem of variation in the active content of plant drugs and the lack of easily available standards for medicinal plants have complicated the issue, which is on the top priority of all Governments in the developing and emerging countries.

With an increasing global demand for medicinal plants, most of the raw materials are being collected widely from forest & natural habitat in an indiscriminate manner. This is posing serious threats to the genetic stocks & diversity of medicinal plants. The present trend demands increased emphasis on commercial cultivation as well as quality standardization of these potential species to meet the demand of the domestic industries and to exploit the bright prospect for export.

In spite of tremendous development of modern medicine, plants still remains as one of the major natural source of drugs in both

modern as well as traditional systems of medicine through out the world. WHO estimated that 80% of the populations of developing countries rely on traditional medicine, for their primary health care programme (Farnsworth *et al.,* 1995). Modern medicines still contain atleast 25% drugs derived from plants and many other synthetic analogues. Many of the traditional drugs have also been the starting point of the discovery of many important modern drugs.

In recent years indiscriminate use of synthetic drugs and antibiotics in health care system has resulted in serious symptoms and side-effects all over the world and the demand of plant based raw materials for pharmaceuticals has increased enormously. Recently, WHO has unequivocally stressed the need to strength the use of traditional in health–care programmes to achieve the goal of health for all. This factor has also collaborated to increase the market potential of medicinal plants (Warrier *et al.,* 1996).

As a result, the demand for plant based raw material has increased enormously in both the national and international markets. Due to overexploitation, a serious threat to the existence of many valuable species has emerged which has necessitated the urgent need of their conservation and systematic cultivation for sustained supply to the user industries (Akerele, 1991; Handa & Kaul, 1996).

The inherent problem of variation in the active content of plant drugs and the lack of easily available standards for medicinal plants have proved a bottle- neck in expanding business opportunities and delivering drugs from developing countries to the world market (Mukherjee, 2002).

Realizing the problems in the national perspective, the Government of India is now paying attention to the conservation, popularization and systematic cultivation as well as standardization of the national wealth (Saraswathy *et al.,* 2002). The regional analytical laboratory at BCKV initiated the work for chemical standardization of important medicinal plants to ensure quality control of crude materials by HPTLC finger print techniques and clearly identify and quantify the active principles through rapid chemical analysis (Wagner & Bladt, 1996).

Medicinal plants have proved their efficacy and safety in therapeutic treatment for alleviation or cure of human diseases since time immemorial. The evidences for the therapeutic action of herbal drugs are documented in Indian, Chinese, European and African system of medicine. Medicinal plants are utilized in two distinct areas of health management *i.e.* (1) Modern system of medicine and (2) Traditional system of medicine. At present about 130 clinically tested prescribed drugs are used in modern system of medicine and

they are solely derived from about 100 spices of higher plants. The traditional systems of medicine still continue to carter the primary health care of 80% world populations. In India a number of Traditional system of medicine are practiced such as Ayurveda, Siddha, Unani, Homeopathy, Yoga and Naturopathy for the total health care. The Global market for herbal products including medicines, health supplements, herbal beauty and toiletry products, is estimated at $62 billion and is growing at a rate of 7% annually. The WHO's forecast is that the global market for herbal products would be of the order of $5 trillion by the year 2050. However India's share in the global export market of medicinal plants related trade is just 0.5%. This is against India's rich biodiversity of 45,000 plant species spread across 16 Agro-climatic zones (Akerele, 2002; Farnsworth *et al.*,1995; Handa & Kaul, 1996; Mukherjee, 2002).

The system of standardization (Saraswathy *et al.*, 2002; Wagner, 1984; Warrier *et al.*, 1996) should be established for every plant medicine in the market because the scope of variation in different batches of medicine is enormous. Knowledge of chemical components of a plant is essential for quality control analysis of a plant, extract or any formulation containing them. A compound or group of compounds present can serve as a "biomarker" and the presence and concentration of the same can be followed to decide on the geniuses of the drug/formulations. Any component other than the biomarkers present indicates adulteration. Not many plants are studied for these markers. A knowledge of this compound and their specific analytical methods will facilitate the herbal industry in checking adulteration and thus raise its standard. Therefore, it is essential that such uses of natural products be documented and studied for systematic regulation and wide spread application. With this objective in view a regional quality control laboratory in the department of agriculture chemicals, BCKV have already standardized the valuable medicinal plants grown in different agro climatic zones of West Bengal to ensure quality of crude materials by HPTLC finger print techniques and clearly identify and quantify the active principles through rapid chemical analysis.

MATERIALS AND METHODS

HPTLC Finger Printing Technique Consists of the Following Steps:

Step-1: Extraction of Plant Material

The dried plant materials were extracted with suitable organic solvents. The extract is then filtered, concentrated and made up to 20 mL volume.

Step-2: Sample Application

Samples along with standards were applied on a HPTLC glass plate coated with 60GF 254 silica gel (20 × 20; 300 μm layer thickness) prepared by HPTLC auto coater (Camag). Samples and analytical standards of known concentrations were applied on the glass plates as 8 mm wide band, positioned 15 mm from the bottom and 20 mm from the side of the plate, using an automated TLC applicator linomat 5 (Camag, Multenz; Switzerland) with nitrogen flow providing a delivery speed 150 nl/s from the syringe.

Step-3: Chromatogram Development

After completion of sample application, the plate was developed in a Camag twin trough chamber which was pre-saturated with the mobile phase. Different compositions of the mobile phase for HPTLC analysis were tested to obtain high resolution and reproducible peaks. The TLC runs were performed under laboratory conditions of 25 ± 5°C and 50% relative humidity.

Step-4: Chromatogram evaluation

After development plates were taken off and dried by drier. The plates were then scanned with Camag, TLCscanner-3 equipped with wincats software (Camag) under the following conditions: slit width 6 × 0.45, absorption and reflection mode, lamps used deuterium and tungsten. The details of HPTLC finger print technique for medicinal and aromatic plants are stated in Table 14.

RESULTS AND DISCUSSION

The medicinal properties of *Andrographis paniculata* (Kalmegh) are due to the presence of a diterpenoid andrographolide. Leaves of *Andrographis paniculata* collected from different agroclimatic zones of West Bengal were analyzed for andrographolide and two less focused chemical actives deoxyandrographolide and dehydroandrographolide (Figs 1a, 1b). The methanol extract of leaves along with andrographolide solution was developed with chloroform: methanol 7:3 and scanned at λ_{max} 230 nm. In HPTLC finger print of *A. paniculata* (leaf) sample, six peaks were observed (Fig 1a). Compound with R_f 0.36 was in highest concentration and was found to be identical with a diterpenoid andrographolide, the chemical active of *A. paniculata*. Concentration of andrographolide was highest in Asanol zone (Table 1). The results found comparable with earlier reports (Hu & Zhou, 1982; Xiao *et al.*, 2006).

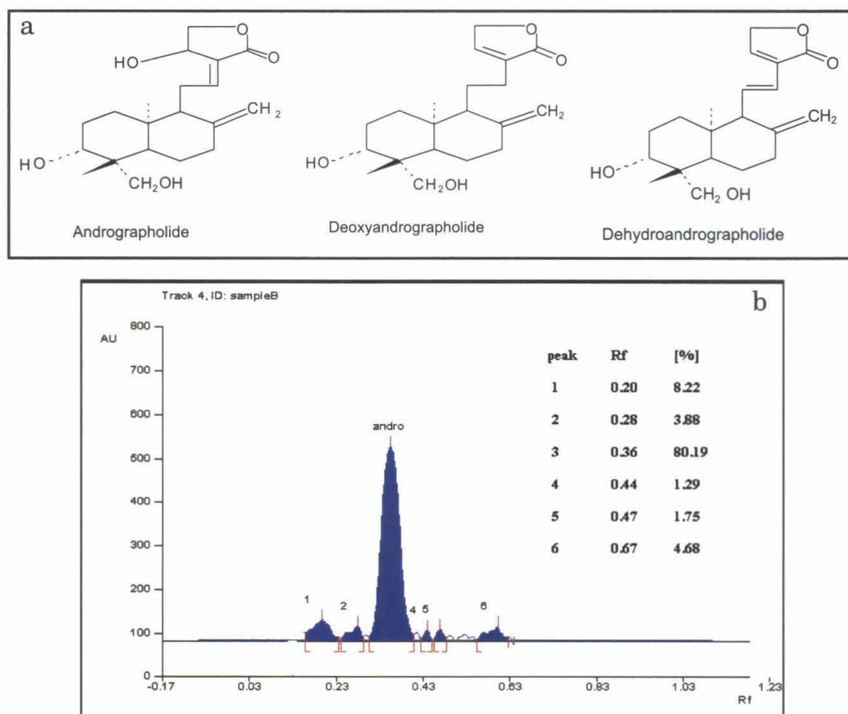

Fig 1. (a) Andrographolide and its two derivatives deoxyandrographolide and dehydroandrographolide (b) Fingerprint of Andrographolide extract at 230 nm

Table 1. Percent of andrographolide and its derivative in leaves of kalmegh

Source	**% of chemical actives present**		
	Andrographolide	**Dehydroandrographolide**	**Deoxyandrographolide**
Mohanpur	1.98	0.984	0.52
Nawadeep	1.33	1.02	0.68
Tarokeswar	2.02	1.08	0.39
Canning	2.23	1.26	0.32
Asansol	2.49	2.11	0.52
Mecheda	2.18	1.38	0.54
Raghunathpur	2.11	1.45	0.49

Table 2. The percent of alkaloids and *Withaferin* A. content in roots of Ashwagandha

Plants parts	**Alkaloid %**	**Withanolide %**	**Withaferin A %**
Root	0.15	1.4	0.1
Stem	0.11	1.2	0.01
Leaves	0.51	2	0.53

The finger print of *Bacopa monnieri* showed the presence of three peaks. Peak with R_f 0.60 was in highest in concentration and was found to be identical with Bacoside A, the active constituent of the plant (Figs 2a, 2b). Matching of the UV spectra of Bacoside A with that of the sample revealed that they are the same sample. The contents of Bacoside A in different plant parts are shown in Table 10. The results found comparable with earlier reports (Chakravarty *et. al.,* 2003; Bhattacharya *et al.,* 2000).

Ashwagandha (*Withania somnifera*), roots are used in the preparation of herbal formulation. *Withaferin A.*, an unsaturated steroidal lactone is the most important withanolide to which the medicinal properties of the plant are attributed. The extraction of roots was followed by approved method. Crude withanolide and the standard *Withaferin A* was applied on a HPTLC plate, developed in chloroform: methanol (9:1) and scanned at λ_{max} 220 nm (Figs 4a, 4b & 4c). In HPTLC finger print of Ashwagandha root samples, six peaks were observed. Compounds with R_f 0.68 was found to be identical with *Withaferin A*, confirmed by superimposing UV spectra of *Withaferin A* with that of spot at R_f 0.68. The percent of alkaloids and Withaferin content was shown in (Table 2). Extraction of total alkaloids and withanolides in different plant parts like Leaves, roots and stem of *Withania sommnofera* were done by methanol. The extract was concentrated and defatted with hexane and then extracted with 1% H_2SO_4, basified with ammonia, extracted with chloroform, evaporated and weighed for total alkaloids. The H_2SO_4 insoluble fraction was extracted with diethyl ether, evaporated and weighed for crude withanolides. The results found were comparable with earlier report (Rekha *et al.,* 2006).

Sennosides A and B, the two anthraquinones isolated from *C. angustifolia (Senna)* have the strong laxative properties. A variety of polyherbal ayurvedic preparations, commercially available in India to relieve constipation were analyzed to quantify these two anthraquinones. The methanol (70%) extract of the sample and standard sennosides A & B were applied on a HPTLC plate, developed in a mobile phase containing 2-propanol: ethyl acetate: water: Formic acid (17:19:12:02.) and scanned at 350 nm (Figs 3a, 3b). Validity of peak was confirmed by comparing UV spectra of standard and that of the standard within the same R_f window. The result (Table 3 & 4)

Table 3. Content of sennoside A and sennoside B in *Cassia angustifolia*

Plant parts used	% of Sennoside A	% Sennoside B
Leaves	1.8	2.3
Pods	2.2	2.5

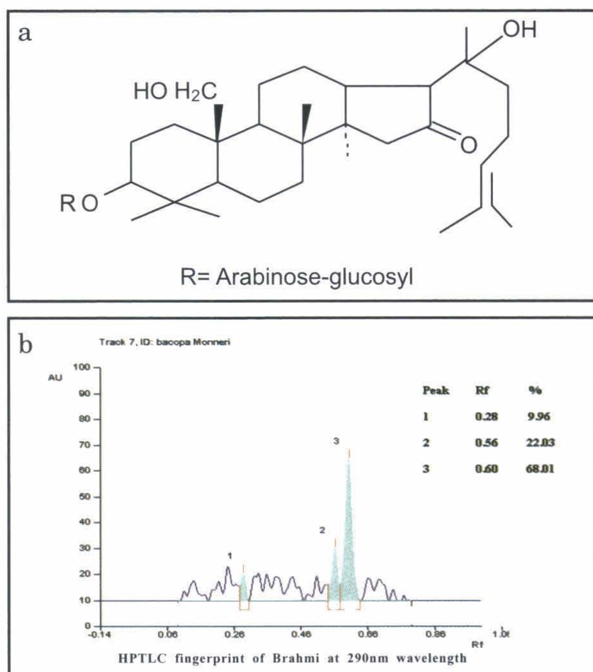

Fig 2. (a) Structure of Bacoside A (b) Fingerprint of Bacoside extract at 290 nm

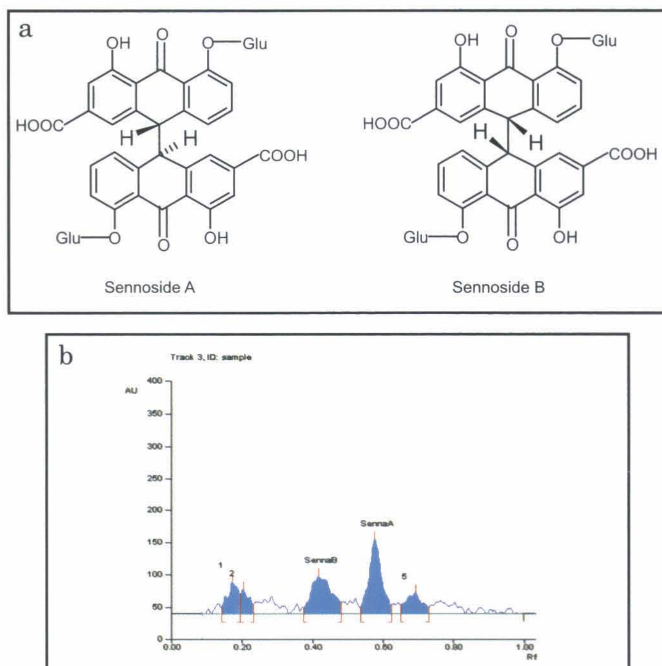

Fig 3. (a) Structure of sennoside A and B (b) HPTLC finger print of senna formulation

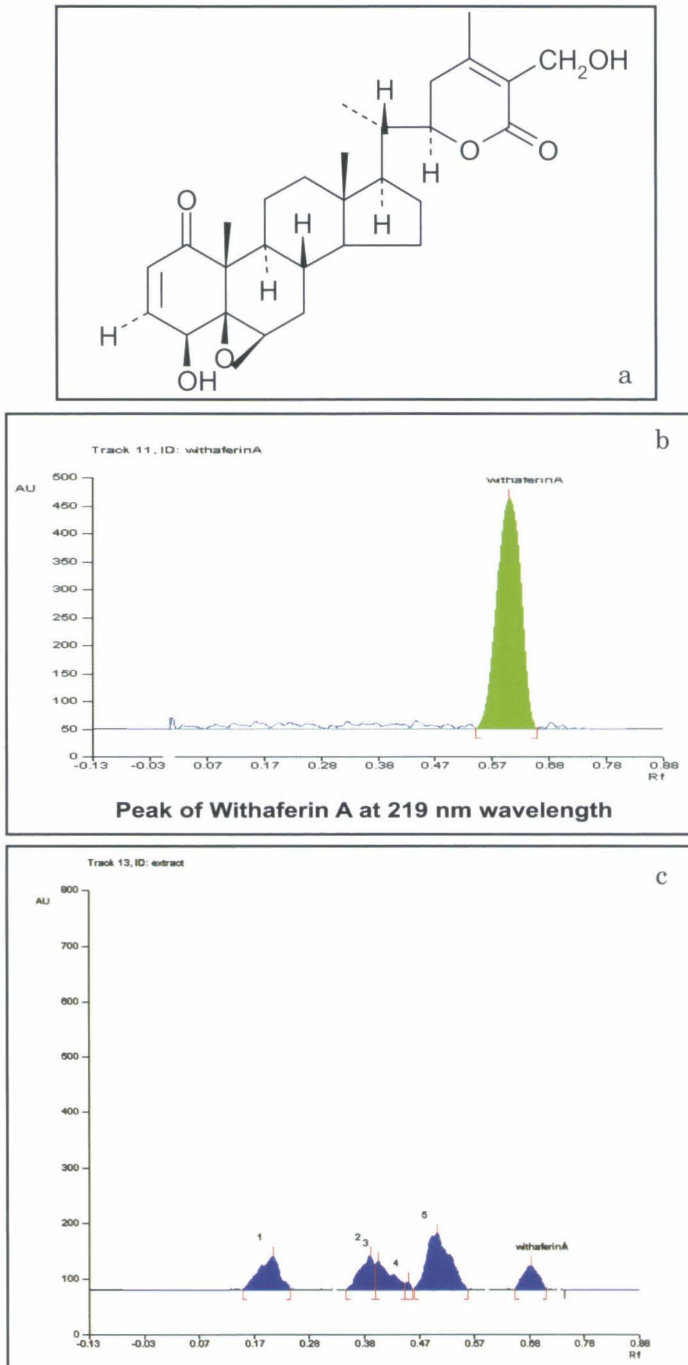

Fig 4. (a) Structure of withaferin A (b) HPTLC finger print of withaferin A analytical standard (c) HPTLC finger print of Ashwagandha root extract

Table 4. Concentration of sennoside A and sennoside B in different ayurvedic formulations

Formulations	Sennoside A [mg/g] of the formulation	Sennoside B [mg/g] of the formulation
Formulation 1	2.5	25.9
Formulation 2	2.3	12.70
Formulation 3	2.1	2.6
Formulation 4	1.8	1.87
Formulation 5	1.62	1.85
Formulation 6	1.58	1.72
Formulation 7	1.4	1.53
Formulation 8	1.2	1.5
Formulation 9	0.92	1.4
Formulation 10	0.91	1.4

Table 5. Aloin content (%) in leaf exudates of *Aloe vera* at different harvesting stages

Harvesting time (months after planting)	(%) Aloin content in leaf exudate			Mean ± S.D.
	R_1	R_2	R_3	
12	4.59	4.72	4.70	4.67 ± 0.07
13	4.81	4.78	4.78	4.79 ± 0.02
14	5.05	4.97	4.92	4.98 ± 0.07
15	5.31	5.28	5.22	5.27 ± 0.05
16	5.76	5.73	5.79	5.76 ± 0.03
17	5.80	5.82	5.81	5.81 ± 0.01
18	5.86	5.91	5.84	5.87 ± 0.04

showed the wide variation of sennosides A and B in different herbal formulations. The results found comparable with earlier reports (Bala *et al.,* 2000; Shah *et al.,* 2000).

Aloe vera Linn. yields two major juice materials; a yellow exduate which is used in traditional medicine as a cathartic agent and a mucilaginous gel that is utilized as cosmetic and as dietary supplement in beverages. A HPTLC method was developed and validated to quantify the aloin content in leaves collected at different time intervals and in different marketed formulations (Table 5 & 6.). The methanol extract of sample and the standard Aloin was developed in a mobile phase containing ethyl acetate: MeOH: H_2O 200: 33: 27 at $\lambda_{max:}$ 370 nm. HPTLC finger print of Gritakumari showed five

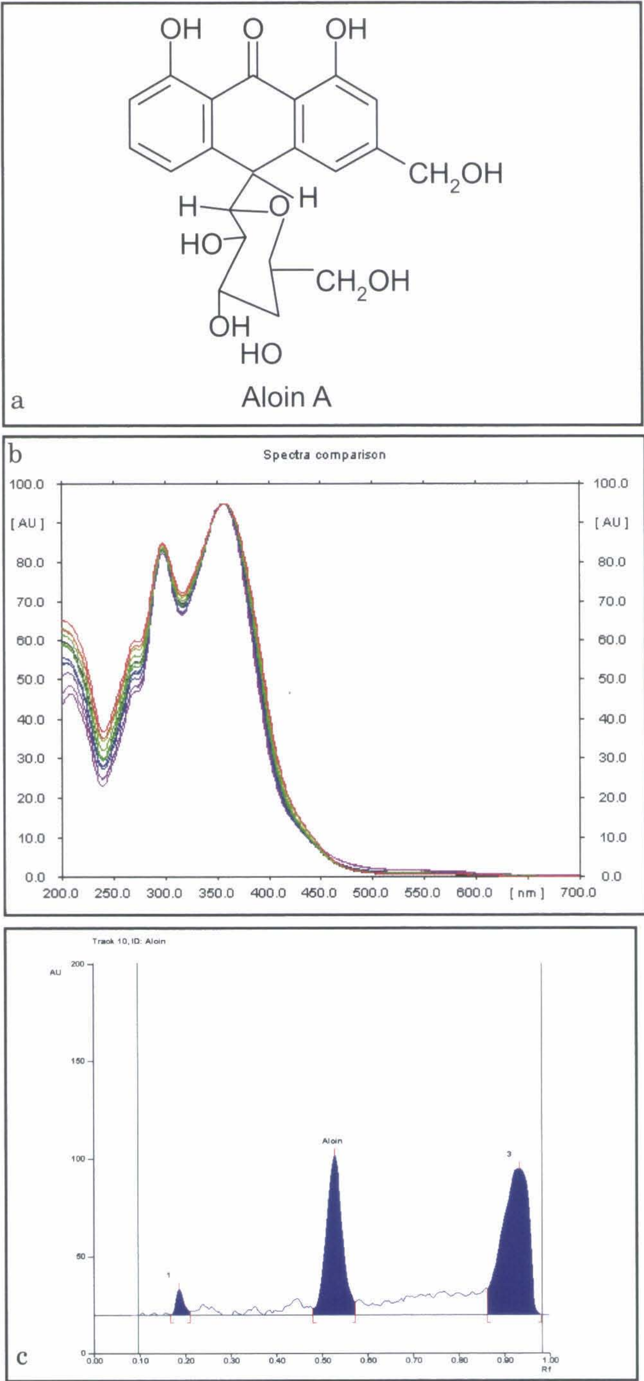

Fig 5. (a) Structure of aloin A (b) Super imposable UV spectra of aloin and the extract of *Aloe vera* (c) HPTLC finger print of *Aloe vera* gel

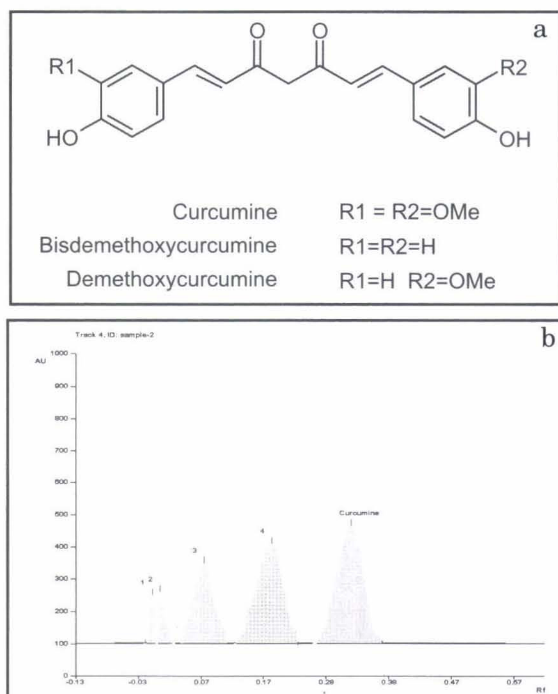

Fig 6. (a) Structure of curcuminoids (b) HPTLC finger print of *Curcuma longa*

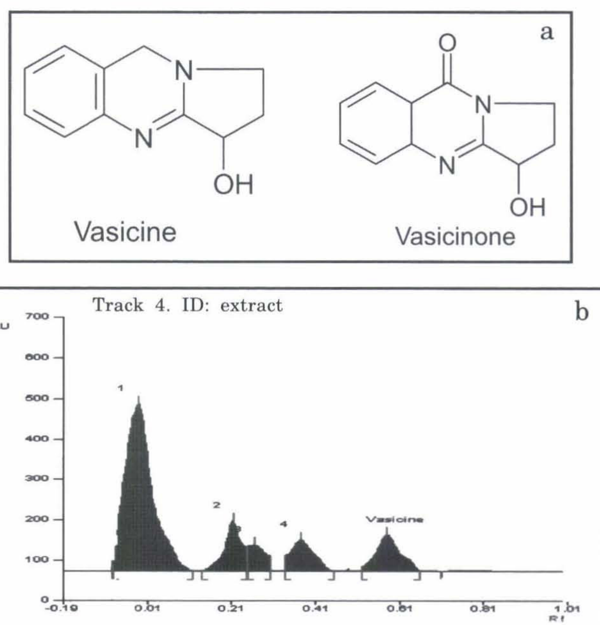

Fig 7. (a) Structures of key alkaloids from *Adhatoda vasica* (b) HPTLC finger print of vasak extract

Table 6. Aloin content in different marketed formulations

Aloe gel supplied	3 mg/100 g of gel
Aloe gel + Na benzoate supplied	3.5 mg/100 g of gel
Gel collected from supplied leaf	4 mg/100 g of gel
Whole leaf juice	45 mg/100 g of whole leaf juice
Tonic	19.75
Capsules	9.75

Table 7. Percent content of vasicine

Plant parts	Vasicine (%)
Leaves	0.65
Stem	0.31
Root	0.062

Table 8. The curcuminoids composition in *Curcuma longa* (collected from medicinal garden, BCKV)

Curcumin %	DMC %	BDMC %
2.2	0.8	0.2

peaks (Figs 5a, 5b & 5c). The major peak at R_f 0.27 was confirmed as aloin by superimposing UV spectra of aloin with that of the spot at Rf 0.27. The results found are comparable with earlier reports (Leung *et al.,* 2004; Shelton, 2007).

Adhatoda vasica (Vasak) is a well known plant drug used in the treatment of various diseases and disorders particularly for the respiratory track ailments. Vasicine, the main active quinazoline alkaloid has been shown to posses bronchodilatory and potent respiratory activities. Samples and standard were developed in MeOH: Toluene: Dioxane: Ammonia 2:2:5:1 and scanned at $\lambda_{max:}$ 270 & 281 nm. HPTLC fingerprint of vasak showed the separation of five peaks (Figs 7a, 7b). Peak at R_f 0.61 was confirmed as vasicine by comprising UV spectra of sample with that of the standard at the same R_f window. Concentration of vasicine was maximum in leaves (Table 7). The results found comparable with earlier reports (Claeson *et al.,* 2000; Shaifali *et al.,* 2001).

Curcuma longa (turmeric), a yellow spice from the family Zingiberaceae have long been used in Ayurvedic to treat a diverse range of conditions. These actions are mainly due to the phenolic fraction containing curcumin, demethoxycurcumin and bisdemethoxycurcumin, collectively known as curcuminoids. A HPTLC finger print technique (Figs 6a, 6b) is developed to standardize the turmeric based

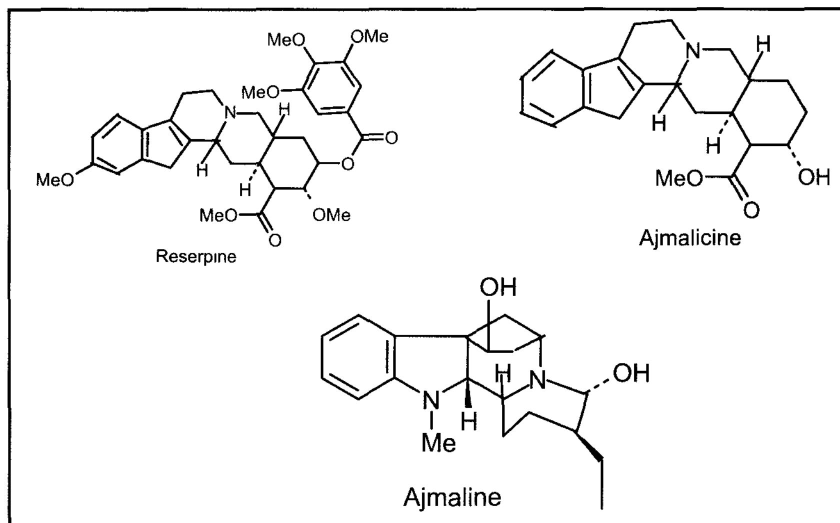

Fig 8. Structure of key alkaloids from *Rauwolfia serpentina*

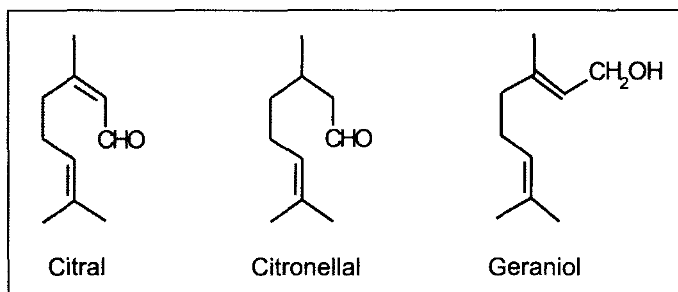

Fig 9. Structure of citronellal, citral and geraniol from *Cymbopogan* sp.

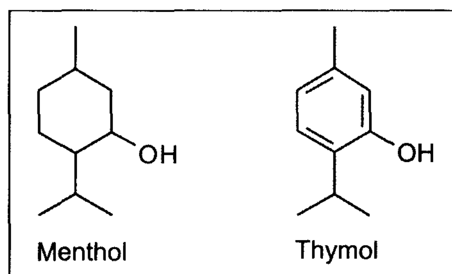

Fig 10. Structure of menthol and thymol in plant spices

formulations (Table 8 & 9). Methanolic solutions of samples and standards were developed in chloroform: methanol (48:2) and scanned at 420 nm. The results found comparable with earlier reports (Chattopadhyay *et al.*, 2004; Schieffer, 2002).

Table 9. The Curcuminoids compositin of some commercial samples of turmeric

Sample	Curcumine %	DMC %	BDMC %
Source 1	70	25	8.5
Source 2	75	20	5.78
Source 3	79	16	3.9

Table 10. The Bacoside A content in *Bacopa monnieri* (collected from medicinal garden, BCKV)

Plant Parts	Bacoside A (%)
Leaves	1.13
Stem	0.86

Table 11. The Alkaloid contents in Sarpagandha (collected from medicinal garden, BCKV)

Plant	Reserpine %	Ajmaline %	Ajmalicine %
Sarpagandha *(Rauwolfia serpentine)*	0.11	0.2	0.03

Table 12. The composition of Lemon grasses (collected from medicinal garden, BCKV)

Plant source	Essential oil %	Citral %	Citronellal %	Geraniol %
Cymbopogan winterianus	1.5%	16	25	40
Cymbopogan flexuosus	1.2%	70	10	15

Table 13. The composition of Mentha and Ajwani (collected from medicinal garden, BCKV)

Plant source	Essential oil %	Menthol %	Thymol %
Menthae arvensis aeth	1.8	–	50
Ajowani fructus aeth	5	42.8	–

The contents of different alkaloids (Fig 8) of Sarpagandha (*Rauwolfia serpentine*) are measured by HPTLC (Table 11). The details of HPTLC finger print technique is stated in Table 14. The results found comparable with earlier report (Youngken, 1975).

The quantitative estimation of Citronellal, Citral and Geraniol (Fig 9) in *Cymbopogan* sp. are done by HPTLC (Table 14) and extraction was done by Steam distillation technique (Table 12). The

Table 14. Finger print profile of Some Medicinal and Aromatic plants

Sl. no.	Common name	Scientific name	Family	Active constituent	Chemical formula	Chemical family	Solvent used for extraction	Mobile phase	λ_{max} (nm)
1.	Kalmegh	*Andrographis paniculata*	Acanthaceae	Andrographolide	$C_{20}H_{30}O_5$	Diterpene	Aq. Methanol	$CHCl_3$:MeOH (7:3)	230
				Deoxy andrographolide	$C_{20}H_{30}O_4$	Diterpene	Aq. Methanol	$CHCl_3$:MeOH (7:3)	230
				Dehydro andrographolide	$C_{20}H_{28}O_4$	Diterpene	Aq. Methanol	$CHCl_3$:MeOH (7:3)	230
2.	Brahmi	*Bacopa monnieri*	Scrophulariaceae	Bacoside A	$C_{41}H_{68}O_{134}H_2O$	Triterpenoid saponin	Aq. Methanol	Ethyl acetate: MeOH:H_2O (1:7:2)	290
3.	Senna	*Cassia angustifolia*	Caesalpiniaceae	Sennoside A and Sennoside B	$C_{42}H_{38}O_{20}$	Anthracene glycosides	Aq. Methanol	2-propanol: ethyl acetate :water:formic acid (17:19:12:02.)	350
4.	Aswagandha	*Withania somnifera*	Solanaceae	Withaferin -A	–	Steroidal lactone	Aq. Methanol	Chloroform: ethanol (95:5)	219
5.	Ghritakumari	*Aloe vera*	Liliaceae	Aloin	$C_{21}H_{22}O_9$	Anthraquinone derivative	Aq. Methanol	Eyhyl acetate: MeOH:H_2O (200:33:27)	370

Table 14. *Contd.*

Sl. no.	Common name	Scientific name	Family	Active constituent	Chemical formula	Chemical family	Solvent used for extraction	Mobile phase	λ_{max} (nm)
6.	Haldi	*Curcuma longa*	Zingiberaceae	Curcumine	$C_{21}H_{20}O_6$	Diferuloylmethane	Aq. Methanol	$CHCl_3$:MeOH (48:2)	420
				Demethoxycurcumine	$C_{19}H_{18}O_4$	Diferuloylmethane	Aq. Methanol	$CHCl_3$:MeOH (48:2)	420
				Bisdemethoxycurcumine	$C_{20}H_{16}O_5$	Diferuloylmethane	Aq. Methanol	$CHCl_3$:MeOH (48:2)	420
7.	Vasak	*Adhatoda vasica*	Acanthaceae	Vasicine	$C_{11}H_{10}N_2O$	Quanizoline alkaloid	Ammoniacal methanol	MeOH: toluene: dioxane: ammonia (2:2:5:1)	270
				Vasicinone	$C_{11}H_{10}N_2O_2$	Quanizoline alkaloid	Ammoniacal methanol	MeOH: toluene: dioxane: ammonia (2:2:5:1)	281
8.	Sarpagandha	*Rauwolfia serpentina*	Apocynaceae	Reserpine	$C_{33}H_{42}N_2O_9$	Alkaloid	Aq. Methanol	$CHCl_3$:MeOH (9:1)	365

Table 14. *Contd.*

Sl. no.	Common name	Scientific name	Family	Active constituent	Chemical formula	Chemical family	Solvent used for extraction	Mobile phase	λ_{max} (nm)
				Ajmaline	$C_{20}H_{26}N_2O_2$	Alkaloid	Aq. Methanol	$CHCl_3$:MeOH (9:1)	365
				Ajmalicine	$C_{21}H_{24}N_2O_3$	Alkaloid	Aq. Methanol	$CHCl_3$:MeOH (9:1)	365
9.	Citronella + Lemon grass	*Cymbopogan* sp	Poaceae	Citral	$C_{10}H_{16}O$	Terpene	Steam	Hexane:EA (18.6:1.4)	220
				Citronellal	$C_{10}H_{18}O$	Terpene	Steam	Hexane:EA (18.6:1.4)	220
				Gerianol	$C_{10}H_{18}O$	Terpene	Steam	Hexane:EA (18.6:1.4)	220
10.	Mentha	*Menthae arvensis aeth*	–	Menthol	$C_{10}H_{20}O$	Terpene	Steam	Toluene:EA (93:7)	
11.	Ajowani	*Ajowani fructus aeth*	–	Thymol	$C_{10}H_{14}O$	Phenylpropane	Steam	Toluene:EA (93:7)	

results found comparable with earlier report (Suman *et al.,* 2005). The active constituents of Mentha and Ajwani (Fig 10) collected from BCKV farm is shown in Table 13. The results found comparable with earlier reports (Ishikawa *et al.,* 2001; Kukreja & Dhawan, 2000).

CONCLUSIONS

Medicinal plants play a vital role in traditional system of herbal medicine as well as in the process of modern drug discovery and economic development. As we have the tremendous potential in respect of flora of medicinal plants which is unique in the World. The system of standardization should be established for every plant medicine in the market because the scope of variation in different batches of medicine is enormous. Adding to this variability is the fact that in herbal medicine several plants may be used together in the same preparation. This means there should be a quality test for the entire preparation to ensure quality of the preparation. Standard procedure should be developed for getting the final product HPTLC finger print techniques of plant materials would help in laying down pharmacopoeia standards to check the identity of the medicinal plant and hence to control the quality of herbal medicine.

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5

Development of a Thin-Layer Chromatography-Densitometric Method for the Quantification of Podophyllotoxin in *Podophyllum hexandrum*

ARCHANA PESHIN RAINA[1,*], S.K. PAREEK[1] AND K.S. NEGI[1]

ABSTRACT

Podophyllum hexandrum Royle (Syn. P. emodi) commonly known, as Indian Podophyllum has become an endangered species due to extensive exploitation of this plant by the pharmaceutical industry for the synthesis of anticancer drugs. It is therefore, necessary that research and development of this plant should be intensified to save the crop from extinction. A prerequisite to this endeavor is the development of a reliable procedure to determine rapidly the content of podophyllotoxin in minimum possible amount of plant material. Therefore, a quantitative high-performance thin-layer chromatographic method was developed to quantify podophyllotoxin from Podophyllum hexandrum. Separation was performed on precoated silica gel 60 F_{254} HPTLC followed by scanning of the spots at 210 nm detection mode using a Camag Scanner 3. The relation between the concentration of standard podophyllotoxin and corresponding peak areas was found to be linear within the range of 100 ng to 1000 ng/spot. The proposed TLC method was found to be precise, specific, sensitive and accurate and can be used for routine quality analysis. The range of variation in podophyllotoxin content of the collections from different regions was between 1.09% to 4.76%.

Key words : HPTLC, podophylloresin, *Podophyllum hexandum*, podophyllotoxin

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INTRODUCTION

Indian Podophyllum (*Podophyllum hexandrum* Royle), a perennial herb, grows wild in the forests of interior Himalayan ranges of India, particularly Himachal Pradesh, Jammu and Kashmir and Sikkim hills (Chatterjee, 1952). The roots and rhizomes of *Podophyllum hexandrum* contain various lignans (Jackson & Dewick, 1984; Kamil & Dewick, 1986). These lignans are used as important drugs in the treatment of testicular and small–cell lung cancer (Uden *et al.,* 1989). Podophyllotoxin is the most important lignan present in this plant, which serves as a starting material for the semisynthesis of the anticancer drugs (Broomhead & Dewick, 1990; Canal *et al.,* 2000; Stahelin & Wartburg, 1991) namely etoposide (VP-16–213) and teniposide (VM-26). Their cytotoxic action is based on inhibition of topoisomerase II, while podophyllotoxin acts as an inhibitor of the microtubule assembly (Imbart, 1998). Podophyllotoxin is also a precursor for the new derivative CPH-82 (reumacon) being tested in Europe in phase III clinical trials for arthritis. In addition, podophyllotoxin and podophyllin (*Podophyllum* resin) are considered as active constituent in dermatological products for therapy of genital warts. The demand for plant material, however, has endangered the common source of podophyllotoxin, *P. hexandrum* (Nayar, 1990). The roots of *Podophyllum hexandrum* contain a high concentration (4 per cent on dry weight basis) of podophyllotoxin (Jackson & Dewick, 1984) that is a dimerized product of two phenyl propane units linked by β-carbon atoms of their side chains. American podophyllum (*Podophyllum peltatum)* contain very low podophyllotoxin (0.25%) compared to Indian Podophyllum.

Due to its medicinal value, *Podophyllum hexandrum* is in great demand, resulting in reckless collection of roots and rhizomes of *Podophyllum hexandrum* from natural habitats for podophyllotoxin extraction. However, roots and rhizomes of *Podophyllum hexandrum* from different locations have not been systematically evaluated for the selection of high podophyllotoxin producing biotypes. Podophyllotoxin is still extracted from the roots of *P. hexandrum* and *P. peltatum* collected from the wild. Chemical synthesis is also possible, but not economical. Therefore, there is an increasing interest in additional sources for supply of podophyllotoxin and future production of these drugs depends upon cultivation of *P. hexandrum* or the use of tissue/cells culture techniques that provide only small quantities of plant material for analysis. A prerequisite to this endeavor is the development of a reliable procedure to determine rapidly the content of podophyllotoxin in minimum possible amount of plant material. Though HPLC method have been reported for determination of podophyllotoxin (Purohit *et al.*, 1999) but HPTLC

has emerged as a versatile technique for the standardization of herbal formulations. It is simple and economical and requires minimum sample clean up. The objective of this study was to standardize a method suitable for quantitative estimation of podophyllotoxin in roots and rhizomes of *Podophyllum hexandrum* using High performance Thin layer Chromatography (HPTLC) and to study variation in podophyllotoxin content in *Podophyllum hexandrum* collections from different geographical regions.

Distribution and Habitat

The herb is distributed from the Himalayas to South-west China, (India, Bhutan, Pakistan, China). It is found in the interior range of the Himalayas from Kashmir to Sikkim at altitudes of 3000 to 4200 m and thrives as undergrowth in the fir forests rich in humus and decaying organic matter. It is generally associated with species of *Juniperus, Rhododendron, Salix* and *Viburnum*. It also prevails in open alpine meadows where it is less frequent. It is known locally as ban-kakri, ban-wagan etc.

Parts Used

Whole plant, fruits, rhizomes and roots.

Therapeutic Uses

The resin of this species is used as hepatic stimulant, in constipation, cold and bilary fever and anti cancer drugs.

Botanical Description

Podophyllum species are the perennial plants in the family Berberidaceae and have the chromosome number 2n=12. *Podophyllum hexandrum* Royle (*P. emodi*) is an erect glabrous succulent herb, 35 to 60 cm high, with creeping perennial rhizomes and bearing countless roots, leaves 2 or 3, palmate, peltate, orbicular-reniform with lobed segments. Flowers are solitary, cup shaped, white or pink. Flowering time is May. Fruits are elliptic or oblong berry, orange or red, 2.5 to 5 cm diameter, containing several seeds embedded in the pulp.

MATERIALS AND METHODS

Plant Material

Mature individual plants, showing variability were collected during May-June from Sikkim hills and Bhowali from altitudes ranging from

1800 to 3500 m above sea level. Roots and rhizomes of individual populations were washed with a fine jet of water till the soil was completely removed. All the root samples were dried at 60°C for 24 h in an oven. Dried roots were ground to a powder in a pestle and mortar.

Reagents and Solvents

All the solvents used were of analytical grade. Reference standard podophyllotoxin was obtained from Across Organics.

Preparation of Standard Solution

Podophyllotoxin (10 mg) standard was weighed, transferred into a 10 mL standard volumetric flask, dissolved in methanol and final volume was made 10 mL so that 1 ml of standard contains 1 mg of podophyllotoxin.

Extraction

Dry root powder (2 g) of *Podophyllum hexandrum* was extracted with ethanol in a soxhlet apparatus for 12 h on a boiling water bath. The ethanol extract was distilled in vacuum to remove the solvent. The residue obtained was dissolved in the minimum volume of absolute ethanol and precipitated by ice cooled acidulated water at 4°C. The resin obtained was filtered and washed with water to make it free from acid. It was weighed for the total podophylloresin content. The resin (1 mg) obtained was dissolved in 1 mL methanol for application on silica gel HPTLC plates.

Chromatography

Chromatography was performed on 20 cm × 10 cm silica gel 60 F_{254} HPTLC plate, (E. Merck, Germany). Samples along with different concentrations of standards (podophyllotoxin 1 mg/1 mL) prepared in methanol, were applied to the plate as 6 mm wide bands with an automatic TLC applicator Linomat V with N_2 flow (CAMAG, Switzerland), 10 mm from the bottom. The HPTLC plates were developed to a distance of 80 mm in a 20 cm × 10 cm CAMAG twin trough glass tank. A combination of different mobile solvents of varying polarity was used to optimize the composition of mobile phase. A solvent combination of chloroform: methanol (9:1; v/v) was found to give best TLC resolution with pre saturation for 15 min. The HPTLC runs were made in the laboratory conditions of 25 ± 5°C and 50% relative humidity. After development the plates were withdrawn and dried and spots were visualized in UV light (UV cabinet, CAMAG, Switzerland).

Densitometric evaluation was done using CAMAG TLC scanner 3 equipped with Wincat software under the following conditions: slit width 5 × 0.45 mm, wavelength 210 nm UV (deuterium lamp) absorption-reflection detection mode. Different amounts (0.1–1 μg) of stock solution of standard podophyllotoxin were applied in duplicates on HPTLC plate. The plate was developed as above and scanned at 210 nm. Peak areas were recorded for all the tracks. A calibration curve was constructed by plotting concentration versus peak area of the compound. A known amount of root extract was spotted on TLC plate and concentration of podophyllotoxin in *Podophyllum hexandrum* root extract was determined using above calibration curve.

Validation of the Method

For determining recovery of podophyllotoxin, known amount of stock solution of pure podophyllotoxin was added to fixed amount of root extract at three concentration levels and quantitative analysis was repeated three times. The percentage recoveries were calculated from the peak areas of podphyllotoxin in the samples.

The repeatability of the method was affirmed by applying 2 μl aliquots of standard solution (10 μg/10 μl) of podophyllotoxin on a TLC plate (n=5) and analyzing them as described in the preparation of calibration plot. Repeatability was expressed as the RSD of the peak areas.

RESULTS AND DISCUSSION

For the analysis of herbal raw materials and herbal preparations, TLC is superior to other instrumental analytical techniques because it is simple and economical and requires minimum sample clean up. That is why TLC has emerged as an efficient tool for the phytochemical evaluation of herbal drugs. A TLC densitometric technique is therefore, suggested for the determination of podophyllotoxin in *Podophyllum hexandrum* roots and rhizomes.

Of the various solvent systems tried, chromatography on silica gel with chloroform: methanol, 9:1 (v/v) gave good separation of podophyllotoxin ($R_f = 0.60$) from other components of the extracts. A typical densitogram obtained for podophyllotoxin is presented in Fig 1. The identity and purity of the band of podophyllotoxin in the sample solutions was confirmed by overlaying its UV absorption spectrum with that of the standard using densitometer. The spectra of all the tracks were recorded between 190 and 400 nm (deutrium lamp); typical spectra of podophyllotoxin standard and of the corresponding spot from the samples are shown in Fig 2. They are

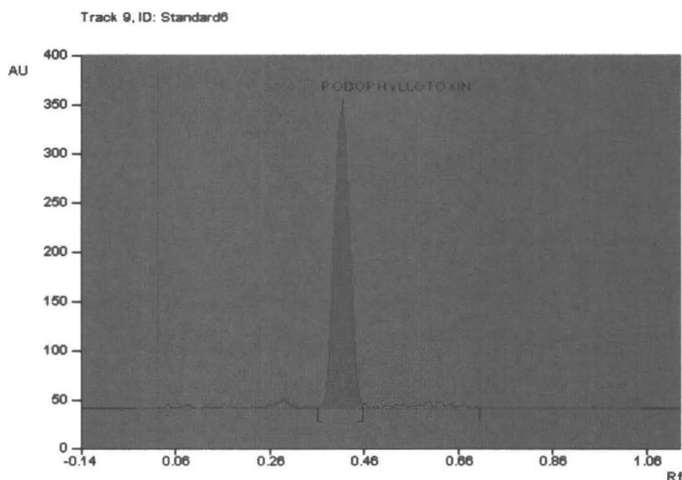

Fig 1. A typical densitogram of standard podophyllotoxin

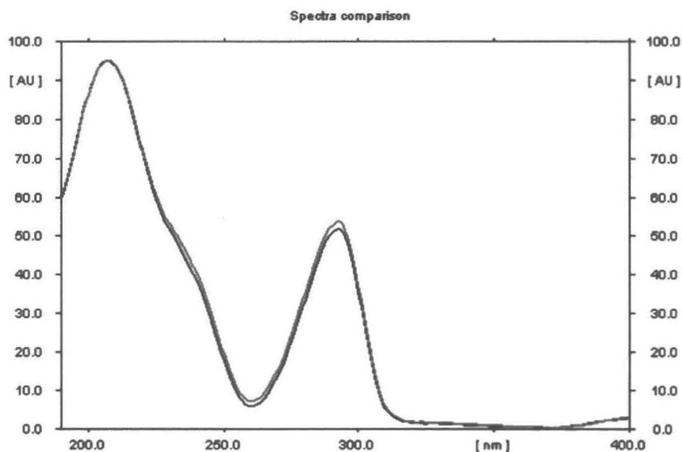

Fig 2. Spectra of podophyllotoxin

Table 1. Recovery study to assess accuracy of the method

Concentration of podophyllotoxin in sample (ng)	Amount added (ng)	Theoretical value (ng)	Experimental value (ng)	Recovery (%)(n = 3)
652.19	100	752.19	761.07	101.2
652.19	200	852.19	827.89	97.14
652.19	300	952.19	912.85	96.00

similar to each other with maxima at 210 nm; this indicates the presence of podophyllotoxin in these samples.

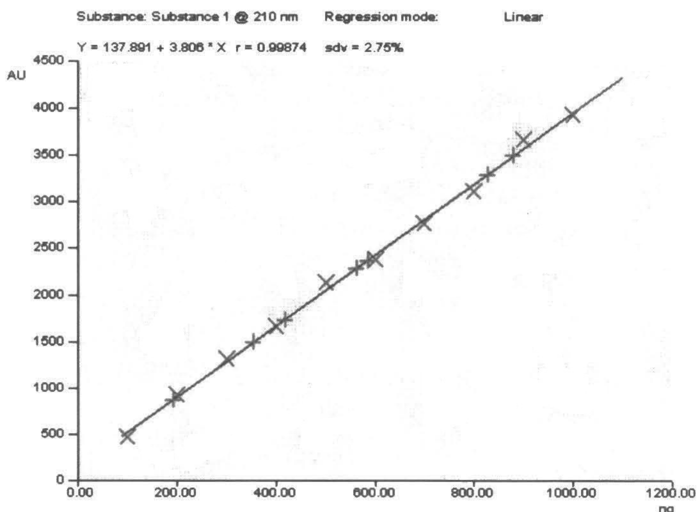

Fig 3. Linear calibration curve obtained for podophyllotoxin

A linear relationship was obtained between peak area and the quantity of podophyllotoxin over the range 100–000 ng, Y = 137.891 + 3.806 *X, r = 0.998, sdv = 2.75% where y is the response and X is the amount of podophyllotoxin (Fig 3). The efficiency of recovery of the compound was in the range 97.10 to 101.2% as shown in Table 1. This indicates the high precision of the method and the non-interference of other compounds. The repeatability of 5 replicates of same sample showed RSD of 1.2%. The limit of detection for podophyllotoxin was 50 ng.

The podophylloresin and podophyllotoxin content in three collections of *Podophyllum hexandrum* from different regions was quantified by the above method (Table 2). Collection VD–25 made from Changu forests of Sikkim at the height of 11000 feet was found to contain maximum content of podophylloresin (6.14%) and podophyllotoxin (4.76%).

Table 2. Podophylloresin and Podophyllotoxin content in *Podophyllum hexandrum* collections by the proposed HPTLC method

Collection number/ Accession number	Site of collection	Podophyllo-resin* (%)	Podophyllo-toxin* (% dry wt basis)
VD-63	Sikkim hills	4.00	1.09
VD-25	Sikkim hills	6.14	4.76
NDGHZ-2400/IC 273896	Bhowali hills	1.84	1.76

*Mean of three replications

CONCLUSIONS

This method is simple, accurate and quick. A large number of samples along with standard can be analysed simultaneously in one TLC plate and solvent requirement is also very negligible, hence making it inexpensive compared to HPLC. In addition it requires very small amount of sample, no sample clean ups and can detect active principle concentration in nanogram level. Thus this method can be conveniently adopted for routine quality control analysis.

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6

Standardisation of Polyherbal Formulations Using Marker Compounds

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ABSTRACT

Through out human history people have relied on drugs derived from plants to promote and maintain good health and to fight sickness, pain and disease. People all over the world have realized that vast plant wealth has still much more to offer in the shape of new, effective and safe remedies accessible to masses. Herbal drugs are having prolonged history of frequent use and documentation in texts of established systems of medicine indigenous to a particular country. However, extremely limited knowledge about the ingredients in the herbal drug formulations and their effects in humans, the lack of stringent quality control and the heterogeneous nature of these preparations all necessitate the continuous monitoring of the safety of the herbal products. Most of the regulatory guidelines for herbal drugs and formulations including WHO guidelines and pharmacopoeias suggest macroscopic and microscopic evaluation and chemical profiling of the botanical materials for quality control. Chemical profiling establishes a characteristic chemical pattern for a plant material, its extracts or formulations. During the last few years a large number of communications on chemical profiling of medicinal plants have appeared but not much work has been reported on polyherbal formulations. In the present communication development of standardization methods for three very common and useful Ayurvedic polyherbal formulations has been described.

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Key words : Polyherbal ayurvedic formulations, standardisation, chemical profiling, sitopladi churna, vasavaleha, hingvatsakachurna

INTRODUCTION

Through out human history people have relied on drugs derived from plants to promote and maintain good health and to fight sickness, pain and disease. While modern (allopathic) medicine has, in many parts of the world, replaced traditional medical practices to the benefit of individual and public health, we are becoming increasingly aware of its limitations, *i.e.* its ineffectiveness in dealing with a large number of conditions and diseases, the often unforeseen negative side effects of synthetic drugs, and the ever-rising costs of medical treatment, including pharmaceuticals. As a result, the public and an increasing number of physicians and public health specialists throughout the world are taking a second look at alternative or complementary medicine generally and traditional plant based drugs in particular. The current resurgence of interest in traditional medicine and plant drugs occurring worldwide, but particularly in the West, is not as revolutionary as it may seem to many. The pharmaceutical industry, for example, has long recognized the therapeutic value of plants that have been used in traditional medicine for centuries. Plants were extracted and pure isolated compounds were produced and eventually synthesized. This eventually led to the development of the pharmaceutical industry where the synthetic approaches to drug design still dominate today, but a large proportion of the pharmaceutical drugs used today are derived from plants or based on the active principles they contain. Numerous plants occurring naturally in India have yielded drugs of major importance of modern medicine. Throughout the world, intensive pharmacological research efforts are underway to screen plants for active compounds and to develop new pharmaceutical products.

The use of drugs of biological origin has increased many folds during the last two decades. People all over the world have realized that vast plant wealth has much to offer in the shape of new and effective remedies which are safe and accessible to masses. The herbal medicines are known to be easily available at low cost, comparatively safe and the people have faith in such remedies too. The additive and synergetic action of poly herbal preparations prevents the side effects of the isolated active compound(s). More over a number of diseases still have no satisfactory cure in modern medicine and certain herbal formulations/traditional medicines are reported to have effective treatment for these ailments. Unfortunately

no serious efforts have been made to verify these claims and to develop validated, standardized herbal formulation(s) for the effective cure or management of these diseases. Although there are many formulations available in the market but neither they are scientifically evaluated nor authenticated or quality controlled. Toxicology or safety data of herbal drugs for all practical purposes is unknown or not available. Further, extremely limited knowledge about the ingredients in the herbal drug formulations and their effects in humans, the lack of stringent quality control and the heterogeneous nature of these preparations all necessitate the continuous monitoring of the safety of the herbal products. Assessment of safety and adverse effects of herbal preparations can be much more complex than the modern pharmaceuticals. The contributing factors to this complexity could be the polyherbal nature of these preparations and unacceptable levels of heavy metals, pesticides/insecticides, microbial load, mycotoxins, presence of adulterants or cheap substituents.

WHO has laid down certain guidelines (1992, 1998) to have uniform approach. Herbal drugs having prolonged history of frequent use and documentation in texts of established systems of medicine indigenous to a particular country with no major side effects reported can be regarded as reasonably safe and no regulatory controls may be needed immediately. The WHO guidelines on herbal/polyherbal formulations cover the following aspects:

A. Quality control of crude drugs material, plant preparations and finished products

B. Stability assessment and shelf life

C. Safety assessment, documentation of safety based on experience or toxicological studies

D. Assessment of efficacy by ethno medical information and biological activity evaluations

The bioactive extract should be standardized on the basis of active principles or major compounds along with the chromatographic fingerprints (TLC, HPLC, HPTLC and GC). The standardization of crude drug materials include the following steps:

1. Authentication (Stage of collection, parts of the plant collected, regional status, botanical identity like phytomorphology, microscopic and histological analysis, taxonomical identity, etc.)
2. Foreign matter (herbs collected should be free from soil, insect parts or animal excreta, etc.)

3. Organoleptic evaluation (sensory characters – taste, appearance, odor, feel of the drug, etc.)
4. Tissues of diagnostic importance present in the drug powder
5. Ash values and extractive values
6. Volatile matter
7. Moisture content determination
8. Chromatographic and spectroscopic evaluation: TLC, HPLC, HPTLC methods will provide qualitative and semi quantitative information about the main active constituents present in the crude drug as chemical markers. The quality of the drug can also be assessed on the basis of the chromatographic fingerprint
9. Determination of heavy metals – *e.g.* cadmium, lead, arsenic, etc.
10. Pesticide residue – WHO and FAO set limits of pesticides, which are usually present in the herbs. These pesticides are mixed with the herbs during the time of cultivation. Mainly pesticides like DDT, BHC, toxaphene, aldrin cause serious side-effects in human beings if the crude drugs are mixed with these agents
11. Microbial contamination – usually medicinal plants containing bacteria and molds are coming from soil and atmosphere. Analysis of the limits of *E. coli* and molds clearly throws light towards the harvesting and production practices. The substance known as afflatoxins will produce serious side-effects if consumed along with the crude drugs
12. Radioactive contamination – Microbial growth in herbals are usually avoided by irradiation. This process may sterilize the plant material but the radioactivity hazard should be taken into account. The radioactivity of the plant samples should be checked accordingly to the guidelines of International Atomic Energy (IAE) in Vienna and that of WHO

The quantitative determination of constituents has been made easy by recent developments in analytical instrumentation. Recent advances in the isolation, purification, and structure elucidation of naturally occurring metabolites have made it possible to establish appropriate strategies for the determination and analysis of quality and the process of standardization of herbal preparations. TLC, HPLC, GC, quantitative TLC (QTLC), and high-performance TLC (HPTLC) can determine the homogeneity of a plant extract. Over-pressured layer chromatography (OPLC), infrared and UV-VIS spectrometry,

MS, GC, liquid chromatography (LC) used alone, or in combinations such as GC/MS, LC/MS, and MS/MS, and nuclear magnetic resonance (NMR), are powerful tools, often used for standardization and to control the quality of both the raw material and the finished product. The results from these sophisticated techniques provide a chemical fingerprint as to the nature of chemicals or impurities present in the plant. A large number of publications on standardizations of herbal medicines have appeared during the last few years (Shaw *et al.*, 2006, Mohapatra *et al.*, 2008). Most of these studies have utilized the modern methods of analysis which include TLC (Wagner *et al.*, 2006; Apers *et al.*, 2006; Bodoki *et al.*, 2005), HPTLC (Shaw *et al.*, 2007; Kaur *et al.*, 2008), HPLC (Cho *et al.*, 2008; Govindrajan *et al.*, 2007; Chopra *et al.*, 2006; Zhang *et al.*, 2005; Sun & Liu, 2007), UPLC (Chan, 2007) and the hyphenated instruments such as GC/MS (Lee *et al.*, 2004), HPLC/MS (Ding *et al.*, 2007), HPLC/NMR.

STANDARDISATION OF FORMULATIONS

Most of the regulatory guidelines and pharmacopoeias suggest macroscopic and microscopic evaluation and chemical profiling of the botanical materials for quality control. Chemical profiling establishes a characteristic chemical pattern for a plant material, its extracts or formulations. In the last two decades there has been an exponential growth in the field of herbal medicine. However further growth is possible only through the development of standardized herbal products with reference to their active phytoconstituents present for commercialization, correct identification and supply of raw material and to avoid adulteration. During the last few years there have been a number of publications on marker based standardization of single herbs and formulations based on them. But no significant progress has been reported for polyherbal formulations. In the present communication development of standardization methods for three very common and useful Ayurvedic polyherbal formulations have been described. The methods were developed as per WHO guidelines.

Following formulations were selected for the studies:

I. Sitopladi churna
Contents (each 1 g contains)

1.	Sitopla (Misri)	=	516 mg
2.	Vamsaro cana	=	258 mg
3.	*Piper longum*	=	129 mg
4.	Ela seed *(Eletaria cardamomum)*	=	64.5 mg
5.	Tvak *(Cinnamomum zeylanicum)*	=	32.5 mg

II. Vasavaleha
Contents (each 10 g contains)

1. *Adhatoda vasica* fresh leaf juice = 7.652 mL
2. Honey = 3.826 g
3. Sarpi (ghrita) cow = 0.956 g
4. *Piper longum* = 0.956 g
5. Misri = 3.826 g

III. Hingvatsaka churna
Contents (each 1 g contains)

1. Dry Ginger (Sunth) = 125 mg
2. *Piper nigrum* (Maricha) = 125 mg
3. *Piper longum (Pippali)* = 125 mg
4. *Trachyspermum ami* (Ajmoda) = 125 mg
5. *Cuminum cyminum* (Savetjiraka) = 125 mg
6. *Carum carvi* (Krsnajiraka) = 125 mg
7. *Ferula asafoetida* (Hingu) = 125 mg
8. Rock salt (Sindhava) = 125 mg

1. Raw Material

The plant materials/Formulations were procured from reputed Ayurvedic company Indian Medicines Pharmaceutical Corporation Ltd, Mohan Nainital, Uttranchal authenticated at the Plant Taxonomy Division of Indian Institute of Integrated Medicine, Jammu. India.

2. Heavy Metal Residue

All the plant materials were tested for heavy metals at the Quality Control and Assurance Division of I.I.I.M., Jammu. Most of the samples were found to have heavy metals well below the permissible limits. Materials with higher levels of heavy metals were rejected.

3. Pesticide Residue

The raw materials were evaluated for pesticidal residues and none were found to contain any of the commonly used pesticides.

4. Microbial Contamination

The raw material as well as the formulations were tested for bioload. The bioload in all the cases was well below the permissible limits. The results of the formulations are given below.

Table 1. Heavy metal residue in raw material (Limits as mg/kg)

S. no.	Sample	Lead as Pb	Cadmium as Cd	Arsenic as As	Mercury as Hg	Copper as Cu	Chromium as Cr	Nickel as Ni
1.	*Piper nigrum* (Kali mirch)	BDL of 0.0001	0.08	BDL of 0.0001	BDL of 0.001	15.5	BDL of 0.003	BDL of 0.006
2.	*Carum carvi* (Kala zeera)	0.42	0.02	0.23	BDL of 0.001	9.93	BDL of 0.003	0.45
3.	*Cuminun cyminum* (Safed zeera)	0.54	0.06	BDL of 0.0001	BDL of 0.001	10.97	BDL of 0.003	1.05
4.	Rock salt	0.07	0.05	BDL of 0.0001	BDL of 0.001	15.39	BDL of 0.003	1.89
5.	*Adhatoda vasica*	BDL of 0.0001	0.08	BDL of 0.0001	BDL of 0.001	15.5	BDL of 0.003	BDL of 0.006
6.	*Trachyspermum ami* (Ajmoda)	BDL of 0.0001	0.45	BDL of 0.0001	BDL of 0.001	BDL of 0.0015	BDL of 0.003	BDL of 0.003
7.	*Cinnamomum zeylanicum* (Tvak)	BDL of 0.0001	0.45	BDL of 0.0001	BDL of 0.001	BDL of 0.0015	BDL of 0.003	BDL of 0.003
8.	*Piper longum* (Pippali)	0.22	0.03	BDL of 0.0001	BDL of 0.001	BDL of 0.0015	BDL of 0.003	BDL of 0.003

Table 1. *Contd.*

S. no.	Sample	Lead as Pb	Cadmium as Cd	Arsenic as As	Mercury as Hg	Copper as Cu	Chromium as Cr	Nickel as Ni
9.	*Eletaria cardamomum* (Ela seed)	BDL of 0.0001	0.84	BDL of 0.0001	BDL of 0.001	BDL of 0.0015	BDL of 0.003	BDL of 0.006
10.	*Ferula asafoetida* (Hingu)	0.18	0.14	BDL of 0.0001	0.63	BDL of 0.0015	BDL of 0.003	0.98
11.	*Zingiber officinale* (Saunth)	0.86	0.13	BDL of 0.0001	BDL of 0.001	15.39	BDL of 0.003	1.88

Permissible Limits As : 0.5 mg/kg (FDA, 1999b) Hg : 1 mg/kg (FDA, 1994) Cd : 0.3 mg/kg (WHO, 1998)
Pb : 10 mg/kg (WHO, 1998) Cr : 120 µg RDI (FDA, 1999c) Ni : 0.1 mg/L (FDA, 1993)

Table 2. Bioload in formulations

S.No.	Sample	Result			
		B_1	B_2	G_1	G_2
1.	Sitopladi choorna Batch no. 1219 dt. April 2006	BDL	BDL	BDL	BDL
2.	Vasavaleha Batch no. 1363 dt. April 2006	BDL	0.11 ppb	BDL	BDL
3.	Hingvashtak choorna Batch no. 1296 dt. April 2006	0.11 ppb	BDL	BDL	BDL

Table 3. Microbial load in Sitopladi churna

S. No.	Result	
1.	Total Bacterial Count (cfu/g) Limits $<5 \times 10^5$	2.3×10^4
2.	Yeast & Mould (cfu/g) Limits $<5 \times 10^3$	2×10
3.	Coliform (cfu/g) Limits $<5 \times 10^3$	$<10^2$
4.	*E. coli* (Limits 10/g)	Absent
5.	*Salmonella* (Limits absent)	Absent
6.	*Staphylococcus* (Limits absent)	Absent
7.	*Pseudomonas* (Limits absent)	Absent

Table 4. Microbial load in Vasavleha

S. No.	Result	
1.	Total Bacterial Count (cfu/g) Limits $<5 \times 10^5$	Nil
2.	Yeast & Mould (cfu/g) Limits $<5 \times 10^3$	Nil
3.	Coliform (cfu/g) Limits $<5 \times 10^3$	Nil
4.	*E. coli* (Limits 10/g)	Absent
5.	*Salmonella* (Limits absent)	Absent
6.	*Staphylococcus* (Limits absent)	Absent
7.	*Pseudomonas* (Limits absent)	Absent

Table 5. Microbial load in Hingvashtak churna

S. No.	Result	
1.	Total Bacterial Count (cfu/g) Limits $<5 \times 10^5$	2×10^5
2.	Yeast & Mould (cfu/g) Limits $<5 \times 10^3$	10
3.	Coliform (cfu/g) Limits $<5 \times 10^3$	Nil
4.	*E. coli* (Limits 10/g)	Absent
5.	*Salmonella* (Limits absent)	Absent
6.	*Staphylococcus* (Limits absent)	Absent
7.	*Pseudomonas* (Limits absent)	Absent

5. Marker Based Standardization: Development of Standardization Protocols

Standardization of Sitopladi *churna* Contents (each 1 g contains)

Sitopla (Misri)	=	516 mg
Vamsaro cana	=	258 mg
Piper longum	=	129 mg
Ela seed	=	64.5 mg
Tvak	=	32.5 mg

Sitopladi churna - Extraction Protocol

The formlation contains Pipali (*Piper longum*), Ella (*Eletaria cardamomum*) and Tvak *(Cinnamomum zeylanicum)*. The main constituent of piper is piperine and the other two plant are mainly essential oil bearing plants. A part of formulation was extracted for estimation of piperine content and the second part was extracted for essential oil determination.

Procedure for determination of piperine content in Sitopladi churna

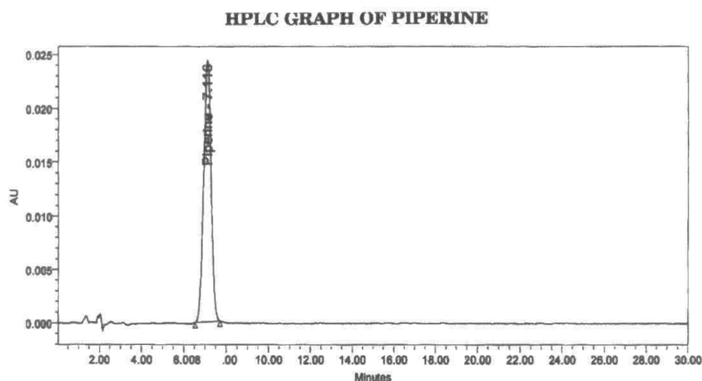
- Take 1 g formulation
- Extract by Soxhlet extraction with $CHCl_3$ (HPLC grade, 100 mL) for 4 h. on steam bath
- Remove chloroform, dissolve residue in methanol
- Make up to 25 mL in MeOH (HPLC grade)
- Filter through Millipore filter 0.45 µm
- Inject 10 microlitre in HPLC

Preparation of standard

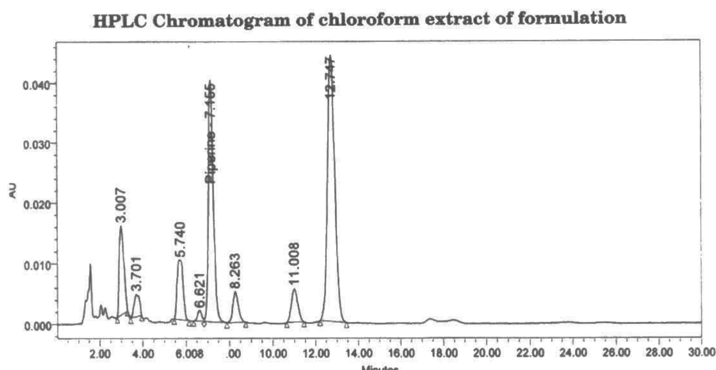
Piperine: 1.2 mg/5 mL methanol HPLC grade from which 5,10,15,20 and 25 µL injected in HPLC system for making standard curve.

Analysis

A Waters HPLC system consisting of two pumps Waters 515 HPLC with Waters pump control module, an automatic sampling unit Waters 717 plus auto sampler, a column oven, a photodiode array detector Waters 2996 and temperature control module II. Waters Empower software was used for data analysis and data processing.



Conditions: Column: RP-18e 5 µm, Column temp. = 30°C
Detector: PDA, λ_{max}: 254 nm. Flow rate: 1 mL/min
Mobile phase: MeOH: Water (70:30)
Run time: 30 min



Determination of Essential Oils

- Take 100 g formulation in a round bottomed flask fitted with a Clevenger apparatus.
- Add 700 mL water into the formulation.
- Take 20 mL hexane (HPLC grade) in Clevenger apparatus.
- Hydro distill for 3 h
- Separate hexane layer
- Distill hexane

RESULTS

Table 6. Piperine concentration (%) estimated by HPLC in Sitopladi churna

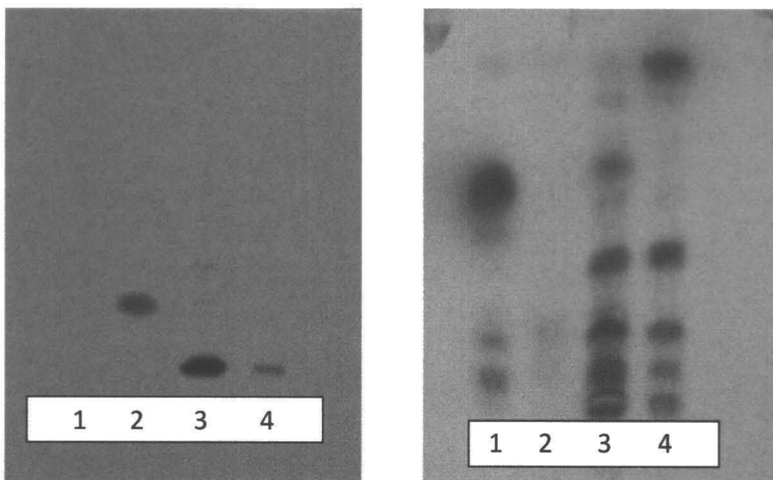
Expt 1	Expt 2	Expt 3	X	SD	CV
0.068	0.065	0.068	0.067	0.00173	2.58

SITOPLADI CHURNA FORMULATION (ESSENTIAL OIL)

Essential oils extractive value of Sitopladi churna ingredients

1. Ela essential oil *(Eletaria cardamomum)* = 2.7733 g from 50 g green illachi
2. Tvak essential oil *(Cinnamomum zeylanicum)* = 1.0339 g from 150 g Tvak bark
3. Pipli essential oil *(Piper longum)* = 0.1172 g from 50 g dried fruits.
4. Sitopladichurna = 0.0871 g from 100 g formulation.

TLC chromatogram of essential oils of Sitopladi churna Formulation and its ingredients



Visualisation under UV at 250 nm

1. Ela
2. Tvak
3. Sitopladi churna
4. *Piper longum*

Visualization by spraying with Cerric ammonium sulfate and heating

TLC plate: Precoated silica gel f254

Developing system: EtOAc: Hexane (1:1)

HPLC grade

GLC FINGERPRINT OF SITOPLADI CHURNA ESSENTIAL OIL

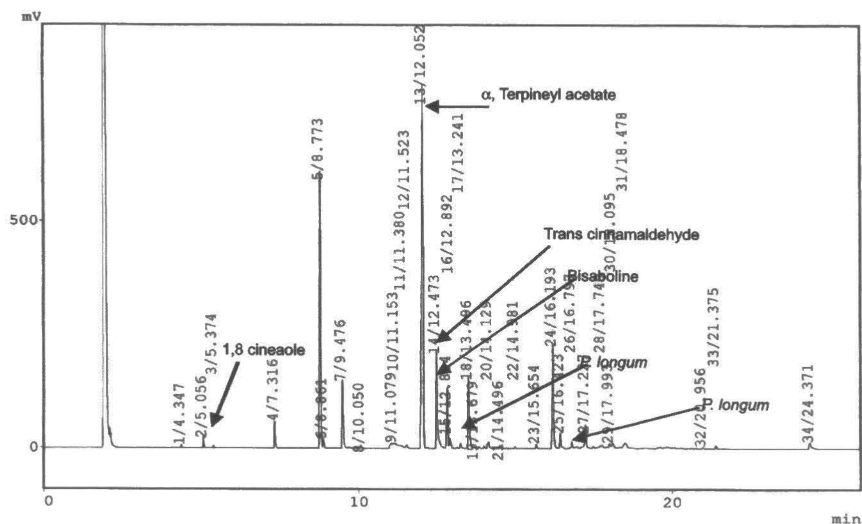
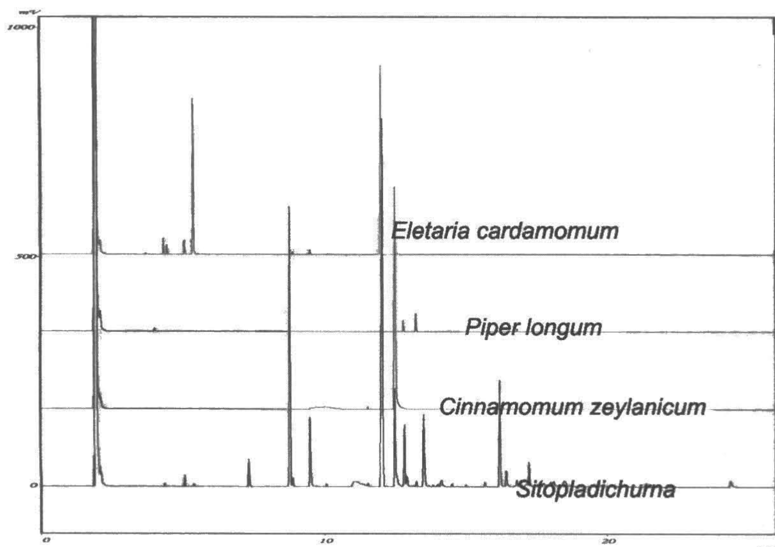


Table 7. GLC results

Retention time (min)	Area percent	Compound	Ingredient
5.374	0.0795	1,8-Cineole	*Eletaria cardamomum*
7.316	1.6514	–	–
8.773	20.4032	–	–
9.476	5.0286	–	–
12.052	35.3413	α-Terpineyl acetate	*Eletaria cardamomum*
12.473	8.0035	Trans cinnamaldehyde	*Cinnamomum zeylanicum*
12.814	4.0880	Bisaboline	*Piper longum*
13.241	0.3408	–	*Piper longum*
13.496	5.7638	–	–
16.193	8.2467	–	–
16.797	0.3570	–	–
17.227	2.1135	–	*Piper longum*

SITOPLADI CHURNA
Super imposable GLC fingerprints



Conditions: Inj. Vol: 0.2 ml GLC: Shimadzu model: GC-17A Column: BP-10 Inj. Temp.-240°C Det temp. 260°C

GCMS ANALYSIS OF SITOPLADI CHURNA OIL

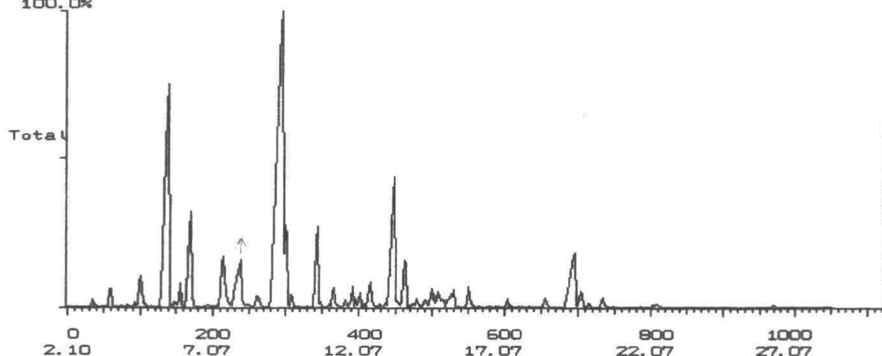


Table 8. Major and identified peaks (Sitopladi churna GCMS)

Retention time (min)	Mass	Area percent	Compound	Ingredient
2.93	136	0.2	Unidentified	*Eletaria*
3.57	154	0.5	Unidentified	*Eletaria*
4.06	136	1.6	Unidentified	*Eletaria*
5.57	152	16.5	Unidentified	–
6.32	136	5.7	α-Terpineol	*Eletaria*
7.42	139	3.6	–	–
8.02	131	4.7	Trans-cinnamaldehyde	*C. zeylanicum*
9.47	181	33.0	α-Terpineol acetate	*Eletaria*
9.60	164	3.9	–	–
10.67	204	3.2	Bisaboline β	*C. zeylanicum*
12.47	206	1.4	–	–
13.27	202	9.2	–	–
13.65	187	2.4	–	–
19.47	223	5.4	–	–

VASAVALEHA

Contents (each 10 g contains)

Adhatoda vasica fresh leaf juice = 7.652 mL
Honey = 3.826 g
Sarpi (ghrta) cow = 0.956 g
Piper longum = 0.956 g
Misri = 3.826 g

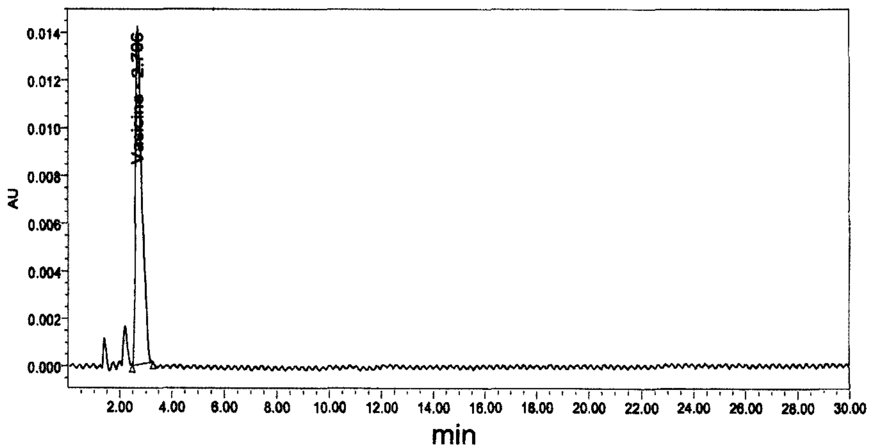
The plant constituents of vasavaleha are *Adhatoda vasica* and *Piper longum*. The active constituents of these plants are vasicine and piperine. Standardisation method was developed for estimation of the compounds in formulation.

Vasavaleha Extraction Protocol

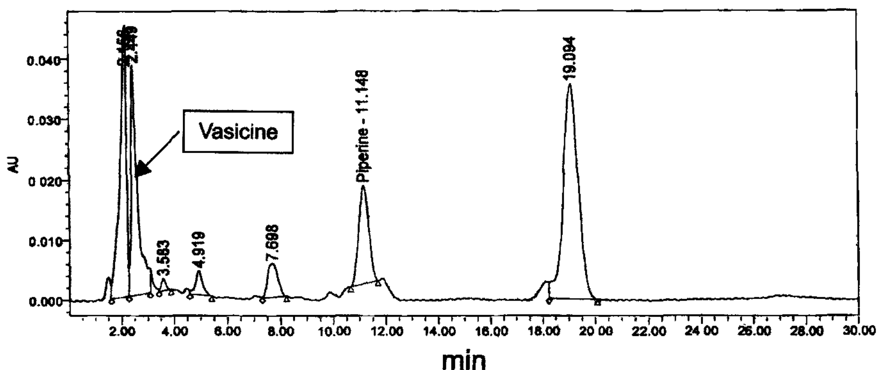
- Take 5 g formulation
- Dissolve in millipore water to total volume 25 mL
- Extract with $CHCL_3$ (4 × 25 mL, HPLC grade)

- Pool all the four extracts
- Desolvnetise
- Dissolve residue in MeOH (HPLC), make up to 25 mL
- Filter through 0.45 μm millipore filter
- Inject in HPLC system for estimation of vasicine and piperine

HPLC GRAPH OF VASICINE



HPLC GRAPH OF VASAVALEH EXTRACT



Conditions:

Column: RP-18 5 μm, Column temp. = 30°C, Detector: PDA, λ_{max}: 254 nm, Flow rate: 1 mL/min, Mobile phase: MeOH: Water (70:30), Run time: 30 min

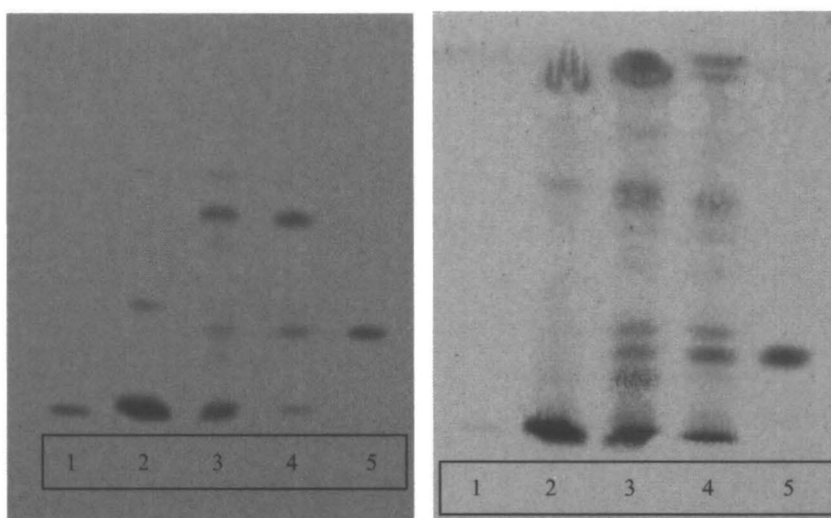
VASAVALEHA - HPLC RESULT

Piperine and Vasicine concentration (%) in Vasavaleha

Table 9. HPLC results based on marker compounds

Marker	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	X	SD	CV
Piperine	0.011	0.011	0.010	0.011	0.010	0.010	0.00054	5.16
Vasicine	0.033	0.030	0.029	0.030	0.027	0.029	0.0216	7.27

TLC chromatogram of $CHCl_3$ extracts of formulation and its ingredients



Visualisation under UV at 250 nm

1. Vasicine
2. *Adhatoda vasica* $CHCL_3$ extract
3. Vasavaleha $CHCL_3$ extract
4. *Piper longum* $CHCL_3$ extract
5. Piperine

Visualisation by spraying with Cerric ammonium sulfate

TLC plate: Precoated silica gel f254

Developing system: EtOAc: Hexane (1:1)

Vasavaleha TLC
(Preparation of extracts for TLC)

Adhatoda vasica	Leaf juice extract (1 g dry wt.), extracted with $CHCl_3$ HPLC (3 × 10 mL). Pooled extract dried and residue reconstituted in 1.5 mL $CHCL_3$.
Piper longum	Seed (1 g dry wt.), extracted with $CHCl_3$ (3 × 10 mL). Pooled extract dried and residue reconstituted in 1.5 mL $CHCl_3$.
Vasavaleha	5 g formulation extracted as per protocol was dried. Residue reconstituted in 1.5 mL $CHCl_3$.

SANDARDISATION OF HINGVATSAKA CHURNA

Contents: Each 1 g contains

1.	Dry Ginger (Sunth)	125 mg
2.	*Piper nigrum* (Marica)	125 mg
3.	*Piper longum (Pippali)*	125 mg
4.	*Trachyspermum ami* (Ajmoda)	125 mg
5.	*Cuminum cyminum* (Savetjiraka)	125 mg
6.	*Carum carvi* (Krsnajiraka)	125 mg
7.	*Ferula asafoetida* (Hingu)	125 mg
8.	Rock salt (Sindhava)	

The formulation was divided into two parts for chemical standardization, one part for essential oils and other for other constituents.

Hingvatsaka churna formulation (Extraction protocol)

1. **For Essential oil**
   - Take 100 g formulation in a round bottomed flask fitted with a Clevenger apparatus
   - Add 700 mL water into the formulation
   - Take 20 mL Hexane (HPLC grade) in Clevenger apparatus.
   - Hydrodistill for 3 h.
   - Separate hexane layer and distill
   - Reconstitute residue in acetone
   - Inject in GLC for estimation of essential oil

2. For other constituents

Formulation was first extracted with ethyl acetate followed by extraction with aq. Ethanol (1:1)

Extraction with ethyl acetate

- Take 1 g formulation
- Extract by Soxhlet extraction with EtoAc for 8 h on steam bath
- Keep marc for subsequent extraction
- Desolventise ethyl acetate extract
- Dissolve residue in MeOH (HPLC grade, mL)
- Filter through millipore filter 0.45 µm
- Inject in HPLC and estimate

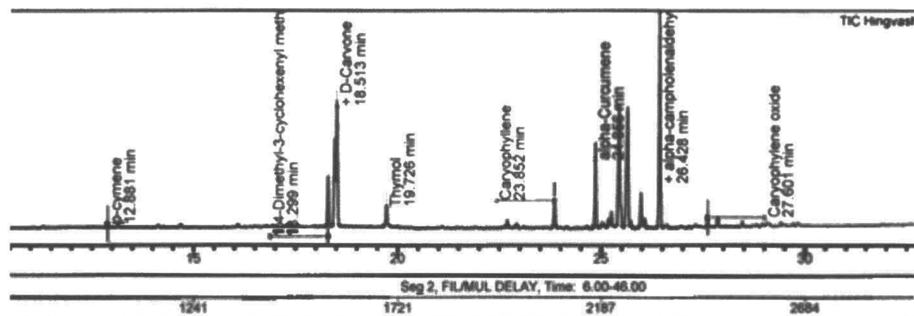
Extraction with aqueous ethanol (1:1)

- Take dry marc in centrifuge tube
- Mix with aqueous ethanol (1:1) 10 ml, sonnicate it for 30 min
- Centrifuge in a refrigerated centrifuge for 10 min at 18°C and 2000 rpm
- Decant supernatant, repeat extraction four times more under similar conditions
- Pool all the extracts and distill off solvents
- Reconstitute residue in HPLC grade MeOH mL
- Filter and inject in HPLC

Essential oils extractive value (g/100 g) in formulation and its ingredients

Hingvatsaka churna	0.3768
Zingiber officinale (dry)	0.6092
Piper longum	0.1172
Piper nigrum	0.1442
Trachyspermum ami	1.1788
Cuminum cyminum	1.9400
Carum species	0.4641
Ferula asafoetida	2.2183

GC/MS GRAPH OF HINGVASHTAK CHURNA OIL



Hingvatsaka churna

Marker based HPLC standardization was done for following plants :

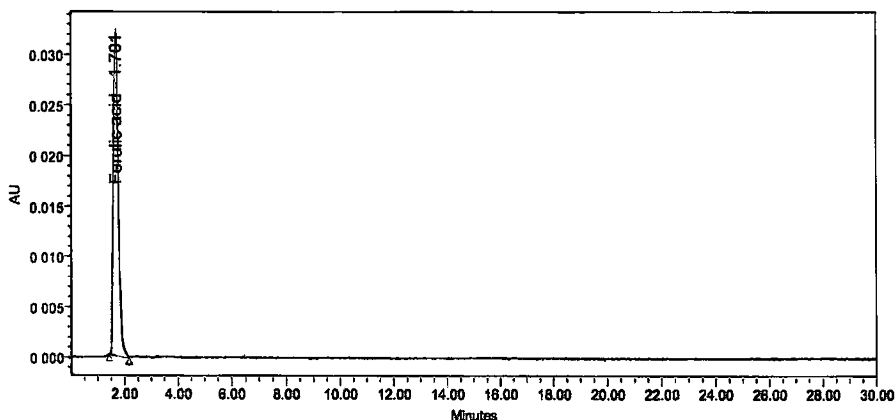
	Name of plant	Marker compounds
1.	*Ferula asafoetida*	Ferulic acid
2.	*Piper longum*	Piperine
3.	*Piper nigrum*	Piperine
4.	*Cuminum cyminum*	Luteolin 7-galacturonide4'-glucoside (K003) Apigenin 7-galacturonide4'-glucoside (K004)
5.	*Piper longum*	Piperlonguminine

Table 10. Major and identified peaks (Hingvashtak churna GCMS)

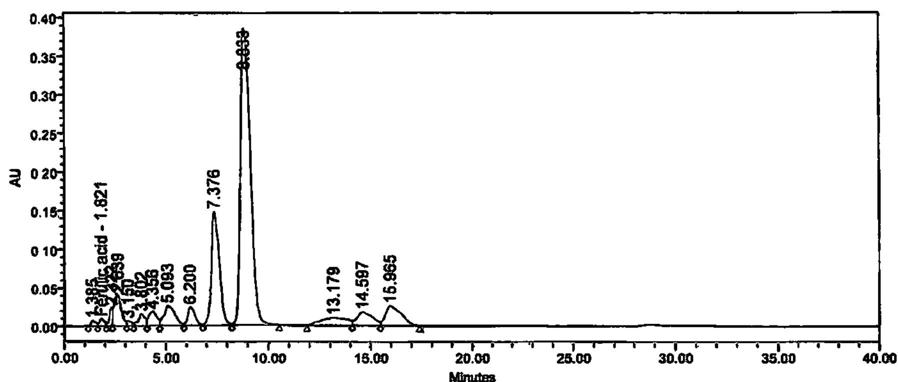
Retention time	Mass	% Total	Marker	Source ingredient
12.88	132	0.9	p-cymene	*T. ammi, C. cyminum, F. asafoetida*
18.29	–	6.7	Unidentified	–
18.45	148	10.72	Cuminal	*Cuminum cyminum*
18.51	150	14.77	Carvone	*Carum carvi*
19.72	150	4.88	Thymol	*Trachyspermum ami*
23.85	204	2.24	Caryophyllene	*P. nigrum, P. longum*
24.85	202	8.75	α curcumene	*Zingiber officinale*
25.43	204	6.54	Unidentified	–
25.634	204	7.21	β bisaboline	*Zingiber officinale*
25.639	204	8.90	Unidentified	–
26.42	152	27.71	Unidentified	–
27.60	220	0.52	Caryophylline oxide	*P. nigrum, P. longum*

Estimation of Ferulic acid

HPLC chromatogram of marker ferulic acid



HPLC chromatogram of EtOAc extract



Condtions

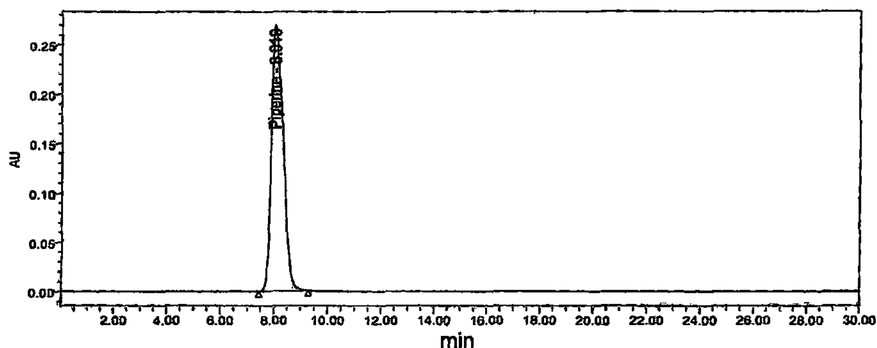
Column: RP-18 5 µm, Column temp. = 300°C,

Detector: PDA, λ_{max}: 340 nm

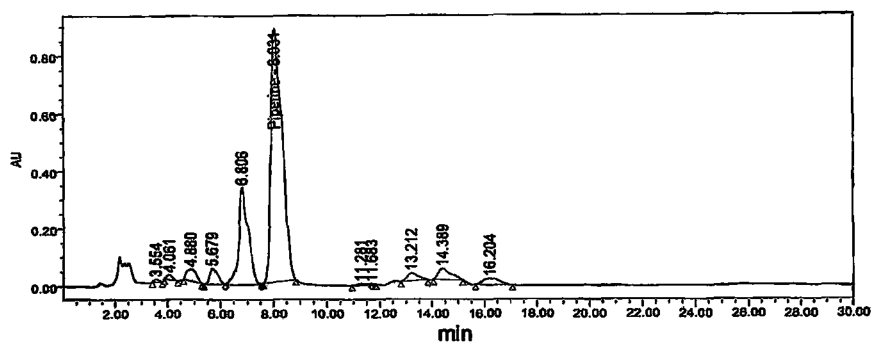
Flow rate: 1 mL/min, Mobile phase: MeOH Water (70:30),

Run time: 30 min

HPLC chromatogram of marker compound piperine



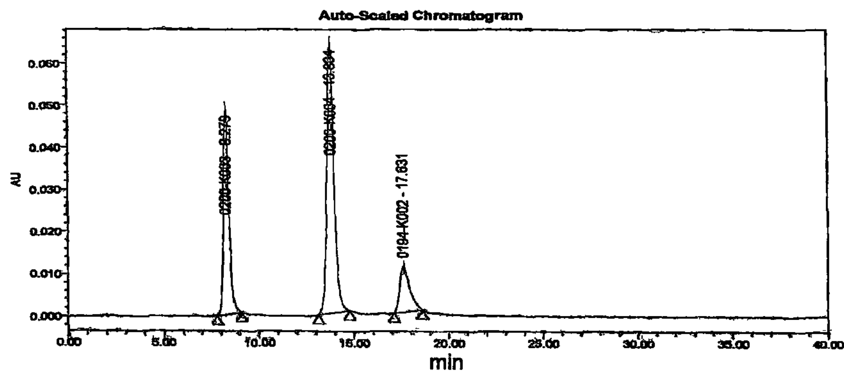
HPLC chromatogram of EtOAc extract for piperine content



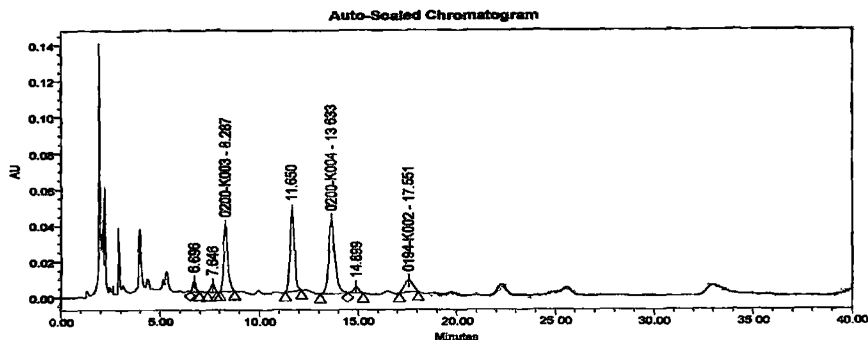
Hingvatsakachurna

HPLC fingerprint: Estimation of Luteolin 7-galacturonide 4'-glucoside (K003) and Luteolin 7-galacturonide 4'-glucoside (K003)

HPLC chromatogram of markers



HPLC chromatogram of hydroalcoholic extract of Hingvatsaka churna



Hingvatsaka formulation

Table 11. HPLC results based on marker compounds

Marker	Expt 1	Expt 2	Expt 3	Expt 4	X	SD	CV
K003	0.08	0.11	0.10	0.08	0.092	0.015	16.3
K004	0.10	0.08	0.13	0.10	0.102	0.020	19.6
Ferulic acid	0.023	0.025	0.026	0.021	0.023	0.002	8.6
Piperine	0.38	0.41	0.37	0.26	0.355	0.065	18.3
Piperlonguminine	0.026	0.026	0.022	0.021	0.023	0.002	8.6

CONCLUSIONS

Standardization and quality control is an essential factor for all herbal medicines. In many cases these medicines are mixtures of several plants. It is very important to establish a system of standardization for every herbal medicine in the market, since the scope for variation in different batches of medicine is enormous. Due to the complex nature and the large number of the herbal preparations, quality standardization is still a difficult task for the regulatory organizations. Plant materials when used in bulk quantity may vary in its chemical content according to different batches of collection *e.g.* collection in different seasons and/or collection from sites with different environmental surroundings or geographical location. It is important that the standards for each formulation as well as its components be set specifying the concentration of some well defined chemical constituents "markers" in the formulations to bring some sort of uniformity in the manufacture of such medicines so as to ensure quality control and quality assurance.

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7

Characteristics Variation of Lavender Oil Produced by Different Hydrodistillation Techniques[†]

G.D. KIRAN BABU[1,*] AND BIKRAM SINGH[1]

ABSTRACT

Lavender (Lavandula angustifolia Mill.) grown in the agro-climatic conditions of western Himalaya was processed for extraction of volatile oil by different hydrodistillation techniques which revealed that water distillation of the herb produced higher yield (1.6%) than that of water-steam distillation (1.1%) and steam distillation (0.9%) methods. The samples were analysed by GC and GC-MS to study and compare the essential oil composition, which revealed that higher monoterpene hydrocarbon (10.05%) and sesquiterpenoid (10.28%) contents were present in the oil produced by steam distillation method followed by water-steam distillation (6.31% and 7.73%) and water distillation (5.62% and 3.18%, respectively) methods. Higher ester content (50.44%) was observed in the oil produced by water-steam distillation followed by steam distillation (41.29%) and water distillation (35.52%). Linalyl acetate, one of the quality determining constituents of lavender oil, was found in higher amount in the oil produced by water-steam distillation (47.1%). The lowest linalyl acetate (30.01%) was recovered in the oil produced by water distillation method followed by steam distillation method (35.28%). On the contrary, the total alcohol content was found to be in higher quantities in the oil produced by water distillation method (52.32%) followed by steam distillation method (35.58%) and lowest in water-steam distillation method (31.86%). These

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variations in the quality of lavender oil produced by different hydrodistillation methods was attributed to the thermal degradations and other chemical reactions such as allylic rearrangements, hydrolysis and elimination reactions of some of the esters and alcohols present. Based on the present experimental conditions, it is recommended that the lavender oil, on commercial scale, may be produced preferably by water-steam distillation method to reduce the by-products formation through above mentioned chemical reactions and to get better oil recoveries. Furthermore, the capital investment for the water-steam distillation unit is much lower than the steam distillation system, as it wouldn't require neither a separate boiler nor a skilled operator.

Key words : Essential oil, hydrodistillation, labiatae, *Lavandula angustifolia* Mill., lavender oil, linalool, linalyl acetate, steam distillation

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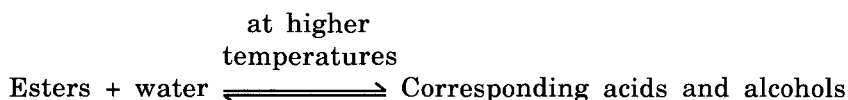
INTRODUCTION

True lavender (*Lavandula angustifolia* Mill. Syn. *L. officinalis* Chainx.) is distributed in the mountainous districts of southern Europe, bordering western half of Mediterranean and the eastern coast of Spain, France, Switzerland, North Italy, Corsica and north Africa. About twenty years back, an effort was made to cultivate lavender on large scale in Kashmir valley, India to meet the requirements of Indian market. However, commercial production did not increase due to political disturbances (Vaze, 2000). India imports about 100 tons lavender oil annually (Shawl *et al.,* 2005). In this regard, Institute of Himalayan Bioresource Technology, Palampur, initiated the extension of the plantation and commissioned distillation units in district Chamba, Himachal Pradesh, India with an objective to produce sufficient quantities of lavender oil for meeting the industry requirement. Lavender oil, obtained from the flowering tips, is used in aromatherapy and has carminative, anti-flatulence, and anti-colic properties (Lis-Balchin & Hart, 1999). The primary use of lavender oils, however, is as raw ingredients in industrial perfume and fragrance materials, with the bulk of this market filled by lavandin oil (McGimpsey & Porter, 1999). Lavender oil is characterized by high levels of linalool and linalyl acetate, moderate levels of lavandulyl acetate, terpinen-4-ol and lavandulol.

Inadequacies in Hydrodistillation

Hydrodistillation of essential oils suffers several limitations in isolating and preserving the composition of natural fragrances. It

causes degradation of thermally labile compounds and hydrolysis of esters. During hydrodistillation, esters present in essential oils are generally hydrolyzed by the release of H^+ ions from water. In the presence of water, at about its boiling point, esters hydrolyze to the corresponding acids and alcohols.



The hydrolysis of esters in geranium oils during hydrodistillation has been discussed extensively by Babu and Kaul (2005). Major ester present in the lavender oil is linalyl acetate, an important quality determining constituent (Wiesenfeld, 1997). Due to high temperatures and unfavorable conditions prevailing during the hydrodistillation process, esters also undergo molecular rearrangements and elimination reactions leading to degradation of molecules. Pickett *et al.* (1975a) examined a model system in which pure linalyl acetate was subjected to steam distillation with pH 4. The composition of the steam distillate was found to contain myrcene (3.7%), limonene (1%), *cis*-ocimene (1.7%), *trans*-ocimene (3.3%), α-terpineol (19.7%), neryl acetate (4.3%), geranyl acetate (5.3%), nerol (1.7%) and geraniol (7%). Linalyl acetate has been found to readily rearrange *in vitro* even when hops are distilled at pH 7. Linalyl acetate was undetectable in oil obtained by steam distillation of hops; whereas it can be easily be detected in oil produced by vacuum steam distillation at room temperature (Pickett *et al.*, 1975b).

Linalyl acetate undergoes thermal degradation during steam distillation, leading to linalyl, geranyl, and neryl carbocation, which induces considerable rearrangement reactions (Fig 1). Hydration of previously formed hydrocarbons yields corresponding alcohols (Morin & Richard, 1985). In plants containing high proportions of linalyl acetate, it is not surprising to encounter recemization of linalool produced during ester thermal degradation in the steam distilled oil (Casabianca *et al.*, 1998). During hydrodistillation, acidic conditions (pH=5.5-6.5) promote the decomposition of linalyl acetate and linalool (Boelens & Sindreu, 1998). For example, linalyl acetate can be converted by allylic rearrangement into geranyl acetate and neryl acetate and by elimination into acyclic monoterpene hydrocarbons such as myrcene, *cis*- and *trans*-β-ocimenes (Fig 2). Linalyl acetate is hydrolyzed in considerable amounts during prolonged steam distillation and under the same conditions it also produces monocyclic monoterpenes hydrocarbon – terpinolene – as the main product from linalyl acetate *via* the α-terpinyl cation and

terpinen-4-yl cation (Fig 3). α-Terpineol and then 1,8-cineole can be formed by hydration of the α-terpinyl cation. So also terpinene-4-ol can be formed by the hydration of the terpinene-4-yl cation. Linalool also showed similar rearrangement and elimination reactions under such conditions. As mentioned earlier, esters are hydrolyzed to their corresponding alcohols and carboxylic acid. Satoh (1987) reported the hydrolysis of linalyl acetate catalyzed by carboxyl esterases yielding linalool, which further produced α-terpineol through allylic rearrangement (Fig 4).

Lavender oil extracted by supercritical CO_2 was observed to possess lower concentration of these by-products formed by above mentioned chemical reactions (Sharma *et al.*, 2008). However, conventionally lavender oil is being produced by steam distillation on commercial scale. There is no report which compares variations in the lavender oil yields and its chemical composition produced by different methods of hydrodistillation *viz.* water distillation, water-steam distillation and steam distillation. Theoretically, there is no fundamental difference between these three methods. However, there are certain variations in practice and the practical results obtained, in some cases have considerable variations because of above discussed chemical reactions occurring during distillation and the same are reported in the present investigation. Therefore, the present investigation is aimed at producing the lavender oils by different hydrodistillation techniques to evaluate the variations in the qualitative and quantitative characteristics. These studies are required to obtain better quality and yield of lavender oils and to reduce the capital investment and recurring costs.

MATERIALS AND METHODS

Plant Material

A plantation of *L. angustifolia* is maintained at Mountain Agriculture Research and Extension Station, Choudhary Sarvan Kumar Himachal Pradesh Krishi Vishwavidhyalaya (CSKHPKV), Salooni, district Chamba, Himachal Pradesh, India. The shoot or flower biomass was harvested in the month of June 2003 from one year old plantation. The experimental location experiences a cold-dry climate, at an altitude of 1800 m above mean sea level (amsl) in the western Himalayas.

Distillation

Water distillation: Two kg flowering tops (spikes) of *L. angustifolia* were charged into a vessel connected to Clevenger-type apparatus

along with 4 l of water to distil by water distillation method. This method involves distilling the essential oil yielding herbs by keeping the biomass in direct contact with the water. The spikes were distilled for 3 h to isolate the essential oil completely. The oil isolated was measured, dried over anh. Na_2SO_4, filtered and analyzed.

Water-Steam distillation: The design and operating procedure of HerboStill™ used for distilling the lavender spikes were described in earlier communications (Babu *et al.*, 2002, 2004, 2005, 2007; Rawat *et al.*, 2007; Babu & Kaul, 2007). Eight kilogram lavender spikes were charged over a false bottom/perforated grid in the HerboStill™, which was adjusted to accommodate 8 l water at the bottom of the vessel to distil the spikes by water-steam distillation method. This method involves, distilling the plant material which was not in direct contact with the water. A distance of 50 mm was maintained between the plant charge and the water. The vessel of the apparatus was heated on liquefied petroleum gas (LPG) to commence the distillation. The water vapor generated below the false bottom was always in the form of saturated steam and never superheated. This saturated steam was used to distil the plant material. The vapors started forming after 15–20 min, were condensed in a condenser and the distillate along with oil was collected in a glass receiver. This process was continued for 3 h. The oil was separated, measured, dried over anh. Na_2SO_4, filtered and used for analysis.

Steam Distillation: Two hundred kg flowering tops of the crop were charged over a perforated grid (false bottom) in a commercial scale stainless steel (SS) distillation tank which was connected to a steam boiler. The herbage was steam distilled by feeding the steam at the bottom of the false bottom in the tank. The steam, evolved through the plant material consisting of water and essential oil, was condensed in a water cooled shell and tube heat exchanger and collected in a receiver/separator. The excess distillate (hydrosol) collected in the receiver was removed continuously from the receiver/ separator and the whole process was continued for 4 h. At the end, 1800 mL oil collected in the receiver was separated.

Gas Chromatography (GC)

GC analysis of essential oil samples was performed on Shimazdu Gas Chromatograph GC-2010 fitted with BP-20 capillary column (SGE International, Ringwood, Australia) length 30 m, internal diameter 0.25 mm, film thickness 0.25 µm using nitrogen as auxiliary carrier gas with flow rate 4 mL/min, equipped with FID. Temperature was programmed from 40-220°C at 4°C/min, held isothermally at 40 and 220°C for 5 min each. Sample injection volume, 2 µl; injector and detector temperatures were kept at 220°C and 250°C, respectively.

Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS (70 eV) data were measured on MS-QP – 2010 series Shimadzu, Tokyo, Japan equipped with MSD, AOC – 20i auto-sampler and BP-20 capillary column (SGE International, Ringwood, Australia) 30 m length, 0.25 mm *i.d.* and film thickness, 0.25 µm (polyethylene glycol). The oven temperature was programmed as mentioned in GC. MS source temperature, 200°C; interface temperature, 220°C, injector and detector temperatures, 220°C. Sample injection volume, 2 µl; split ratio, 1:50 and Mass Scan, 50–600 amu. Helium was used as a carrier gas with 1.1 mL/min flow rate.

Identification

A mixture of *n* alkanes (C_8-C_{32}) was used as reference in calculation of relative retention indices. Identification of constituents was carried out with the help of retention indices and by comparison of mass spectra with the data available in the literature (Adams, 1995; Jennings & Shibamoto, 1980; MacLafferty, 1989), National Institute of Standards and Technology (NIST) (Stein, 1990) and our own created libraries.

RESULTS AND DISCUSSION

Variation in Lavender Oil Yields

Water distillation produced higher oil yield with 1.6% (32 mL from 2 kg) closely followed by water-steam distillation with 1.1% oil yields (88 mL from 8 kg). However, steam distillation produced lowest oil yields with 0.9% (1800 mL from 200 kg). This trend was also observed earlier, in the case of geranium oil production (Babu & Kaul, 2005). The lower oil yields in the steam distillation may be attributed to the conditions prevailed during distillation such as lump formation and agglutination of plant material and not recycling of hydrosol to recover the dissolved oil, which caused incomplete recovery of the oil.

Variation in Lavender Oil Composition

The major constituents characterized in the oil were myrcene, 1,8-cineole, (*Z*)- and (*E*)-*β*-ocimene, linalool, linalyl acetate, *β*-caryophyllene, lavandulyl acetate, *α*-terpineol, neryl acetate, geranyl acetate and geraniol. Lavender oil produced by water distillation method was found to be richer in linalool (39.74%), *α*-terpineol (8.01%), geraniol (2.78%), geranyl acetate (2.16%) and neryl acetate (1.25%). The oil produced by water-steam distillation method contained higher percentage of linalyl acetate (47.1%), 1,8-cineole (2.3%), *α*-pinene

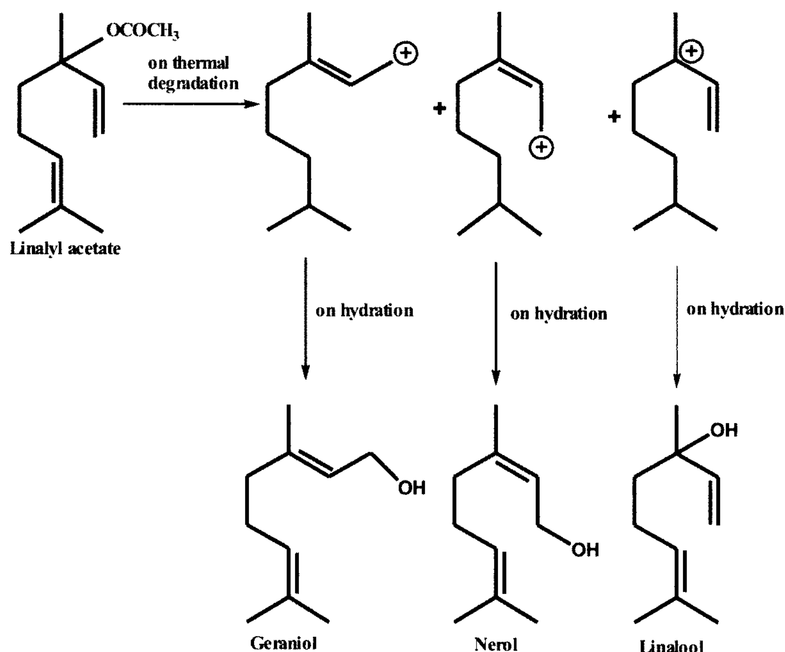


Fig 1. Thermal degradation of linalyl acetate

(0.29%), δ-3-carene (0.28%), β-pinene (0.2%) and sabinene (0.14%). The oil produced by the steam distillation method contained higher amounts of β-caryophyllene (6.27%), (*E*)-β-ocimene (3.03%), myrcene (2.03%), (*Z*)-β-ocimene (2.28%). The composition of oil recovered from the hydrosol collected during steam distillation was also analysed and compared with direct oil and reported elsewhere (Babu & Singh, 2007).

Higher monoterpene hydrocarbons (10.05%) and sesquiterpenoids (10.28%) content were observed in the oil obtained by steam distillation method followed by water-steam distillation method (6.31% and 7.73%) and water distillation method (5.62% and 3.18%, respectively). This may be attributed to the non-polar nature of the constituents possessing strong lipophillic bondages with the fatty (oil) components (Koedam *et al.*, 1979), which require higher energy to break the bondage (Babu & Kaul, 2007). This energy can be met by the higher enthalpy of the steam which is generated in a separate boiler and delivered at a higher pressure in the distillation tank during steam distillation method. The steam at higher pressure also easily distills the higher boiling point constituents such as sesquiterpene hydrocarbons. Hence, the steam distilled oil possessed higher

monoterpene hydrocarbons and sesquiterpenoids. Contrary to this, water distillation (5.62%) and water-steam distillation (6.31%) methods produced oils having lower monoterpene hydrocarbons and sesquiterpenoids, as the steam generated in the still is always saturated and never be superheated, which possess lower enthalpies. The highest monoterpene cyclic ethers were found in the oil produced by water-steam distillation method (2.66%) closely followed by water distillation method (2.29%) and steam distillation method (1.43%). Out of 2.66% of monoterpene cyclic ethers (in the oil produced by water-steam distillation method), 1,8-cineole was found to be the major component (2.3%).

Higher total ester content (50.44%) was also observed in the oil produced by water-steam distillation method followed by steam distillation method (41.29%). The lowest total ester content was observed in the oil produced by water distillation method (35.52%). The major esters present in all the oil samples produced by these different hydrodistillation techniques were linalyl, lavandulyl, neryl and geranyl acetates. Linalyl acetate is one of the most important ester which determines the quality of lavender oil. The oil produced by water-steam distillation method possessed highest linalyl acetate (47.1%). The lowest linalyl acetate (30.01%) was found in the oil produced by water distillation method followed by steam distillation

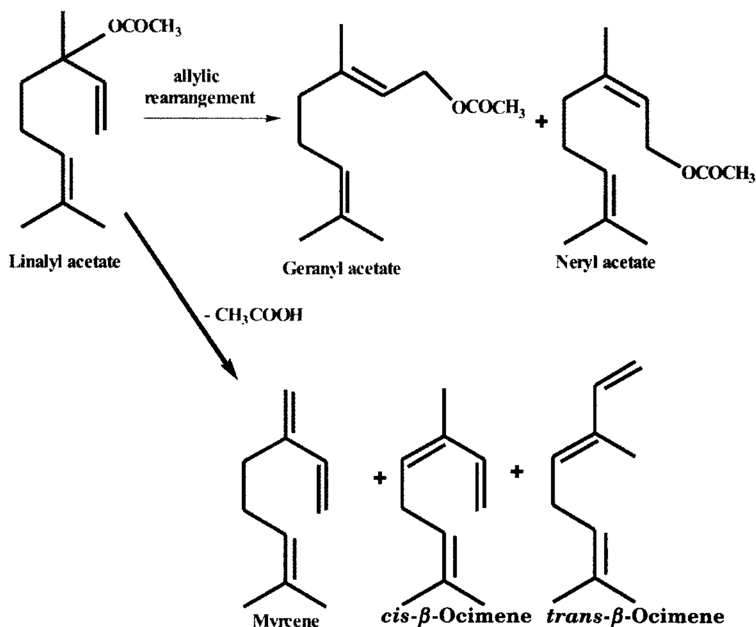


Fig 2. Rearrangement of linalyl acetate during distillation

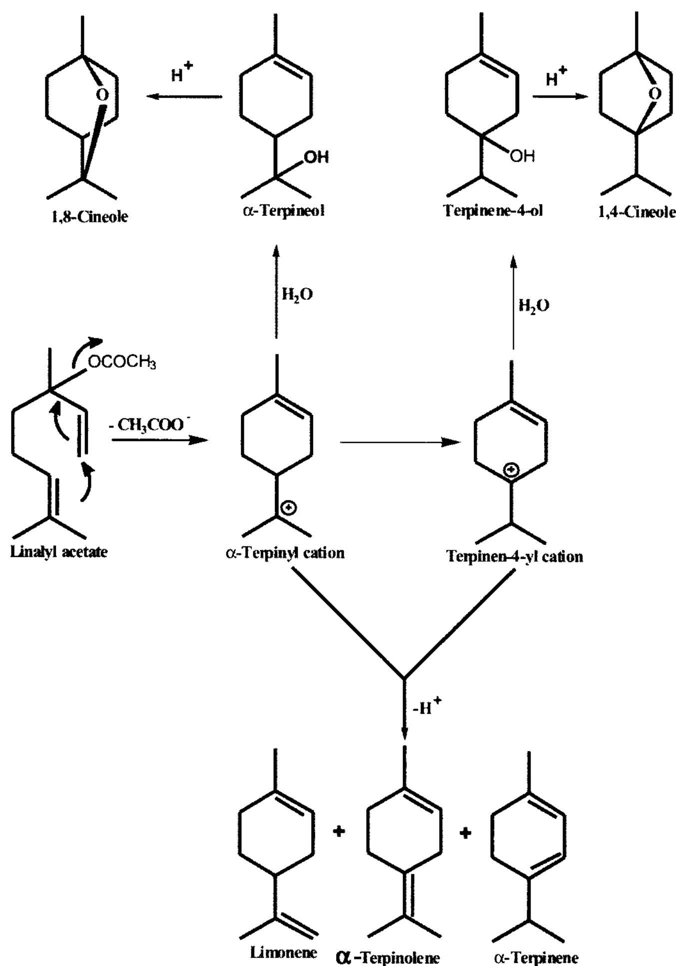


Fig 3. Hydrolysis of linalyl acetate

method (35.28%). On the contrary, neryl acetate and geranyl acetate were found to be higher in the water distillation method (1.25% and 2.16%) and steam distillation (1.1% and 1.97%) than the water-steam distillation method (0.51% and 0.8%, respectively). This can be attributed to the fact that the linalyl acetate under acidic conditions gets rearranged into neryl and geranyl acetates (Fig 2) (Mastelic, 2000).

In addition to experiments described above, the spent water (generally brown in color) discharged from the vessels after completion of the distillation process was tested for its acidity. The pH in the tank during water distillation and steam distillation process was recorded as 5 and 5.5, respectively. However, the spent water in

the water-steam distillation tank possessed pH=6.8. This variation in the pH can be correlated to the extent of these chemical reactions that had taken place during distillation. The water content in the still during water distillation method is generally kept high as the plant material has to be dipped/immersed into the water. Although, initially there was no water present in the tank during steam distillation, as distillation proceeds, steam gets condensed through heat losses and the level of water content increases. Higher the water contents in the vessel, higher the degraded product formation as suggested by the reversible reaction presented in the introduction part. Hence, the pH of spent water in water distillation and steam distillation methods possessed higher acidity (pH is 5 and 5.5, respectively). However, in the case of water-steam distillation method, the water was kept below the false bottom and the level was maintained constant through out the process, which helps in keeping the pH near neutral.

Contrary to the total ester content, the total alcohol content was found to be higher in the oil produced by water distillation method (52.32%). The lowest total alcohol content was observed in water-steam distillation method (31.88%) followed by steam distillation method (35.58%). Similar trend was also observed in the distillation of geranium oil due to the hydrolysis of esters discussed elsewhere (Babu & Kaul, 2005). Therefore, it can be concluded that the more water content in the distillation tank/vessel, the more hydrolysis of esters takes place, thereby increasing the total alcohol content and acidity as the reversible reaction carry forward (towards right hand-side/product side) until the equilibrium is established. The products formed during hydrolysis of esters, for example linalyl acetate, can be divided into two categories (i) oxygenated monoterpenes and (ii) non-oxygenated monoterpenes. The oxygenated monoterpenes for example 1,8-cineole, α-terpineol and terpinene-4-ol are formed by hydration of the α-terpinyl cation and terpinene-4-yl cation. Similarly, the non-oxygenated monoterpenes *viz.* terpinolene, limonene and α-terpinene were formed through the same cations (intermediates) but

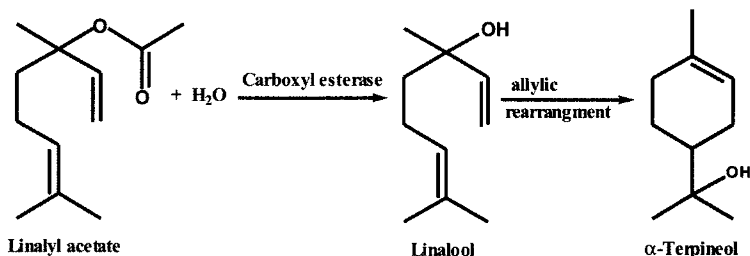


Fig 4. Hydrolysis of linalyl acetate by carboxyl esterase

Table 1. Lavender oil distilled by different hydrodistillation techniques

Compounds	RI#	Water distillation	Water-steam distillation	Steam Distillation
α-Pinene	1011	0.19	0.29	0.16
Camphene	1049	0.20	0.31	0.35
β-Pinene	1114	0.13	0.20	0.14
Sabinene	1123	0.13	0.14	0.12
δ-3-Carene	1149	0.19	0.28	0.16
Myrcene	1162	1.10	0.97	2.03
Limonene	1203	0.72	0.92	1.10
1,8-Cineole	1211	1.83	2.30	1.43
(Z)-β-Ocimene	1235	1.12	1.39	2.28
(E)-β-Ocimene	1252	1.47	1.50	3.03
p-Cymene	1269	0.14	0.17	0.30
α-Terpinolene	1283	0.23	0.14	0.38
1-Octen-3-yl acetate	1383	0.73	0.67	0.90
cis-Linalool oxide	1444	0.28	0.20	—
trans-Linalool oxide	1472	0.18	0.16	—
Camphor	1509	0.26	0.25	0.23
Linalool	1557	39.74	26.06	28.78
Linalyl acetate	1564	30.01	47.10	35.28
α-Santalene	1567	—	—	0.74
Bornyl acetate	1578	0.24	0.14	0.24
α-*trans*-Bergamotene	1584	—	0.16	0.25
β-Caryophyllene	1590	1.27	5.30	6.27
Terpinen-4-ol	1602	0.79	0.72	0.53
Lavandulyl acetate	1611	1.13	1.22	1.80
α-Humulene	1659	—	0.19	0.27
(E)-α-Farnesene	1666	1.02	1.38	1.64
Lavandulol	1682	—	0.42	0.47
α-Terpineol	1699	8.01	3.55	3.78
Neryl acetate	1730	1.25	0.51	1.10
γ-Cadinene	1747	—	0.13	0.19
Geranyl acetate	1760	2.16	0.80	1.97

Table 1. *Contd.*

Compounds	RI#	Water distillation	Water-steam distillation	Steam Distillation
Nerol	1805	1.00	0.29	0.56
Geraniol	1854	2.78	0.82	1.46
Caryophyllene oxide	1971	0.61	0.44	0.69
τ-Cadinol	2169	0.28	0.13	0.23
Total	—	99.19	99.25	98.86
Monoterpene hydrocarbons	—	5.62	6.31	10.05
Monoterpene cyclic ethers	—	2.29	2.66	1.43
Carbonyls	—	0.26	0.25	0.23
Esters	—	35.52	50.44	41.29
Alcohols	—	52.32	31.86	35.58
Sesquiterpenoids	—	3.18	7.73	10.28
Oil yields (%)	—	1.60	1.10	0.90

#RI = retention indices on BP-20 column; —, absent

by eliminating the proton (Fig 3). Linalool also shows similar reactions of rearrangement and elimination under the same conditions as well (Mastelic *et al.*, 2000). Therefore, linalool by allylic rearrangement gives rise to geraniol and/or nerol and so on.

Therefore, depending upon the conditions prevailed in the distillation tanks, the reactions progressed and different products of varying quantities were formed. For example, neryl acetate (0.51%) and geranyl acetates (0.8%) were found minimum in the water-steam distillation and maximum content was found in water distillation (1.25% and 2.16%, respectively). This excess amount of neryl and geranyl acetates might have formed by the allylic rearrangement of linalyl acetate (Fig 2). Hence, the conditions prevailing during water distillation favored the formation of neryl and geranyl acetates. Similarly, allylic rearrangement of linalool also more favored in water distillation and steam distillation as higher nerol (1% and 0.56%, respectively) and geraniol (2.78% and 1.46%, respectively) contents were found than in the water-steam distillation (0.29% and 0.82%, respectively). Similarly, elimination (of CH_3COOH or CH_3COO^- from linalyl acetate and OH or O^- from linalool) reactions were also favored in water distillation and steam distillation as the formation of

myrcene, α-terpinolene and α-terpineol were found in higher concentration than in the oil produced by water-steam distillation. The formation of acyclic monoterpene hydrocarbons *viz*. (*Z*)- and (*E*)-β-ocimene (2.28% and 3.03%, respectively) were very high in steam distillation method whereas the conditions might not be favorable in water distillation as they are in lower quantities (1.12% and 1.47%, respectively). Similar trends were also observed in the case of limonene. The formation of linalool from linalyl acetate is higher in the water distillation method than in the water-stream distillation. Hence, the oil produced by water distillation method contained higher amount of linalool (39.74%) and lower content of linalyl acetate (30.01%) in contrary to the higher content of linalyl acetate (47.1%) and lower content of linalool (26.06%) in the water-steam distillation method.

CONCLUSIONS

It is well known that conventionally the lavender oil is being steam distilled on commercial scale. However, the present experimental findings reveal that water-steam distillation method produced higher yields and better quality lavender oil in terms of higher ester content than the steam distillation method. In general, the by-products content formed by different chemical reactions were higher in water distillation and lower in water-steam distillation method followed by steam distillation method. Therefore, it is recommended that the lavender oil, on commercial scale, may be produced preferably by water-steam distillation method to reduce the by-product formation by various chemical reactions and to get better oil yields. Furthermore, the production cost can be reduced as water-steam distillation doesn't require steam generator, skilled operator, large space for unit installation etc.

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Quality Control Methods for Herbal Medicines

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ABSTRACT

Quality control activities in industry and practice are fundamental in maintaining the safety and efficacy of complementary medicines for customer and patient usage. Internationally, however, there is still great variation and inconsistency of active components in herbal medicines. In order to address these issues, there is an urgent need to develop internationally recognised facilities and methods of quality control for herbal medicines. This review covers the multiple interdisciplinary nature of quality control, including study of traditional Chinese medicine practises, the development in pharmacognosy and the respective analytical methods for fingerprint and quality standardisation of herbal medicines. A combination of these analytical methods will therefore provide a quality control framework for the evaluation of all herbal medicines.

Key words : Capillary electrophoresis, chemometrics, fingerprinting, herbal medicines, high performance liquid chromatography, liquid chromatography mass spectrometry, pharmacognosy, quality control, thin layer chromatography, traditional Chinese medicines

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INTRODUCTION

The prevalent use of herbal medicines has raised concerns of their quality, efficacy and safety due to their ready availability. Many herbal products are sold as over-the-counter medicines and are often used as a decoction of a mixture of herbal materials defined in a formula. Thus, the clinical application of a particular herbal medicine consists of the synergistic effect of multiple chemical components. In this case, the pharmaceutical approach of analysing a single component can not be applied in discerning the quality of a herbal preparation. Thus, quality control methods which reflect the holistic approach of complementary medicine have to be developed in order to determine the chemical basis of herbal medicines (Liang *et al.,* 2004). Incorrect preparation and usage of herbal medicines can potentially cause serious life-threatening effects. Toxic herbs need to be regulated, processed and monitored appropriately. Many homeopathic herbs such as Deadly nightshade (*Atropa belladonna*) contain leaves and berries that are extremely toxic. This is attributed to atropine (a terpene alkaloid). In correct application, *A. belladonna* is a powerful antispasmodic in intestinal colic and spasmodic asthma (Chevallier, 1996). Aconite (*Aconitum napellus*) contains highly toxic aconitine alkaloids and requires special processing and decoction procedures. It is used to diminish the pain of neuralgia, pleurisy and rheumatism (Chevallier, 1996). Herbal medicines have been reported to contain heavy metals and synthetic prescription or non-prescription drugs. They may originate from mineral components, contamination and adulteration (Ernst, 2002). Heavy metals found in large quantities include lead, mercury and/or arsenic from Ayurvedic medicine (Saper *et al.,* 2004). Adulterants in herbal medicines is a serious issue as it can cause poisoning, especially if patients are on similar medications or unknowingly have drug interactions. Plant samples in the Indian market are stored under undesirable conditions over many years and often contain a mixture of unidentified plant species (Dubey *et al.,* 2004).

There is also the danger of misidentification and substitution of plant species. Some species of plants with similar Chinese names differ in their indication and toxicity and cannot be used interchangeably. *Aristolochia fangchi* (Guang fang ji) and *Stephania tetrandra* (Han fan ji) have similar names and clinical indications in Traditional Chinese Medicine (TCM), but *Aristolochia fangchi* contains the highly toxic aristolochic acids which are carcinogenic (Nortier & Vanherweghem, 2002). In a manufacturing error, *Stephania tetrandra* was inadvertently replaced by *Aristolochia fangchi* in weight-loss pills, causing urothelial carcinoma in patients (Nortier *et al.,* 2000). Herbs of high toxicity should be banned or, when necessary, should be used

with special caution, at appropriate dosages, by highly qualified practitioners. Thorough and well documented quality control procedures of herbal plants throughout their production into therapeutic medicines are required to guarantee their identity, consistency and authenticity.

Currently, a limiting factor in the quality control of herbal medicines is the quantitative determination of a single active compound, marker compound or a class of active components (*e.g.* hypericins, total flavonoids). The standards do not reflect the quantitative range of individual active components and the synergistic nature of multiple active components, in the context of traditional or clinical application (Evans *et al.,* 2002). Quality control involves correct identification of a plant with reference to its accepted scientific name(s), good agriculture practice (GAP), good harvesting and processing practice, good manufacturing practice (GMP) and good clinical practice procedures. Pharmacognostic (including comparison with published descriptions or with authentic samples), chemical and physical methods have been employed to define and control the quality of herbal medicines and include the following specific methods: variety identification (plant taxonomy), macroscopic identification (morphology), microscopic identification (anatomy), physical and chemical analysis, quality evaluation with quantitative analysis of active components, biological evaluation and clinical evaluation. Thus, the complexity of a herb requires a comprehensive assessment of the chemical constituents (phytochemicals), in the form of a fingerprint which will enable reliability of product and reproducibility of clinical effects.

It is vital that the fingerprint of a herbal sample is compared to a published reference product, ideally one that has therapeutic viability. Documented quality standards enable specification for dry medicinal plants and their products. Standards of common herbs can be found in Commission E Monographs (Blumenthal *et al.,* 1998), British Herbal Pharmacopoeia (1996), Pharmacopoeia of the People's Republic of China (2005), guidelines from The European Agency for the Evaluation of Medicinal Products (2001) and Australian Therapeutic Goods Administration (TGA) (2004). However, pharmacopeial methods show many inconsistencies of the active component(s) and many procedures have no international uniformity, lack complete characterisation of the herbal species or are simply out-dated.

Thus, we have proposed a multiple disciplinary platform (Fig 1) for the quality evaluation of traditional medicines entailing three dimensions: medicinal herb herbarium and database; medicinal herb

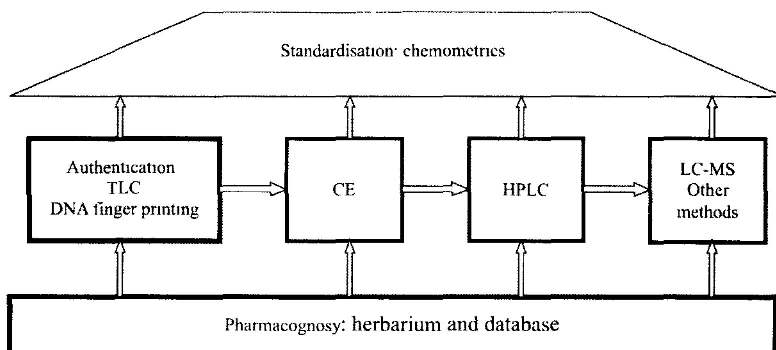

Fig 1. Multiple disciplinary quality control platform for herbal medicines

authentication and fingerprint database; and quality standardisation studies. The platform, involving a combination of efficient pharmacognostic and analytical methods aided by infometrics, is required for the quality standardisation of all herbal medicines.

QUALITY CONTROL OF TRADITIONAL CHINESE MEDICINE

In TCM, reference herbs are essential for identification and research as they are required for identification of many items in Chinese Pharmacopoeia. Raw herbal materials, rather than processed materials, are normally used as reference materials. There are some basic requirements in determining a raw herbal material as a reference material. The botanical Latin names must be identified. Materials of quality herbs from different locations should be compared with fingerprinting and plant materials from the commercial market should not be used as reference materials. A homogeneous powder of the full plant is recommended to eliminate the variability of chemical contents in different parts of the plant and other uncontrollable factors. All the materials must be sealed properly and used before the expiry date to ensure the consistency in quality (Wang, 1994).

In TCM, 'Genuine traditional Chinese herbs' describe materials of high quality originating from specific regions. The term has developed from the comparison and observation of the quality, efficacy and safety applied throughout clinical practice in TCM history. This traditional approach to standardising the quality of herbal materials deals with the variations of herbal materials influenced by variety, geography, climate, season, soil, harvesting and processing. Growers from specific regions know the defined collecting or cultivation techniques to produce a plant material of consistent properties and

quality. Recently, the background, basis and research methods on region suitability of genuine traditional Chinese herbs was discussed (Suo *et al.,* 2005). Analytical methods have been used for the quality assessment of the herb *Ligusticum chuanxiong* (Li *et al.,* 2007), and molecular methods were employed for variety authentication of the popular Chinese herb rhizome, *Dendrobium officinale* (Ding *et al.,* 2008).

The chemistry of Chinese herbal medicines is the study of structure, physical and chemical properties which entail extraction and separation, structure determination and biogenensis of the components in Chinese herbs. The study correlates chemical composition and Chinese medicinal properties under the guidance of TCM theory and practical skills, incorporating herbal pharmacology, TCM clinical and modern science.

Analysis of chemical changes may help to understand the principles of processing. Processing of raw herbal material is the ancient pharmaceutical technology in China and is the summary of clinical experience of TCM pattern differentiation. The purpose of processing is to enhance the efficacy and reduce toxicity of the herb. Processing changes the property, taste and function of Chinese herbs (Sionneau, 2000). Traditional processing of *Paeonia lactiflora* was simulated and the content of paeoniflorin in water extracts among different preparations was assayed by high performance liquid chromatography (HPLC). The results showed that processing procedures were controllable and the heating temperature influenced the content of paeoniflorin (He *et al.,* 2006).

During the past few years, fingerprint method has been developed for quality control of Chinese herbal medicine and recommended by Chinese State Food and Drug Administration to control batch to batch consistency (Mok & Chau, 2006). The recent Pharmacopoeia of the Peoples' Republic of China 2005 edition (volume 1) includes 1146 items of raw herbal materials and processed herb, lipids, extracts, formulary and single herb products. Quality control methods total 1523 thin layer chromatography (TLC) methods for identification and 45 for quantitative testing; 479 HPLC methods for quantitative analysis and 47 gas chromatography (GC) methods for identification and quantitative analysis. For *Morinda officinalis*, a TLC method is included to detect the presence of anthraquinones in raw herbal material in comparison with the standard herb. In addition, the water soluble extract is more than 50% polysaccharides. TLC method for *Centella asiatica* compares asiaticosides with a standard herb. A new addition in this edition is *Hypericum perforatum* (St John's wort). Identification and quantitation via TLC and HPLC, respectively,

requires the hypericin content to be no less than 0.10% (State Pharmacopoeia Commission of the People's Republic of China, 2005). This is compared to the Australian, British and European requirements of 0.08% in all herbal preparations (British Pharmacopoeia Commission, 2007; Council of Europe, 2007).

In the last two decades, the Chinese government has strongly supported research on quality of herbal medicines and improved the standard of Chinese *Materia Medica*. A recent project has lead to a publication of 27 herbs, including *Rheum palmatum, Crataegu pinnatifida, Salvia miltiorrhiza, Artemisia annua, Acanthopanax senticosus, Lonicera japonica, Scutellaria baicalensis, Astragalus membranaceus and Coptis chinensis. Lonicera japonica* is a common Chinese materia medica, with 47 species of *Lonicera* used medicinally in China. Thus, the current Pharmacopoeia quantitative method based on the analysis of the single component, chlorogenic acid, is insufficient. Although the plant contains flavonoids and triterpenoid glycosides, the chemometric study chose 4 major components: chlorogenic acid, isochlorogenic acid, sweroside and secoxyloganin as marker compounds. The quantitative analysis was carried out against the 4 reference compounds and significant variations were found for the 11 different samples. 8 samples were chosen to construct a reference fingerprint and the similarity index was compared via statistical analysis. It was recommended that 0.95 similarity with the reference fingerprint would be a standard for *Lonicera*. In some herbs, Cluster analysis was also carried out using SPSS software, between-group linkage, and Euclidean distance (Huang, 2006).

Quality control methods have been developed for Chinese medicine formulas. Quantitative analysis of active components has become routine procedure in the quality control of manufacturing of herbal products. Yinhuang Injection consists of *Lonicera japonica* and *Scutellaria baicalensis* and contains chlorogenic acid and baicalin as the main active components, respectively. UV spectrophotometry is used to test the content of chlorogenic acid and baicalin in manufacturing (Xiao, 1998). HPLC coupled with diode array detection (HPLC-DAD) has recently become a popular analytical method for Chinese herbal preparations. This method has been employed for monitoring the manufacturing processes of a Chinese herbal preparation, Qingfu Guanjieshu (QFGJS) capsule. Fingerprint and quantitative analysis is based on five marker compounds: sinomenine, paeoniflorin, paeonol, curcumin and hypaconitine (Xie *et al.*, 2007). Another similar method is based on quantitative analysis of eight active components (berberine, aloe-emodin, rhein, emodin, chrysophanol, baicalin, baicalein and wogonin) in Chinese medicine Yiqing capsule (Qu *et al.*, 2007). HPLC-DAD method has been used

to identify peaks in herbal formulations against its individual ingredients. Identification and quality evaluation of a complex traditional Chinese medicine preparation of Baoji pills were carried out using 'Back-tracking' method. The HPLC fingerprint of the Baoji pills consisted of 44 peaks, whereby 35 peaks were assigned by parallel comparison with the fingerprint of the 10 corresponding crude drugs (including pueraria, pummelo peel and magnolia bark) in the formula. The established HPLC fingerprint is a powerful tool for assessing the complete profile of the pills and is more effective than selecting any individual marker for qualitative or quantitative testing target (Wang *et al.,* 2007b).

TLC and HPLC methods are also applied to identify and quantify key herbs in the popular formula Six Ingredient Rehmannia Pill (Liuwei Dihuang Wan). HPLC is used to quantify the content of marker compounds in *Cornus officinalis* and *Paeonia suffruticosa* in the formula (State Pharmacopoeia Commission of the People's Republic of China, 2005). Thus, extensive analytical methods have been applied in the production of TCMs.

PHARMACOGNOSY APPROACHES

The safety and efficacy of herbal medicines is closely correlated with the quality of the source materials used in their production. The quality of the source materials is, in turn, determined by intrinsic factors (genetic) and extrinsic factors (environmental conditions, cultivation and harvesting, field collection and post harvest/collection transport and storage) (WHO, 2005). Therefore, implementing quality control on the raw materials of herbal medicine is an extensive process (Table 1).

Biochemical ecology studies the variation of chemical components of herbs. Many attempts to generate the fingerprints of individual botanical species from different locations and seasons, or even distinct parts of plant used, have been published. The determination of chemical markers for St. John's wort is a prime example. During each phonological stage, the amount of certain constituents (such as hypericin, flavonoids, hyperforin) differs, as well as the parts of plants largely responsible for the variation of each constituent. Fruit and flower part of St John's wort contain significantly large amounts of non-polar constituents such as hypericins and hyperforin, whereas the leaves contain a large amount of flavonoid derivatives (Muller, 2005). As a result, variations of constituents according to phonological stage and portion of different parts may partly contribute to the overall quality of St. John's wort. In addition, "Production of High Quality Australian Ginseng" (Wills & Stuart, 2001) examines changes

Table 1. Typical procedure for quality standardisation of herbal medicines

Research components	Details
Cultivation	Climatic condition, season, light, temperature, rain fall, soil, altitude
Sample collection	Different locations or countries
Collection seasons	Each month or season
Plant parts	Root, stem, leaf, flower, fruit, seed
Processing methods	Drying: sun dry, dry under shade, heated dry, freeze dry, etc.TCM processing: steam, stir fry, quench, ferment, parch, germinate, frost, etc.
Extraction	Solvents such as methanol, water, butanol, chloroform etc.
Analytical method	Pharmacognosy, TLC, HPLC, GC, LC-MS, CE, carbohydrate analysis, chemometrics
Method validations	Precision, accuracy, calibration, inter-day, intra-day, recovery, stability, reference compounds
Reference material	Monographs and pharmacopoeias from China, Europe, Britain, U.S.A; Regulatory authorities such as WHO, TGA, FDA.
Product survey	Products from different companies

in the levels of ginsenosides during plant growth, post-harvest handling, processing and in marketed-products. In a paper by Razmovski-Naumovski *et al.* (2005), growing *Gynostemma pentaphyllum* outside its native China produced a different saponin profile compared to other Gynostemma products. As a consequence, the various growing conditions affecting standardisation should be taken into account in order to establish the quality control in herbal medicines. In regards to the harvesting processes, cultivation has the advantage of confined species, controlled environment and better facilities for treatment after collection. The most advantageous time of collection is during that period when the plant part for drug use and its content of active principle is highest. Recommended periods for plant part collection is summarised in Table 2.

The next step is the preparation of herbal plants. Traditional medicine employs a variety of processing and preparation methods such as decoctions, tinctures and powders for clinical application. Solvents are chosen to yield concentrated dry extracts having medicinal properties similar to traditional applications and to achieve maximum content of the active component(s).

Table 2. Recommended periods for plant collection (Evans *et al.*, 2002)

Plant parts	Recommended periods for collection
Root and rhizomes	Fall (after the vegetative process has ceased)
Bark	Spring (before the vegetative process begins)
Leaves and flowering tops	The time of flowering and before the maturing of fruit and seed (belladonna leaf, sage) (when photosynthesis is most active)
Flowers	Prior to or just about the time of pollination (marigold, honey suckle)
Fruits	Either before or during the ripening period (black pepper, citrus), or when fully ripe (anise, fennel)
Seeds	When fully mature

The effectiveness of an extraction is greatly affected by the polarity of the solvent compared to the polarity of the solute. Polarity is measured by the dielectric constant and can be predicted from the structure of the herbal components. Polar compounds have ionic or partial ionic hydrophilic groups and have relatively higher dielectric constants. Polar components are generally alkaloids, flavonoid glycosides, saponins, polysaccharides, amino acids, phenols and tannins. Non-polar compounds have large hydrophobic region(s) and have lower dielectric constants. Non-polar components found in herbal plants consist of essential oils, fatty acids, coumarins, anthraquinones, certain alkaloids and flavonoids. Common non-polar solvents used in herbal extractions include hexane, chloroform and ethyl acetate; medium polarity solvents are butanol and isopropanol; polar solvents include ethanol, methanol and water (Harborne, 1998).

Ultimately, extraction procedures will depend on the intended purpose. In a commercial product, toxicity of the solvent is of prime importance and thus ethanol or hot water extractions are preferred. In analytical research of active constituents, accuracy and reproducibility are the main factors; for phytochemical and pharmacological tests, sufficient amounts are required for further studies.

The emergence of the term "Pharmacognosy" in 1815 replaced the previous term "Materia Medica" which linked the herbal constituents to their pharmacological activities (De Pasquale, 1984). In recent years, pharmacognosy is so-called a "multi-disciplinary science" and intertwines the studies of phytochemistry, microbial chemistry, biosynthesis, biotransformation, chemotaxonomy and other biological and chemical sciences (Phillipson, 2007). Many reviews outline the

advances in pharmacognosy (Huang *et al.,* 2000; Kinghorn, 2002; Jones *et al.,* 2006) and focus on high-throughput automated bioassays and the combination of novel hyphenated procedures, spectrometric and chemometric approaches (Liang *et al.,* 2004).

Pharmacognosy is the first step of quality control assessment, as GAP guidelines define that the quality and authenticity of the final botanical product is directly related to the proper identification and authenticity of the source material. In other words, the whole process should start with good voucher specimens that act as reference materials, in order to establish quality standardisation.

Herb authentication is a quality assurance process that ensures the correct plant species and parts are used as raw materials for herbal medicines. The proper authentication of herbal raw materials is critically important to the safety and efficacy of herbal medicines. Recent publications in authentication of herbal medicines with pharmacognostic approaches are shown in Table 3.

The World Health Organisation (WHO, 1998) recommends that medicinal plant materials are categorised according to sensory, macroscopic, microscopic and molecular characteristics. Macroscopic identity of medicinal plant materials involves comparison of morphological features that are visible with the naked eye or under low magnification. It is based on shape, size, colour, surface characteristics, texture, fracture characteristics and appearance of the cut surface (WHO, 1998). This rapid method offers a physical reference sample of both wild and cultivated plants that may be referred to at any time and independently identified by other researchers. In addition, the reference sample also supplies an authenticated sample for further microscopic and chemical trials. In terms of quality assessment, the appearance, organoleptic characteristics of particular parts of plant and recognising the presence/absence of obvious contaminants, such as sand, gravel, or morphologically distinct plant material can be simultaneously determined (Bisset, 1989). Microscopic examination of medicinal plants focuses on anatomical structures visible only under a microscope. Histologic studies are made from very thin (20 µm) transverse (radial) or longitudinal (tangential) sections properly mounted in suitable stains, reagents, or mounting media. Typical tissues types include epidermis, mesophyl, cambium, phloem and xylem (Techen *et al.,* 2004).

Molecular markers generally refer to biochemical constituents, including primary and secondary metabolites, and other macromolecules. Secondary metabolites have been extensively used in quality control and standardisation of plant drugs (Joshi *et al.,*

Table 3. Recent publications in authentication of herbal medicines with pharmacognostic approaches

Herbs	Parts	Macroscopic character	Microscopic character	Reference(s)
Phyllanthus amarus *P. fraternus* *P. maderaspatensis*	Whole plant	Stem, branchlet, leaves, flowers and sepals	Sclerenchymatous cells	(Khatoon *et al.*, 2006)
Cassia angustifolia	Seed	Wedge-shaped with transverse ridges and furrows	Palisade cells	(Srivastava *et al.*, 2006)
Berberis asiatica	Root	Odour phenolic	Stone cells	(Srivastava *et al.*, 2004)
Uncaria tomentosa *U. guianensis*	Bark and leaves	Slightly bitter astringent flavour (bark)	Anticlinal walls(leaves)	(Gattuso *et al.*, 2004)
Actaea racemosa	Rhizome and root	Cup-shaped stem scars	Long narrow vascular bundle	(Applequist, 2003)
Coleus forskohlii	Root	Pale brown, tapering with few rootlets	Calcium oxalate crystals	(Srivastava *et al.*, 2002)

2004). Various molecular markers such as Random Amplified Polymorphic DNAs (RAPDs), Restriction Fragment Length Polymorphisms (RFLPs), Microsatellites and Polymerase Chain Reaction (PCR)-based DNA markers such as Sequence Characterised Amplified Regions (SCARs), Sequence taggedsites (STS) and Inter-simple Sequence Repeat Amplification (ISA), Amplified Fragment Length Polymorphic DNAs (AFLPs) and Amplicon Length Polymorphisms (ALPs) are currently used for plant drug analysis (Schinde & Dhalwahl, 2007). From Joshi's work (2004), it appears that DNA markers may have several benefits over typical phenotype markers, such as reliability for informative polymorphisms, owing to its individual uniqueness for each species which is not governed by age, physiological conditions and environmental factors. The assessment of the physical form for each sample has no restrictions since DNA can be extracted from fresh or dried organic tissue. Additionally, small amounts of sample are sufficient for the analysis, which is particularly relevant for Chinese medicinal materials that are expensive or in limited supply (Kelvin & Henry, 2002). DNA analysis of closely related species and/or varieties and common botanical adulterants and contaminants is essential to establish a marker for identification of a particular species. Advantageously, a DNA fingerprint will remain the same, irrespective of the plant part used, whereas the botanical constituents will differ with the plant part used, physiology and environment. Thus, DNA fingerprinting certifies existence of the correct genotype but does not disclose the phytochemical or active principle content. Consequently, it is suggested to be used as a complement tool with other pharmacognostic techniques. More details related to this subject can be found in the references there in (Hess, 2000; Verpoorte, 2000; Kapteyn & Simon, 2002; Do & Bernard, 2004; Techen *et al.,* 2004). Examining these extrinsic and intrinsic properties is the first step towards establishing the identity and the degree of purity of such materials, and should be carried out before any further tests are undertaken. Nevertheless, the application of macroscopic/microscopic methods leads to some difficulties, mostly concerned with the access to a photographic reference library which requires expensively sufficient quality standard reference materials. Additionally, the limitation of sensitivity in determining subtle gradation of some plant species, and the requirement of optimal storage conditions of voucher specimens and macroscopic/microscopic samples should be also taken into account (Techen *et al.,* 2004). Also, the high cost investment and maintenance of microscopic instruments and lack of trained personnel working in these particular areas are still challenging deterrents (Techen *et al.,* 2004). The application of DNA analysis has been restricted to academic circles and needs a large amount of money and time investment.

Good-quality DNA isolation appropriate for examination from semi-processed or processed botanicals also exists with some technical hitches.

There are established centres for herbal authentication around the world. The Centre of Phytochemistry and Pharmacology at the Southern Cross University (N.S.W., Australia) houses a Medicinal Plant Herbarium containing nearly 300 medicinal herbs, with ongoing work continually adding new specimens. The reference herb collection is used to authenticate raw medicinal herbs and extracts thereof. The Chinese Medicinal Plants Authentication Centre (CMPAC) at the Royal Botanic Gardens, Kew, England offers an authentication service for the wide and increasing range of Chinese herbs currently available on the international market.

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is the one of the earliest methods employed in the analysis of herbal plants and its application has now extended to extracts and herbal products. Many pharmacopoeias and monographs now include TLC fingerprint identification of herbal drugs. In industry, TLC is often the first line tool in quality control and screening of herbal plants (Reich & Blatter, 2003). TLC can give a comprehensive profile of the plant as it can separate different classes of compounds at the same time. The variety of compounds that TLC can be utilised for include alkaloids, saponins, cardiac glycosides, anthracenes, essential oils, flavonoids and coumarins (Wagner & Bladt, 1996). Silica gel is predominantly used due to its extreme versatility and high performance (HP) TLC plates provide better separation, sensitivity and reproducibility. Derivatisation and visualisation of the developed profile is usually achieved by spraying or dipping a reagent, followed by heating on a plate. The chromatogram can then be electronically documented and evaluated with scanning densitometry (Hahn-Deinstrop, 2007). TLC is particularly useful for different sample evaluation, stability testing and identification of adulterants. New and improved techniques in TLC endeavour to achieve higher sensitivity and reproducibility in herbal products. In a recent paper, Zarzycki, (2008) introduced thermostated micro-TLC which was capable of separating more than 10 spots in one direction, or up to 180 spots for two dimensional runs in complex samples obtained from natural products. Planar chromatography coupled with direct analysis in real time time-of-flight mass spectrometry (DART-TOF-MS) obtained instantaneous mass spectra from substance zones and detectability limits down to the pg-range for caffeine (Morlock & Ueda, 2007). The non-uniformity illumination of a charge-coupled device (CCD) camera in quantitative evaluation of TLC was rectified

by applying Catmull-Rom spline curve to reconstruct the image background. The estimated background was subtracted from the original image to make the image segmentation. The gray level integration for astragaloside (from Radix *Astragali*- which is proportional to the value of samples) was then calculated (Zhang & Lin, 2006).

A number of studies using TLC/HPTLC for determination of marker components in herbal extracts and pharmaceutical dosage form are listed in Table 4. Aescin, a saponin component in a herbal product containing *Aesculus* and *Vitis* dry extracts was determined by HPTLC. From previous studies, colorimetric method could not determine the aescin content in the capsule due to interference of polyphenols in the *Vitis* extract. Due to poor UV absorbance of triterpene glycosides, HPLC-UV could not be used for detection. From the results, the validated HPTLC method was applicable for both quality control and stability evaluation (Apers *et al.,* 2006). A fast and accurate quantitative determination of colchicines from meadow saffron seed and pharmaceutical formulations was developed by using TLC-densitometry. This validated method provided accurate quantitative determination of colchicines due to elimination of interferences caused by degradation or bioactive compounds. The results were compared with that of a HPLC method and a recommended spectrophotometric method by the Romanian Pharmacopoeia. The results for colchicine content in tablets by TLC-densitometry, HPLC and spectrophotometric determination were 1.03, 1.016 and 1.03 mg/tablet respectively, which indicated a good correlation (Bodoki *et al.,* 2005).

A qualitative and quantitative analysis employed HPTLC to detect the fingerprint marker caffeic acid phenethyl ester (CAPE) in the screening of propolis for regional verification (Fig 2). Based on the results, the New Zealand propolis contained the highest amount of CAPE, whilst Brazilian and Indian propolis did not show CAPE (Peng *et al.,* 2002).

Therefore, planar chromatography (TLC/HPTLC) is a powerful, flexible and inexpensive analytical method that is often the preferred and first-line tool for qualitative and quantitative analysis of herbal plants. TLC/HPTLC analysis can simultaneously compare the profiles of various extracts of a particular herb and thus assess extraction or processing procedures. At the same time, marker compounds (or reference standards) can be employed to determine their presence and respective amounts in the plant. In comparison to other chromatographic methods, TLC is relatively simple to learn and use. TLC equipment is inexpensive and the consumption of solvents is low. The wide variety of stationary and mobile phases promotes high

Table 4. Recent publications on TLC fingerprinting of herbal medicines

Method	Herb	Active compound	Reference(s)
HPTLC **Mobile phase:** toluene-ethyl acetate-diethyl amine (6.5:2.5:1, v/v/v)	*Holarrhena antidysenterica*	Conessine	(Kaur *et al.*, 2008)
HPTLC **Mobile phase:** *n*-propanol-methanol-water (4:1:4, v/v/v)	*Trigonella foenum-graecum*	Trigonelline	(Chopra *et al.*, 2006)
TLC **Mobile phase:** acetic acid-water-butanol (10:40:50, v/v/v)	*Aesculus hippocastanum*	Aescin	(Apers *et al.*, 2006)
HPTLC **Mobile phase:** hexane-chloroform-methanol (5:5:0.5, v/v/v)	*Boswellia serrata*	Boswellic acid	(Shah *et al.*, 2007)
TLC **Mobile phase:** chloroform-acetone-diethylamine (5:4:1, v/v/v)	*Colchicum autumnale*	Colchicine	(Bodoki *et al.*, 2005)

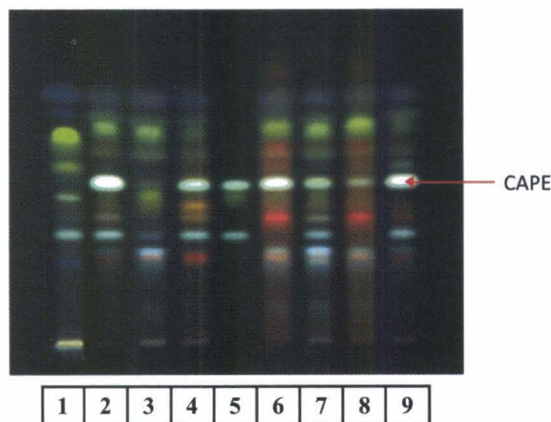

Fig 2. HPTLC profiles of propolis from different regions under UV 366 nm (Peng *et al.,* 2002). Track 1: Raw propolis from Brazil; Track 2: Raw propolis from New Zealand; Track 3: Raw propolis from India; Track 4: Raw propolis from Tasmania (Australia); Track 5: Reference mixture: Caffeic acid-Kaempferol-CAPE; Track 6: Processed propolis from Guangzhou (China); Track 7: Raw propolis from China; Track 8: Ethanolic extract from China; Track 9: Water extract from China

selectivity. The TLC mobile phase is not governed by pH, unlike HPLC, in which the pH of solvents should be between 2 and 8. TLC is also ideal for compounds that do not exhibit a strong chromophore. The duration of development is short and a large number of samples can be analysed simultaneously (Chopra *et al.,* 2006). Compared to other analytical methods, however, there is less sensitivity, reproducibility and separation power in TLC. TLC results are also affected by other factors, including small concentration changes in the solvent system (such as evaporation), the amount of solvent in the chamber, size of the chamber, chamber saturation and ambient temperature (Hahn-Deinstrop, 2007). The results, however, show that TLC/HPTLC method for quality control of herbal medicines still has significant importance for research and industry.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Prior to development of the high performance liquid chromatography (HPLC) technique, other analytical methods such as open-column, paper or thin-layer chromatography techniques have been the dominant means for phytochemical separation (Marston, 2007). HPLC technique not only provides superior precision and resolution but also is well suited for analysing thermal liable and non-volatile constituents. As a consequence, HPLC has become one of the dominant chromatographic techniques in the analysis of herbal medicine.

HPLC chromatographic fingerprints which provide information on chemical composition of crude extract (or sample) are also extensively applied in herbal medicine for the purpose of authentication of species, adulteration and quality control. Recent applications of HPLC in herbal medicine are shown in Table 5. Common separation mechanisms of LC are classified in accordance with specific interactions modes, such as adsorption, partition, ion-exchange, gel permeation (size exclusion) and chiral interaction.

In practice, reversed-phase (RP) chromatography, which is based on partition, is commonly applied in phytochemical separation due to its versatility and wide range of applicability (Zhang *et al.,* 2005; Heard *et al.,* 2006; Wang *et al.,* 2007b). Stationary phase (column) conditions and composition of mobile phase are regarded as the main factors influencing the separation of compounds of interest. However, other factors that need to be considered include pH of mobile phase, pump pressure and column temperature for the optimal chromatographic fingerprint. Choosing optimal condition(s) is important and challenging for the analysis of herbal plants since they contain mixtures of complex compounds with wide ranges of polarities. For example, it has been suggested that the anti-depressive activity of St John's wort is due to the synergistic effect of the flavonoid derivatives of hypercin and hyperforin. A number of studies have produced a phytochemical fingerprint of these compounds (rutin, hyperoside, isoquercitrin, quercitrin, quercetin and pseudohypericin) by HPLC method using C-18 RP column and mobile phase with gradient elution of water-acetonitrile-methanol-tetrafluoroacetic acid (Li & Fitzloff, 2001). However, the resolution of hyperoside and isoquercitrin was relatively poor due to their close structural resemblance. By optimising the mobile phase condition and column chemistry, it has been shown that complete resolution of hyperoside and isoquercitrin was achieved with gradient elution (ammonium acetate buffer: acetonitrile/methanol) on a C-12 RP column (Ganzera *et al.,* 2003). This demonstrates the importance of selecting the optimal condition in HPLC analysis.

Predominantly, C-18 bond phase is applied as the stationary phase in the analysis of herbal medicines. However, it is important to note that shorter carbon chain bond phase or columns with other bond phase chemistries may also be applicable to increase chromatographic performance on specific compounds of interest. Furthermore, classical HPLC columns are packed with silica particles which restrict higher mobile phase flow rates. Alternatively, silica-based monolithic HPLC columns contain a novel chromatographic support with a single, continuous network (monolithic rods) of porous silica. The main advantage of monolithic column is decreased backpressure due to

highly porous (2 µm) metal free silica throughout the network. This allows fast turnaround time and better chromatographic performance that are unattainable with traditional particulate columns. Although application of monolithic column in herbal medicine is currently very limited, a recent study, (Alaerts *et al.,* 2007) demonstrated that monolithic column was compatible with organic modifiers isopropanol and successfully applied to analyse different herbal extracts.

Even though HPLC is a powerful tool to separate constituents of interest in a sample or to produce an entire fingerprint profile of the sample, the analytical procedures are rather tedious and require extended run time for complex herbal materials. Recently developed LC, termed "ultra performance liquid chromatography" (UPLC), has shown many superior aspects such as enhanced peak capacity, separation, speed and sensitivity compared to conventional HPLC (Wilson *et al.,* 2005). Briefly, the principle of UPLC is based on the van Deemter equation, with the columns packed with sub–2 µm particles and resulting dramatic improvements in peak resolution. One of the few applications of UPLC on herbal medicine includes the investigation of different ginsenosides from raw and steamed root of *Panax notoginseng* (Chan, 2007). In this study, separation of constituents was performed on a small 1.7 µm particle size (100 × 2.1 mm) column by Waters Acquity UPLC coupled with time-of-flight mass spectrometry (TOFMS) detection. The results showed that UPLC achieved higher reproducibility when compared with traditional HPLC, with ten times less run time.

The choice of detector also needs to be considered in order to maximise output efficiency and sensitivity. In herbal medicine, commonly used HPLC detectors are UV-diode array detector (DAD) and evaporate light scattering detector (ELSD) (Yan *et al.,* 2005; Qi *et al.,* 2006; Yan *et al.*, 2006; Yi *et al.,* 2007). Since UV-DAD measures analyte spectrum at a range of wavelengths simultaneously, HPLC coupled to DAD provides additional sample fingerprint spectra which is not available from a single UV detector. Herbal compounds that consist of aromatic groups and conjugated double bonds respond well to the UV range, hence HPLC-DAD is commonly applied in herbal medicine for determination of phenols, polyphenols (flavonoids, tannins) and alkaloids (Ding *et al.,* 2007; Cho *et al.,* 2008).

As most compounds in herbal medicines lack a prominent chromophore, ELSD is also suggested as an alternative detector for detecting poor UV absorption compounds such as terpenes or saponins (Tang *et al.,* 2003). ELSD detection does not rely on the UV absorbance of the analytes, but largely depends on the size, shape and number of eluate particles. Park *et al.* (1996) compared HPLC-

Table 5. Recent HPLC applications in determination of secondary metabolites in herbal medicine

Method	Herb	Active compound	Reference(s)
HPLC-DAD **Stationary phase:** 4.6 mm × 250 mm *i.d.*, Symmetry C-18 (Waters Corp) **Mobile phase:** Gradient of (A) 0.1% aqueous formic acid and (B) acetonitrile-methanol-0.1% formic acid (7:2:1, v:v:v)	*Xanthosoma brasiliense*	Flavonoids, phenolic acids	(Cho *et al.*, 2008)
HPLC-ELSD **Stationary phase:** 4.0 mm × 250 *i.d.*, Diamonsil C-18 **Mobile Phase:** Isocratic methanol-water (33:67, v/v)	*Ginkgo biloba*	Terpenes Ginkgolides A, B and C; bilobalide	(Tang *et al.*, 2003)
HPLC-PAD **Stationary phase:** 150 mm x 2.1 mm *i.d.*, Hypersil GOLD 3 u C-18 **Mobile phase:** Gradient of (A) 10% acetonitrile and (B) 60% acetonitrile	*Panax notoginseng*	Saponin Glycosides Ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, Rg1	(Kwon *et al.*, 2008)
HPLC-DAD-MS/MS **Stationary phase:** 4.6 mm x 200 mm *i.d.*, Diamonsi C-18 **Mobile phase:** Gradient of (A) 0.2% acetic acid, adjusted with triethylamine to pH 5 and (B) acetonitrile	*Corydalis yanhusuo*	Alkaloids	(Ding *et al.*, 2007)
UPLC/TOFMS ES-TIC **Stationary phase:** 100 × 2.1 mm C-18 1.7 µm column **Mobile phase:** Gradient of (A) 0.1% formic acid in water and (B) acetonitrile containing 0.1% formic acid	*Panax notoginseng*	Ginsenosides	(Chan, 2007)

ELSD to HPLC-UV in the analysis of major ginsenosides in Ginseng, since ginsenosides have poor UV absorbance. The study concluded that HPLC-ELSD was superior to HPLC-UV, with respect to separation and sensitivity in analysing ginsenosides.

Hyphenated instruments, such as HPLC-MS and HPLC-NMR, provide additional structural information and are suggested as more reliable techniques for analysis of herbal medicine (Charchoglyan *et al.*, 2007). Moreover, combinations of multiple hyphenated instruments such as HPLC-DAD-MS, HPLC-ELSD-DAD and HPLC-UV-MS/MS have been published recently for fingerprinting herbal products (Yu *et al.*, 2007; Zhao *et al.*, 2007; Su *et al.*, 2008a).

In essence, HPLC is well recognised and routinely applied in the quality control of herbal medicines. With established chromatographic conditions (*e.g.* mobile and stationary phase) and identification strategies, HPLC is well suited for analysing complex sample matrices indicative of herbal medicines. However, it requires extended time for complete separation of the complex herbal mixture. The fast speed and high-throughput novel UPLC is now considered as an alternative chromatographic technique for analysing herbal medicines. Moreover, multiple hyphenated techniques are extremely powerful for characterisation of complex herbal compounds. It is anticipated that its application in phytochemical studies will be dramatically increased in the near future.

GAS CHROMATOGRAPHY

Gas chromatography is a separation technique which involves a very small amount of sample being vaporised and injected into a long column. The sample is then carried through the column by the flow of an inert gas, such as helium, as the mobile phase. Separation is based essentially on the volatility of sample molecules and its partition between the carrier gas and the liquid film stationary phase within the column. Therefore, GC presents significant restrictions for analysing only those relatively volatile and thermally stable constituents in herbal medicine. Volatile essential oils in complex herbal mixture are highly suited for GC analysis. The extraction methods commonly used for the isolation of these volatile components are generally based on hydrodistillation.

Essential oils consist of a large group of chemical compounds with diverse functional groups and widely ranging physiochemical properties. It mostly comprises of volatile organic compounds such as monoterpenes, sesquiterpenes and their oxygenated derivatives of aromatics, phenols, alcohols, aldehydes, ketones, esters, lactones and ethers (Shaw, 1979). The advantage of GC separation, notably, is

simple and amenable for subsequent high sensitivity detection for these volatile compounds. GC coupled with MS is widely used for characterisation and profiling of chemical components in essential oils. The identification of an individual compound is based on its unique mass spectrum as reference to MS spectral library. Over the past decades, substantial articles have been published on characterisation of essential oils in various herbal mixtures by GC-MS method (Kunert *et al.*, 2002; Ahn *et al.*, 2006; Marie *et al.*, 2007; Qi & Armstrong, 2007; Zhang & Li, 2007; Su *et al.*, 2008b). To further overcome the difficulty of resolving the volatile compounds present in highly complex herbal matrices, comprehensive two-dimensional (2D) gas chromatography (GC x GC) was introduced to increase the peak resolution. The technique employs two columns of different selectivity directly connected through the thermal modulator (Marriott *et al.*, 2000). The eluates from the first column are trapped and focused by the modulator. Sharp chemical pulses are produced into the second column, resulting in higher peak capacity, as compared to conventional single-column GC (Beens *et al.*, 2000). 2D-GC coupled with Flame Ionisation Detector (FID) (Di *et al.*, 2004) and mass spectrometry (MS) (Shellie *et al.*, 2003) have been sucessfully applied for profiling volatile oil in American ginseng. 2D-GC also provides fast chiral analysis. By using enantoselective capillary columns as second dimension, the enantiomeric distribution of monoterpene compounds in bergamot essential oil was reported (Shellie & Marriott, 2002).

The analysis of volatile compounds by GC is very important in the chemical profiling of essential oil in herbal products. One of the key advantages of GC is its high sensitivity of detection (FID and MS) for most of the volatile compounds. 2D-GC provides further peak resolution and chiral selectivity of volatile constitutes in the complex herbal mixture. Therefore, GC-MS has become a popular and useful analytical tool in authentication and profiling of herbal products.

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Although different chromatographic techniques, particularly HPLC-DAD, have been widely employed, liquid chromatography mass spectrometry (LC-MS) has played an increasingly important role in chromatographic fingerprinting in the authentication and quality assessment of herbal medicine. Atmospheric pressure ionisation mass spectrometry (API-MS) became commercially available in early 1990. The technique generates soft ionisation, thereby preserving the molecular ion for accurate quantitative analysis. More detailed structural information of the analyte can subsequently be obtained by resorting to tandem MS (MS/MS) for qualitative analysis. The

basic MS/MS principle can be described as mass selection of a parent ion in the first stage of MS, and then analysis of the daughter ion(s) formed by the collision induced dissociation (CID) process in a second MS for structural elucidation of parent ion. Atmospheric pressure chemical ionisation (APCI) and Electrospray ionisation (ESI) are two basic ionisation techniques for API in LC-MS analysis. Ionisation of sample molecules at atmospheric pressure is a very efficient approach due to the high collision frequency between the ions and molecules. The mass spectrum provides the molecular weight information of the specific analyte and, therefore, not only offers better selectivity, but also specificity than spectrophotometric absorption (DAD) techniques. This is particular useful when measuring compounds in complex matrices like herbal extract.

ESI-MS has been the mainstay for analysing polar higher molecular weight constituents in herbal medicine. On the contrary, APCI is applicable to analysing neutral and less polar compounds of lower molecular weight than ESI. In general, basic compounds are readily ionised in positive ionisation (PI) mode at pH below 7. A trace of formic acid is often added to aid protonation of the sample molecules in PI mode. The basicity of alkaloids varies greatly and depends entirely on the availability of the lone pair electrons on the nitrogen atom. A substantial body of work describes the use of MS/MS in the analysis of alkaloids found in natural products, both in the confirmation or refutation of previous work. Several groups have shown that ESI(+) ion mode is applicable for analysing alkaloids because of the higher proton affinity (PA) of amine moieties (Wong *et al.,* 2002; Koo *et al.,* 2006; Kontrimaviciute *et al.,* 2007). Ding and colleagues (2007) has recently quantitatively determined alkaloids in *Corydalis yanhusuo* and shown that all tertiary alkaloids yield prominent $[M + H]^+$ while quaternary alkaloids yield $[M^+]$ ions in the first order spectra. All these studies suggest ESI(+)-MS not only provides rapid screening but also structural characterisation of alkaloids in herbal medicine. It is anticipated that there will be an increase in published literature on the application of MS for analysing various alkaloids in near future.

Flavonoids are widely distributed in the plant kingdom. They constitute an important class of secondary metabolites and commonly occur as flavonoid *O*-glycosides, in which one or more hydroxyl (OH) groups of the aglycone are bound to a sugar. The use of both APCI and ESI has been generally accepted for the analysis of flavonoids. Several studies have reported the application of MS in structural analysis of flavonoid conjugates and utilisation of LC-MS methods in profiling flavonoid glycosides (Cuyckens & Claeys, 2004; Wang *et al.,* 2004; Lee *et al.,* 2005; Stobiecki & Kachlicki, 2006). Although both

PI and negative (NI) modes are amenable, better sensitivity for the detection of flavonoids has generally been achieved in NI mode and provides unique fragmentation behaviour of the analytes (Saracini *et al.*, 2005; Stobiecki *et al.*, 2006). NI mode offers an additional advantage of selectivity, because chemical background is usually lower compared to PI mode. The most useful fragmentations for flavonoid aglycone identification are those that require cleavage of two C-C bonds of the C-ring, providing information on the number and type of substituents in the A- and B-rings. With MS detection, it is possible to unambiguously identify the glycosylation patterns of the aglycones. Detailed MS techniques for analysing flavonoid glycosides have been reviewed by Stobiecki (2000).

The terpenes are the largest group of plant secondary metabolites and built from a specific 5-carbon unit (up to 40-carbons in their structures). Triterpenoids have similar configurations to steroids and posses a framework of approximately 30-carbons. Saponins are ester glycosides of triterpenes that have soap-like properties. Structural elucidation of these compounds can be quite complicated. Separation and identification of individual saponins by HPLC-DAD can be difficult due to poor sensitivity and specificity of chromophores. In addition, most saponins appear as complex mixtures and contain multiple oligosaccharide chains at different positions in the molecule. ESI-MS permits individual fragmentation pathways to be followed and are most applicable for analysing these high molecular weight polar constitutes. Ionisation in the NI mode is generally reported in the literatures (Balsevich *et al.*, 2006; Ye *et al.*, 2006; Chapagain & Wiesman, 2007). In the MS spectra, the saponins exhibited predominantly their quasi-molecular ion $[M\text{-}H]^-$. Lai and co-workers identified 27 saponins and 9 of them including notoginsenoside R1, ginsenoside Rb1, Rb2, Rb3, Rc, Rd, Re, Rf and Rg1 were quantified (Lai *et al.*, 2006). The MS/MS spectra demonstrated an unique fragmentation pattern corresponding to loss of the glycosidic units $[M\text{-}H\text{-glycoside}]^-$ and aglycone of *m/z* 475. Such MS techniques are particular suitable for identification of different saponins and now commonly apply for glycosylation studies. PI ionisation has also been reported for saponins in *Gynostemma pentaphyllum* (Razmovski-Naumovski *et al.*, 2008). Parameters such as stationary and mobile phase, buffer, temperature and fragmentation patterns were discussed.

All the above studies clearly indicate that MS/MS is a powerful technique for quantitative analysis and characterisation of glycosylation pattern in herbal medicine. It is capable of high throughput analysis and does not require much sample or a rigorous sample extraction strategy. A new generation dual ion source to perform simultaneous ionisation with ESI and APCI will allow a wide

range of compounds of low to high polarity to be ionised in a herbal extract and subjected to structural elucidation. However, ionisation of very low polarity phytosterols (free and conjugated β-sitosterol, campesterol) are a great challenge for the ESI or APCI source due to poor ionisation efficiency. Recent development is a novel atmospheric pressure photo ionisation (APPI) technique (high energy photon) and provides high sensitivity LC-MS analyses for low polar herbal constituents such as phytosterols that are not feasible by ESI or APCI source (Kostiainen & Kauppila, 2005; Lembcke *et al.,* 2005).

CAPILLARY ELECTROPHORESIS

In recent years, capillary electrophoresis (CE) is becoming more popular in the separation and quantitation of mixed natural compounds with excellent efficiency, making itself one of the most powerful methods in quality control in the herbal industry (Suntornsuk, 2002). Due to its simplicity and high efficiency, it has been widely used in qualitative and quantitative analysis, purity testing, chiral purity and stoichiometric determination (Suntornsuk, 2007). Currently, registered CE methods have been proposed by several Pharmacopoeias, including the British Pharmacopoeia (BP) (2001) and United States Pharmacopoeia (USP) (2002). Routine CE methods have been successfully submitted to regulatory authorities, such as the US Food and Drug Administration (FDA) (Altria and Elder, 2004) and the Australian TGA.

The mechanism of the separation in CE is based on the differential migration velocities of analytes in the capillary. This is dependent on the sizes, charges and degree of ionisation of the analytes, and viscosity, temperature and dielectric constant of the background electrolyte (BGE) (Fig 3).

Whilst travelling inside the capillary, analytes are driven by the electrophoretic migration and the electro-osmotic flow (EOF) upon application of high voltage supply, and then separated based on their different mobility. Separated compounds are detected in the outlet end of the capillary and the signal is transmitted to the computer processing system, with the output in the form of peaks. The most commonly used online detectors include UV-diode array spectrophotometer, spectrofluorometer and electrochemical detector. MS, due to its advantage of high sensitivity and obtaining molecular weight or structure information, is also playing an increasing role as an external detector. The most widely used CE techniques for herbal medicines include capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), non-aqueous CE (NACE), chiral CE, CE-MS, capillary gel electrophoresis (CGE) and capillary isoelectric focusing (CIEF), capillary isotachphoresis (CITP), affinity

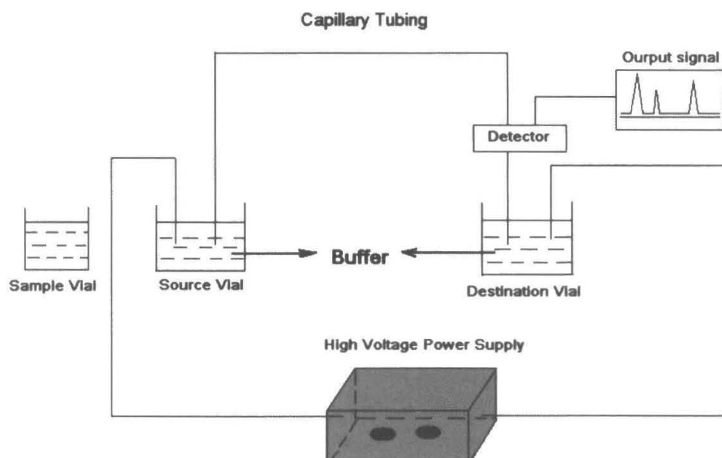

Fig 3. Schematic of capillary electrophoresis system. Sample travels under high voltage inside the capillary from the source vial to the destination vial, and is detected at the end of the capillary by a detector, which records the electrophoregram output on an integrator or computer

capillary electrophoresis (ACE), microchip CE and multiplexed CE (MCE) (Suntornsuk, 2007). Different CE methods have been performed for the analysis of a wide range of natural compounds in herbal medicines and these are summarised in Table 6. Applications of CE for the analysis of phytochemical substances have been recently reviewed (Suntornsuk, 2007).

The main advantage of CE is the large surface-to-volume ratio which allows a greater electric potential to be applied across the capillary. At the same time, this maintains effective heat dissipation, which results in superior resolution (Suntornsuk, 2007). Other advantages of CE include high peak capacity, low sample consumption, low reagent consumption, high speed analysis, high efficiency, excellent mass sensitivity and cost-effectiveness.

A major drawback of CE is the relatively poor precision and reproducibility, which is due to the irreproducible electro osmotic flow (EOF) caused by the unstable surface condition of the inner wall of the capillary (Mayer, 2001). In addition, marker compounds or internal standards are commonly needed in CE for both qualitative and quantitative analyses because the separation efficiency of CE highly depends on the concentration of the analytes (Guzman *et al.*, 2006). For example, linearity of anthraquinone-1-sulphonate by CE and HPLC was excellent, but the precision and detection limits by HPLC were better than CE. However, several methods have been introduced to improve the precision in CE analyses, such as the introduction of multiple injections or uses of internal standards.

Table 6. Recent applications of CE methods in herbal medicines

CE Method	Herb	Active compounds	CE conditions	Reference(s)
Capillary zone electrophoresis (CZE)	*Centella asiatica*	Flavonoids (rutin, kaempferol, quercetin, myricetin and apigenin)	20 mm NaH2PO4-Na2HPO4 (pH 8.0) with 10% v/v acetonitrile and 6% v/v methanol; 25 kV; 30°C; 220 nm.	(Suntornsuk & Anurukvorakun, 2005)
	Strychnos pierrian	Alkaloid (brucine, aconitine, hypaconotine, mesaconotine)	40 mm ammonium acetate and 0.1% acetic acid in 80% methanol; 15 kV; 200 nm.	(Feng & Li, 2002)
	Garcinia atroviridis	hydroxycitric acid and hydroxycitric acid lactone	30 mm Na2B4O7, 90 mm NaH2PO4 and 0.5 mm tetradecyltrimethyl ammonium bromide, pH 9.2; -20 kV; 25°C; 200 nm.	(Muensritharam *et al.*, 2008)
Micellar electrokinetic chromatography (MEKC)	"Shuangdan" granule	protocatechuic aldehyde, salvianolic acid B and gallic acid	5.0 mm borate, 15 mm phosphate, 35 mm SDS and 10% (v/v) acetonitrile; 15 kV; 25°C; 210 nm.	(Yu *et al.*, 2006a)
	Cortex moutan	Paeonol and paeoniflorin	10 mm borate and 25 mm SDS, pH 9.5; 15 kV; 25°C; 233 nm.	(Yu *et al.*, 2006b)
CE-mass spectrometry (CE-MS)	*Triticum aestivum* L.	Secoisolariciresinol and arctigenin, pinoresinol, lariciresinol, hinokinin	40 mm ammonium acetate, pH 9.5; 25 kV; 214 nm (UV-ESI-MS).	(Dinelli *et al.*, 2007)
			Aconitum alkaloids: 40 mm ammonium acetate and 0.1% acetic acid in methanol.	

Table 6. *Contd.*

CE Method	Herb	Active compounds	CE conditions	Reference(s)
	Strychnos pierrian	Aconitum alkaloids (hypaconine, aconine, mesaconine)	Alkaloids in *S. pierrian*: 80 mm ammonium acetate and 0.1% acetic acid in 40% water and 60% methanol. 15 kV (CE-UV) 200 nm and 30 kV (CE-MS).	(Feng *et al.*, 2003)
Non-aqueous CE (NACE)	*Thalictrum atriplex*; *Thalictrum finetii*	Isoquinoline alkaloids (palmatine, jatrorrhizine, (+)-tetrandrine, northalfine)	Methanolic solution of sodium acetate (75 mm) and acetic acid (1 M); 30 kV; 25°C; 200 nm.	(Su *et al.*, 2002)
	Crocus sativus	Seven crocin metabolites	25:75 mixture of 50 mm H3BO3 in methanol and 50 mm Na2B4O7 in methanol; 16 kV; 20°C; 245, 335 and 435 nm.	(Zougagh *et al.*, 2005)
	Hypericum perforatum	Anthraquinone derivatives (hypericin) and phloro-glucinol derivatives (hyperforin)	Methanol, dimethylsulfoxide and Nmethylformamide (3:2:1, v/v/v) containing 50 mm ammonium acetate, 150 mm sodium acetate and 0.002% (w/v) hexadimethrine bromide, –25 kV; 25°C; 300 nm (hyperforins), 590 nm (hypericins), 350 nm (flavonoids).	(Jensen & Hansen, 2002)
Capillary isotachphoresis (CITP)	*Hypericum perforatum*	Flavonoids and phenolic acids (quercitrin, chlorogenic acid)	50 mm boric acid and 25 mm β-hydroxy-4-morpholinopropanesulfonic acid, pH 8.3.	(Hamoudova *et al.*, 2006)

Currently, the predominant tool in quality control of herbal medicines remains to be HPLC. However, CE will take on an indispensable and complementary role. Now-a-days, tremendous efforts aimed at improvements in separation capabilities, detection sensitivity, reliable quantitation and commercial instrumentation are bringing widespread acceptance of this technique worldwide. Thus, CE will significantly contribute to a better understanding of the solution behaviour of herbal medicines, especially when coupled with other powerful analytical methods.

METHOD VALIDATION

Analytical data is used to screen potential herbal candidates, aid in the development of a herbal drug, support formulation studies, monitor the stability of bulk herbal products and test final products for release. The quality of analytical data is a key factor in the successful development of an herbal product for the market. Likewise, the process of method development and validation has a direct impact on the quality of these data (Green, 1996).

Method validation is the process of proving that an analytical method is acceptable for its intended purpose. It is important that all steps are documented in detail in order to minimise any problems or variations that may arise from changes in equipment, laboratories and personnel (Green, 1996). During each validation study, key method parameters are determined and then used for all subsequent validation steps (Table 7). For pharmaceutical methods, the U.S. Pharmacopoeia 23 (1994), International Conference on Harmonisation (ICH) (1995) and the FDA (FDA, 1987; Center for Drug Evaluation & Research, 1994) provide guidelines for performing method validations.

To perform validation studies, a standard compound that appears in the plant is usually required. The compound is then diluted to a range of concentrations and measured via the various chromatographic or spectrometric instruments available (densitometer (TLC), HPLC, GC, CE or LC-MS). A linear response in the working range is usually attained via regression analysis, with an r^2 value ideally greater than 0.999. Quantitation is based on a comparison of the peak area/height (HPLC or GC) or spot intensity (TLC) of the sample to that of a reference standard of the analyte of interest (Center for Drug Evaluation & Research, 1994; Reich & Blatter, 2003). Recent publications of validation of herbal products using various analytical methods are shown in Table 8.

Performing a thorough method validation can be a time-consuming process, but the quality of data generated from a particular method is directly related to the quality of the process. Performing proper

Table 7. Summary of parameters involved in method validation

Parameter	Description
Accuracy	Degree to which the determined value of the analyte in the sample corresponds to the true value
Precision (repeatability, reproducibility)	Expresses the closeness of agreement between a series of measurements
Limit of detection (LOD)	Lowest amount of analyte that can be detected (+3SD or 3S/N ratio)
Limit of quantification (LOQ)	Lowest amount of analyte that can be quantified (+10SD or 10S/N ratio)
Range	Interval between upper and lower concentrations
Linearity	Test results proportional to the analyte in the sample

Table 8. Recent publications of method validation of herbal products

Plant	Compound	Method	Reference(s)
Artemisia annua	Artemisinin	TLC and HPLC	(Marchand *et al.*, 2008)
Chamomilla recutita	Apigenin	CE	(Fonseca & Tavares, 2004)
Plantago palmata	Acteoside	HPTLC	(Biringanine *et al.*, 2006)
Gardenia herbs	Geniposide	LC-MS/MS	(Wang *et al.*, 2007a)
Scutellaria lateriflora *Scutellaria baicalensis*	Baicalin, baicalein and wogonin	HPLC	(Gao *et al.*, 2008)
22 traditional medicinal herbs including *Angelicae dahuricae*, *Bupleuri*, *Clematidis* and *Salviae miltiorrhizae radix*	Isoflavonoids, lignans and mycoestrogens	GC-MS	(Lee *et al.*, 2004)

validation studies initially will eliminate problems encountered during the analytical process. Thus, method validation is becoming a crucial aspect in the optimisation of herbal products.

CHEMOMETRICS

Chromatographic fingerprints not only show comprehensive chemical information of constituents, but also show ratios of them. Since fingerprints obtained from herbal extracts are complex systems, adequate multivariate statistical methods are necessary to evaluate the data obtained from a fingerprint. A number of chemometric methods such as similarity evaluation, principle component analysis and hierarchical clustering analysis have been applied to evaluate complex fingerprint chromatography data for authentication species and thus quality control of herbal medicine (Liang *et al.*, 2004). Similarity evaluation compares the degree of similarities between chromatographic fingerprints with certain references, such as mean or median spectrum of fingerprints. For measurement of similarity between two chromatograms, different measures for similarity such as correlation coefficiency, congruence coefficiency and cosine value of vectoral angle are commonly applied (Ni *et al.*, 2008).

Even though proposed methods of similarity evaluations are simple and easy to implement in fingerprint analysis, they also present certain limitations. The need for definite objective criterion in evaluating the similarity was pointed out by Gan and Ye (2006). They proposed a new method for evaluating similarity of chromatographic fingerprints by using Bayesian hypothesis test for similarity evaluation, and by using scalar means of the difference vector to describe dissimilarity between fingerprints. In conclusion, both t-test and Bayesian hypothesis test could provide the basis for one-to-one comparisons of fingerprints and, thus, it could offer better evaluation criterion. Another problem of existing methods is the limitation of quantitative assessment. Previously, Sun *et al.* (2003) proposed a series of quantitative evaluation parameters attempting to provide the general quantitative assessment. However, all parameters exhibited only one function of the quantitative or qualitative assessment. To overcome this issue, Sun and Liu (2007) again proposed "Involution Similarity" which enabled simultaneous quantitative and qualitative assessment.

Due to the uncertainty of secondary metabolites in herbal medicine, subjective outcome might arise if sample fingerprints are evaluated with the mean or median of their fingerprints (Liang *et al.*, 2004). Also, swamping and masking effects could arise from similarity evaluation. To avoid this, pattern recognition methods, such as principle component analysis (PCA) or hierarchical clustering analysis are often combined with similarity evaluation. For example, Ni *et al.* (2008) studied similarities in the chromatograms of 46

Eucommia Bark samples derived from eight different provinces in China. In the study, data matrix obtained from LC-DAD was auto scaled and analysed by Computer Aid Similarity Evaluation System (CASES). Similarities of each chromatogram were based on calculating the cosine value between two data vectors, where the mean value of the chromatogram was chosen as a standard reference (Fig 4, 5).

The study indicated strong similarity (>0.90) between samples obtained from Sichuan, Hubei, Shanxi and Anhui province, whereas less similarity (<0.87) was shown in other provinces. Further evaluation was done by applying principle component analysis biplots and dendrograms from hierachial cluster analysis using MATLAB 6.5 software.

In principle component analysis, samples from Sichuan, Hubei, Shanxi and Anhui province were observed to cluster together (Fig 5), whereas other samples were scattered widely. The study was able to differentiate fingerprint samples derived from different provinces according to pattern and peak shape of the chromatograms.

Obtaining good chromatographic fingerprints with optimised signal intensity, retention time, peak area and peak height are a necessary requirement for chemometric calculation. However, acquiring a clear chromatogram of an extract mixture is quite challenging. Factors such as overlapping peaks and/or shift of peak retention time may produce errors in evaluating peak area and actual peak appearance time, respectively.

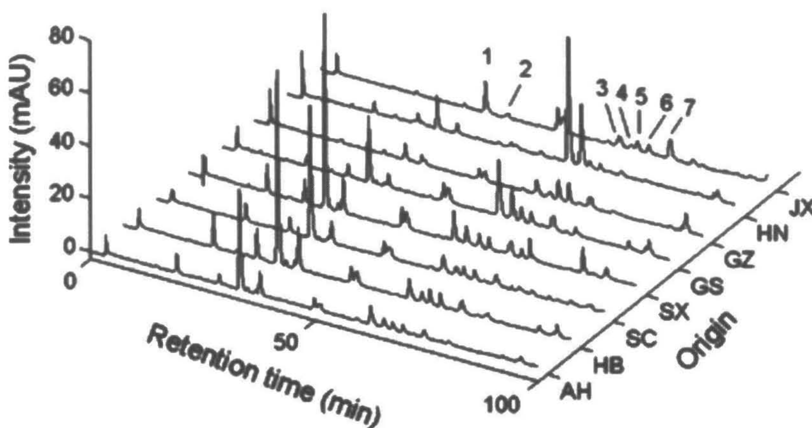

Fig 4. Mean chromatograms of the *Eucommia* bark extracts from samples collected in different provinces: Anhui (AH), Hubei (HB), Sichuan (SC), Shanxi (SX), Gansu (GS), Guizhou (GZ), Henan (HN) and Jiangxi (JX). The seven common peaks (1–7) are labeled (Ni *et al.,* 2008)

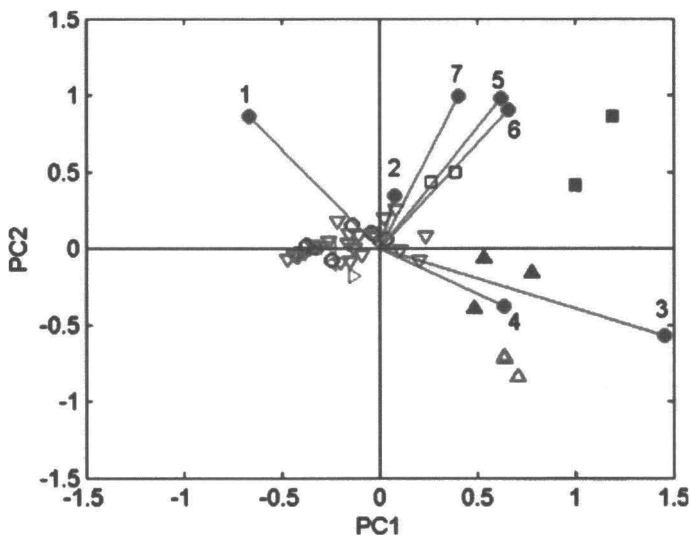

Fig 5. Biplot of the 46 chromatographic responses of the *Eucommia* bark samples (83% data variance explained) from Sichuan (▽), Hubei (O), Shanxi (▷), Anhui (◁), Henan (△), Gansu (▲), Jiangxi (□) and Guizhou (■). The seven loadings vectors (●) are numbered 1–7 (Ni *et al.*, 2008)

Changes in retention time could be due to degradation of the stationary column, changes in the composition of the mobile phase and other unknown factors. In order to minimise these errors, pretreatment of the data is necessary before statistical evaluation is attempted. The treatment of vertical splitting has been commonly used to approximate areas of overlapping peaks. However, overlapping peaks gives reduced areas under the peak. Chromatographic peaks could also be regarded as a continuous signal determined by its chromatographic shape. Liang *et al.* (2004) proposed that all peaks will produce maximum height and correspond to a normal distribution shape. Calculation of peak area based on the normalisation of the chromatogram would avoid unnecessary identification of the retention time, peak intensity and peak width for all peaks.

Correction optimised wrapping (COW), dynamic time wrapping (DTW) and parametric time wrapping (PTR) have been proposed for correcting retention time. However, these methods do not efficiently match with hyphenated chromatographic instruments such as HPLC-DAD, LC-MS and are often time consuming. Recently, Xu *et al.* (2006) proposed target peak alignment approach (TPA) to correct retention time shift presented in hyphenated chromatographic data, and multiplicative scattering correction (MSC) to reduce the variations in response.

Chemometrics offers a different approach to herbal medicine fingerprint quality evaluation. One advantage is that individual components that comprise the fingerprint can be unknown. Thus, there is no need for reference standards that may be difficult to obtain. Also, the synergistic effect of a herbal preparation can be evaluated by comparing the proportion of constituents in different samples. Fingerprint analysis via chemometric can also be applied to assess the effects of harvesting time, various locations and storage time on herbs (Yang *et al.,* 2005).

Coupled with chemical analytical techniques, differentiation of fingerprint patterns with chemometric methods would allow authentication of species and enhance the quality assessment of herbal medicines.

CONCLUSIONS

The rapid growth of herbal and complementary medicines around the world has instigated a thorough review of current practices in the quality assurance of starting materials through to finished products. However, due to the complex nature and the large number of herbal materials and products world wide, the quality standardisation of herbal medicines is still an arduous task for researchers, industry and regulatory organisations. Herbal medicines are characterised by many varieties, numerous components, multiple pharmacological actions and variable clinical applications. Thus, the current analytical methods using one or two marker compounds do not reflect the synergistic nature of herbal medicines. As alternatives to the conventional approaches of quality control of herbal medicines, various pharmacognostic and chromatographic techniques have been applied to define the quality standards of a specific herbal extract.

Genetic, environmental and geographical conditions will ultimately decide the quality of herbs. Pharmacognosy and molecular techniques reveal the effects of these intrinsic and extrinsic factors. Comparisons of plant materials from different sources are essential to define the qualitative and quantitative variations of herbs. HPTLC is a versatile and rapid method for qualitative and quantitative fingerprinting of herbal medicines. CE is an efficient technique and can be further developed as a first line method for quality control analysis in the herbal industry. Chemometric methods can be applied to HPLC, LC-MS, CE and other methods to generate multiple patterns to define the quality standards of herbal medicines. Since herbal medicine represents a multivariate system, this statistical approach is well suited in the concept of phytoequivalence as the pattern of the whole chromatographic fingerprint can be measured in an objective manner.

The multiple disciplinary approaches described in the chapter can be extensively applied for authentication, adulteration, quality standardisation and quality control of all herbal medicines.

Finally, quality standards need to integrate pharmacology and clinical evidence. Whilst quality is the foundation of the efficacy and safety of herbal medicines, herbal quality is based on specific efficacy and clinical applications. Different pharmacological activities and clinical effects of a single herb will require different amounts and combination of active compounds. Eventually, active components or fractions responsible for different pharmacological activities need to be defined. It is essential that the quality standards documented in various pharmacopoieas and monographs are continually revised. Therefore, a long term quality control platform requires a multidisciplinary and standardised international approach to pharmacognosy, chemistry, pharmacology and clinical study. However the priority step forward for herbal medicines remains that all marketed herbal products have quality standards encompassing the many available techniques in order to provide safe and effective therapeutic benefits to patients.

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Evaluation of Biological Activity in Quality Control of Herbal Medicines

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ABSTRACT

Evaluation of biological activity is a fundamental step in evaluating the quality, safety and efficacy of herbal medicines. To determine the complex biological activities caused by multiple chemical components in herbal medicines, new scientific research methods and comprehensive documentation are required. This review covers the significance and methodology of the biological evaluation in relation to quality control of herbal medicines. Vigorous and validated in vitro, in vivo and clinical studies of herbal medicines will need to be applied in order to ascertain active extracts or compounds, their potential pharmacological activity and synergistic effect of active compounds. This systematic biological evaluation and quality standardisation research platform concept is related to the bioequivalence theory in pharmaceutical science. The concept is further discussed in reviewing the development in the assessment of anti-inflammatory and anti-diabetic activities of herbal medicines.

Key words : Anti-diabetic, Anti-inflammation, bioequivalence, biological evaluation, herbal medicines, metabonomics, quality control

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INTRODUCTION

Several diverse lines of evidence indicate that medicinal plants represent the oldest and most widespread form of medication (Sigerist, 1961; Sharma, 1992; Jingfeng & Yan, 2003). Herbal medicines have become an important part of human health care throughout the world and, in fact, are the original sources of many synthetic drugs. Until the last century, most medicines were derived directly from plant or animal sources. Despite the increasing use of factory-made synthetic drugs, herbal medicines have persisted as the "treatment of choice" for a multitude of health problems in populations throughout the world. According to a recent estimate of the World Health Organisation (WHO), 70-80% of the world population, especially in developing countries, rely on traditional medicine, mostly herbal drugs, for their primary healthcare needs (WHO, 2002). Medicinal plants play a vital role in the traditional medical system of different countries due to traditional knowledge and relative unavailability and high cost of Western medicines (Burke *et al.*, 2005; McHughes & Timmermann, 2005). The size of the worldwide market of herbal medicines is estimated to be around US $80 billion to US $100 billion and this market is expected to reach US $250 billion by the year 2010 (Mathur *et al.*, 2003).

Many patients turn to herbal medications as many mainstream prescription medicines exhibit unwanted side effects, such as gastric irritation and ulcers from NSAIDS, myopathy from statins and increased risk of breast cancer from hormone replacement therapy. Many popular herbal products including plant sterols (for reduction of cholesterol levels), cranberry juice (for urinary tract maintenance and combating infection) and red clover (relief of menopausal symptoms) have been tested for safety and efficacy using *in vitro*, *in vivo* and clinical studies.

Millions of people today use herbal medicines along with prescription and non-prescription medications. This is evident with the supplementation of Chinese herbal medicine and the reduction of chemotherapy-reduced nausea (Mok *et al.*, 2007). In recent years, Western countries have focused their attention on commercially available traditional herbal remedies (Fisher & Ward, 1994; Ni & Simile, 2001). These remedies are now widely used for chronic diseases for which synthetic medicines show poor therapeutic effects such as artemisinin for the treatment of malaria (Astin, 1998; Burg *et al.*, 1998; Yeh *et al.*, 2002). Furthermore, the therapeutic effectiveness of these remedies such as St John's wort (*Hypericum perforatum*) for the treatment of mild to moderate depression has been well recognised in patients who do not respond well to synthetic medicines (Clement *et al.*, 2006).

The therapeutic effects of herbal drugs are due to the presence of active components that differ widely in terms of structure and biological properties and possess distinct mechanisms of action or work together synergistically (Miller *et al.*, 2000). Herbal medicines are often used as a mixture of herbal materials in a defined formula, which contains multiple components. Hence, the clinical application of a herbal medicine may not necessarily be related to a single active component but rather the synergistic effect of multiple active ingredients. Thus, the pharmaceutical approach of testing for the content of a single component does not reflect the quality of an herbal preparation. Therefore, quality control methods which reflect the holistic approach of complementary medicine have to be developed in order to determine the chemistry foundation of herbal medicines (Liang *et al.*, 2004). It is well accepted in modern pharmaceutical science and traditional practice that quality is the foundation of efficacy and safety. On the other hand, it must be recognised that quality could not be determined alone without the support of efficacy and safety evidences of the herbal medicines under evaluation (WHO, 1993; WHO, 1998).

There are many avenues available that advocate the efficacy and safety of herbal preparations on an international basis. Monographs and pharmacopoeias continually review experimental data in order to update formulae for herbal preparations. Regulatory authorities such as Food and Drug Administration (FDA) and Therapeutics Goods Administration (TGA) outline and follow codes of good laboratory practice (GLP) and good clinical practice (GCP) which promote strict documentation. Medicinal standard is a government technical regulation on medicinal quality and identification methods in China. It is a legal document which must be followed by all manufacturers, businesses, applications, testing and regulatory bodies. The establishment of quality standard must match three preconditions: consistent formula ingredients, reliable source materials and dependable production procedures (Wang, 1994). The Cochrane Collaboration enlists the help of over 90 countries in documenting randomised controlled trials in the Cochrane reviews.

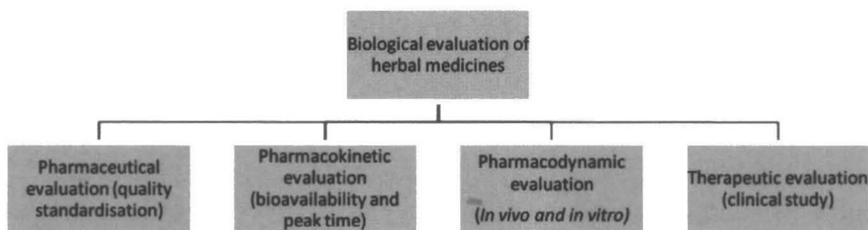

Fig 1. Level of evidence in the bioequivalence study of herbal products

Thus, in order to complement analytical quality control of herbal medicines, we propose a biological evaluation platform of herbal medicines (Fig 1) which encompasses traditional clinical usage and modern day approaches of *in vitro, in vivo* studies and controlled clinical trials.

UNDERSTANDING BIOLOGICAL ACTIVITIES IN TRADITIONAL CHINESE MEDICINE

Traditional Chinese medicine (TCM) is a medical system based on theory, pathology, diagnosis, treatment and herbal pharmacology principles which differ from those of orthodox/conventional medicine or Western naturopathy. TCM practice has developed from knowledge accumulated through clinical observation and treatment practice over several millennia. Text books and monographs can be consulted for basic knowledge and modern research on TCM (Chan & Lee, 2002; Bensky *et al.*, 2004; Maciocia, 2005).

The basis of TCM treatment is *via* the four natures (qi) of Chinese herbal medicines: cold, cool, hot and warm properties derived from the different actions of the drugs on the human body and their therapeutic effects. Some herbs are slightly cold or warm and are called neutral nature. Generally, the principle of prescribing drugs which are used to treat hot syndrome are mostly cold or cool in nature; drugs having the therapeutic effect on cold syndrome are warm and hot. Modern research has shown some common features on the scientific nature of the four natures: for example, the warm and hot herbs have positive effect on the heat generating procedures. *Aconitum carmichaeli* is an internal warming, hot and pungent herb and is used to restore yang. Its active substance is identified as higenamine, a β-agonist with structure similar to catecholamines such as adrenaline. Ephedrine can also directly stimulate β-receptor. There are many other herbs containing catecholamine analogues or relevant compounds (Tang & Eisenbrand, 1992). Table 1 summaries a selection of herbs and active components according to their five natures in TCM practice.

Thus, traditional knowledge of herbal plants should be consulted when assessing their quality, safety and pharmacological activities.

IMPORTANCE OF BIOLOGICAL STUDIES IN QUALITY CONTROL OF HERBAL MEDICINES

The primary objective of quality control of herbal medicines is to ensure its safety and efficacy (Gong *et al.*, 2003; Liang *et al.*, 2004; Li *et al.*, 2008a). The complexity of herbal cultivation and manufacturing process, from planting to selling, not only affects its

Table 1. Active components of herbs of the five natures (active component in brackets)

Cool	Cold	Warm	Hot	Neutral
Salvia miltiorrhiza (cryptotanshinone)	*Coptis chinensis* (berberine)	*Morinda officinalis* (monotropein)	*Aconitum carmichaeli* (higenamine (demethylcoclaurine)	*Lycium barbarum* (polysaccharides)
Paeonia suffruticosa (paeonol)	*Phellodendron amurense* (berberine)	*Panax ginseng* (ginsenosides)	*Ephedra sinica* (ephedrine)	*Glycyrrhiza uralensis* (glycyrrhizin)
Momordica charantia (cucurbitane triterpenoids)	*Scutellaria baicalensis* (baicalin)	*Crataegus pinnatifida* (flavonoids- *e.g.* vitexin, rutin and quercetin)	*Citrus reticulata* (synephrine)	*Dioscorea opposite* (dioscin)
Pueraria lobata (puerarin)	*Rehmannia glutinosa* (raw rehmanniosides)	*Tripterygium wilfordii* (triptolide)	*Cinnamomum cassia* (cinnamaldehyde)	*Codonopsis pilosula* (polysaccharides, phenylpropane glycosides *e.g.* syringin & tangshenoside I)
Forsythia suspensa (forsythin)	*Lonicerae japonica* (luteolin)	*Astragalus membranaceus* (astragalosides)	*Zingiber officinale* (gingerol)	*Gastrodia elata* (gastrodin)

quality but also its efficacy and safety. The efficacy of materials obtained from different sources is an important criterion for their quality. Therefore, herbs from different sources should be assessed and compared with their special pharmacological effects for the quality control.

Modern research has revealed the ways in which many herbs act through one or more than one active component. Many active components have been isolated from herbal medicines and some of these have become an important part of modern pharmaceutical development. They include ephedrine for asthma (from ephedra (*Ephedra sinica*), artemisinin for malaria (from Chinese wormwood (*Artemisia annua*), berberine, an antibacterial and antidiabetic component (from Chinese goldthread (*Coptis chinensis*), anthraquinone glycosides in rhubarb (*Rheum officinale*) and gingerols in Ginger (*Zingiber officinale*) (Tang & Eisenbrand, 1992). Also, the whole plant is usually used for convenience, however, the active compound(s) may be located in the respective plant parts including roots, leaves, flowers, stems, fruit, seeds, rhizomes and bark. In many cases, compounds may work synergistically, whereby two or more compounds work together in order to achieve the desired therapeutic outcome. The additive synergistic effect combined with multiple botanical treatment has been shown *via* a large number of biological studies. The combined treatment of medicinal mushroom *Ganoderma lucidum* and the herb *Duchesnea chrysantha* extracts (GDE) caused a synergistic induction of mitochondrial damage and apoptosis in human leukaemia HL-60 cells. It appeared that a single exposure to *Duchesnea* or *Ganoderma* extract exerted minimal effects on the apoptotic protein level or caspase activity, which, by itself, was insufficient to activate the mitochondria-dependent apoptotic pathway (Kim *et al.*, 2007). *Scutellaria baicalensis* and grape seed proanthocyanidins acted synergistically to scavenge ROS and potentially enhancing their antioxidant efficacy. However, the active principle(s) remain inconclusive. This finding allows lower dosages of each drug to be feasible and therefore eliminating the risk of side effects when higher doses of the single herb is used (Shao *et al.*, 2004). In the case of the Feverfew extract, individual flavonoids (apigenin and luteolin) may have moderate to weak synergistic effects with parthenolide on the inhibition of cancer cell growth of Hs605T, MCF-7 and SiHa cancer cell lines (Wu *et al.*, 2007). In the study on prostate cell viability, findings from Adams and colleagues (2006) have revealed that a combination of *Scutellaria baicalensis* and *Dendranthema morifolium* were additive with a trend toward synergy, whereas Dendranthema morifolium and *Rabdosia rubescens* together were additive. However, *Scutellaria baicalensis* and *Glycyrrhiza*

uralensis extract combinations showed antagonism. The extracts inhibited each other to the point of rendering their action insignificant. The four extracts together were significantly more effective than the two-by-two combinations and the individual extracts alone. Some further information about the synergistic interactions in phytomedicines can also be found in a review by Williamson (2001).

Thus, the active components of most herbal medicines and the synergistic nature of active components in herbal extracts remain largely unknown. Hence, biological studies are tremendously useful for illustrating the efficacy of botanical components, proving synergistic effects of multiple herbal treatments and establishing safety parameters. Nonetheless, it appears that biological methods fail when saponin preparations are used for biological tests, since the biological activity of saponin is closely correlated with the chemical structure of individual saponins and also their concentration may vary with different factors such as growth locality and extraction methods. Furthermore, poor characterisation is the main factor in interpreting findings and differences in the data obtained on the same preparation by different laboratories (Oleszek, 2002). Therefore, the efficacy and safety profiles of herbal preparations can not be achieved without the application of chromatographic fingerprinting approaches.

INTEGRATING CHEMICAL FINGERPRINTING WITH PHARMACODYNAMIC AND PHARMACOKINETIC STUDIES

The construction of chromatographic fingerprints aims to evaluate the quality of herbal medicines *via* authentication and identification of chemical profile. The application of chromatographic fingerprints as quality control approaches is based on the concept of phytoequivalence. The authenticity, sameness and stability of herbal medicines can be based on their active compounds and standardised herbal materials. However, lack of reference compounds and knowledge of bioactive chemical ingredients are the limits for chromatographic fingerprinting for quality control of herbal medicines. The method on its own does not reflect the pharmacodynamic and pharmacokinetic characteristics (Li *et al.*, 2008a).

The complex nature of the compounds in herbal medicines can be illustrated by propolis. Using HPTLC, propolis from various geographical regions showed profound variability in the active compound caffeic acid phenethyl ester (CAPE) (Peng *et al.*, 2002). The HPTLC fingerprint of propolis is a measurement of characteristic ingredients of the propolis sources, but not their therapeutic efficacy. Little information is available to correlate the fingerprints of propolis

to its bioactivity, stability, bioavailability, clinical efficacy and safety. Biological studies are still required to establish its quality standards based on its therapeutic value.

Hypericum japonicum is widely used as a Chinese herbal medicine for the treatment of bacterial diseases, infectious hepatitis, gastrointestinal disorder, internal haemorrhage and tumours. The herb contains xanthones, chromenes, flavonoids, dipeptide derivatives and phloroglucinol derivatives. Of these components, flavonoids are commonly considered as the major bioactive constituents (Su *et al.*, 2008). Chemical chromatographic fingerprint profiles of *Hypericum japonicum* from different habitats have been previously studied. Variations among different province regions have been observed and indicate the existence of 2 to 3 fingerprint patterns (Yang *et al.*, 2005; Su *et al.*, 2008). Its anti-cancer and anti-inflammation activities have been documented (Morikawa *et al.*, 2003; Chen *et al.*, 2006b; Morikawa *et al.*, 2007; Rogerio *et al.*, 2007). However, no information is available to correlate *Hypericum japonicum* from different regions, chemical fingerprint profiles, biological activities such as anti-inflammation and anti-cancer activities, pharmacokinetic characteristics and clinical efficacy.

Ideally, chemical fingerprinting analysis, in combination with pharmacological assessment, is suggested to be an essential tool fulfilling a herbal quality control task. This amalgamated approach enables reproducible quality and efficacy of botanical products and is particularly useful when the active compound(s) have not yet been identified in medicinal plants (Piersen *et al.*, 2004). Development of a red clover (*Trifolium pratense* L.) standardised extract for evaluation in Phase I and Phase II clinical trials from The National Institutes of Health (NIH) Office of Dietary Supplements, University of Illinois at Chicago (UIC), is a prime example of standardisation of bioactives, implying a correlation between the chemical content and bioassay activity. It should be noted that this correlation does not always guarantee efficacy at a medicinal target and this relationship must be substantiated by clinical testing (Piersen *et al.*, 2004). This ideal concept of quality standardisation has also been used in a large number of studies as shown in Table 2.

With regard to herbal medicines, most research works have focused on the pharmacological effects of the ingredients. Little has been done on the metabolism of herbal medicine in the body. To evaluate the influence of the herbal compounds and their beneficial health effects, it is important to monitor their concentration occurring in biological samples. Despite the benefits of these components, their bioavailability after oral administration is considered to be a limiting factor. After ingestion, flavonoid glycosides are thought to be first

Table 2. Recent chromatographic and biological activity studies of herbal medicine products

Herb	Active compound(s)	Chromatographic approach(es)	Biological activity test(s)	References
Tanacetum parthenium	3,5-, 4,5- and 3,4-di-*O*-caffeoylquinic acids (DCQAs)	Open column chromatography, HPLC, GC-MS, LC-MS and NMR	*In vitro* bioassay-orientated antioxidant tests	(Wu *et al.*, 2007)
Polygonum cuspidatum	Emodin	TLC and HPLC	Recombinant yeast screening (YES) assay	(Zhang *et al.*, 2006a)
Piper longum	Pipataline, pellitorine, sesamin, brachystamide B and quineensine	HPLC	α-glucosidase-I inhibitory assay (chromogenic method)	(Pullela *et al.*, 2006)
Pueraria lobata	Puerarin	HPLC	Recombinant yeast screening (YES) assay	(Zhang *et al.*, 2005a)
Scutellaria baicalensis	Baicalein and/or baicalin	HPLC	Cell viability assay and PGE_2 enzyme immuno-assay	(Ye *et al.*, 2004)

hydrolysed by microorganisms in the gastrointestinal tract to aglycones. Parent compounds such as Aconitum alkaloids decompose rapidly in the human body and are difficult to detect. Clinical dosage concentrations can also be low (pg/mL). Thus, metabolites provide chemical information for identification (Zhang *et al.*, 2005b). Metabonomic (or metabolomic) analysis of possible active compounds and their metabolite profiles in human or animal serum has been explored as a new method for quality control of herbal medicines. Extremely sensitive preparative and analytical methods are therefore required (Ding *et al.*, 2006). Solid-phase extraction (SPE) is a powerful sample preparation technique currently available for rapid clean-up and enrichment of sample analytes preceding chromatographic analysis. Compared to traditional liquid-liquid extraction (LLE), SPE provides major advantages in terms of simplicity, high throughput, robustness, and, in most cases, greater cost effectiveness. A wide range of SPE resin chemistries is now available for various applications. The sampling format has also extended from simple packed disposable syringes to cartridges, disks, SPE pipette tips, 96-well and 384-well micro-plates. Therefore, SPE sample preparation techniques provide improved assay standardisation and hence better reproducibility (Li *et al.*, 2006a). Validated analytical methods such as HPLC, LC-MS and CE with improved sensitivity have been developed to determine the fate of herbal medicines in the body after oral intake. Animals or humans are given a particular dosage of the extract. Plasma, blood or urine samples are usually taken. Blood samples are collected prior to dose and at subsequent intervals, usually to 24 h. Pharmacokinetic models with statistical analysis have also been designed and used to study the parameters of quercetin in human blood and urine samples (Moon *et al.*, 2008). Using the metabolomic method, two flavonoids, quercitrin and isoquercitrin from *Hypericum japonicum* have been identified in rat serum (Li *et al.*, 2008b). Prasain *et al.* (2004) reviews the determination of flavonoids in biological sample. Table 3 summarises recent analytical methods used to determine the compound's fate in the body

In conclusion, chemical fingerprints are vital in the study of many herbal medicines and should be included with any biological study involving extracts. There is a need to correlate the fingerprinting data with pharmacodynamic and pharmacokinetic parameters in order to standardise herbal medicines. Different quality standards may be required for different biological activities and clinical application.

BIOEQUIVALENCE

Unlike chemically defined drugs, herbal medicinal products contain complex mixtures of different compounds. In many cases, the active

Table 3. Analytical methods employed to detect herbal compounds in biological fluids

Herb	Compound	Disease	Analytical method	Biological system	References
Artemisia annua L.	Artemisinin	Malaria	HPLC	Human plasma	(Rath *et al.*, 2004)
Andrographis paniculate	Andrographolide phase II glucuronide conjugate metabolites	Infectious diseases (cold, fever)	Prep-HPLC, NMR, MS	Human urine	(Cui *et al.*, 2005)
Arctostaphylos uvaursi (L.) bearberry leaf	Arbutin	Lower urinary tract infections	CE	Rat urine	(Glockl *et al.*, 2001)
Ginkgo biloba	Flavonoids and terpene lactones	Antioxidant activity (brain, nerve cells)	HPLC/MS	Human urine	(Ding *et al.*, 2006)
Aconitum medicinal herbs	Aconitum alkaloids	Inflammation (arthritis)	LC-ESI-MS	Human urine	(Zhang *et al.*, 2005b)
Rheum undulatum	Anthraquinones	Inflammation, bacterial	TLC, HPLC and LC-MS	Human plasma	(Lee *et al.*, 2003)
Quercetin-500 Plus®	Quercetin	Antioxidant activity	HPLC	Human blood and urine	(Moon *et al.*, 2008)
Boswellia serrata	11-keto β-boswellic acid	—	HPTLC	Human plasma	(Pozharitskaya *et al.*, 2006)

constituents responsible for efficacy are unknown. A marker compound is a characteristic compound often used to represent the quality standard for a standardised extract - it is often, but, not necessarily, one of the pharmacologically active compounds.

Thus, it is a challenge to assess and compare the quality of these products, as pointed out in a review by Loew and Kaszkin (2002). For extracts containing known active markers, the concept of essential similarity used with chemically defined substances can be applied. However, if the active components are not known, then data on selected chemical substances within an extract may not be sufficient to prove bioequivalence in terms of therapeutic effect. Therefore, to be effective, herbal medicinal products must meet comparable standards concerning the assessment of not only quality, but also efficacy and safety as standard preparations. In other words, herbal preparations should exhibit bioequivalence in a range of aspects including pharmaceutical equivalence (standardisation), biopharmaceutical equivalence (*in vitro* dissolution) and comparable biological effects (*in vitro* and *in vivo* models).

According to the Note for Guidance on the investigation of bioavailability and bioequivalence, medicinal products are pharmaceutically equivalent if they contain the same amount of active substance(s) in the same dosage forms that meet the same or comparable standards (EMEA, 2001). This principal is applied to herbal medicinal products as quality and quantity standardisation is essential to guarantee pharmaceutical equivalence. Hence, essentially similar extracts should be standardised to a defined content of active constituents, as well as equivalent, in terms of starting herbal materials, extractions solvents, manufacturing process and chromatographic fingerprints. In terms of biopharmaceutical equivalence, they should contain the same extract or extract fractions, the same presentation form, the same dose and *in vitro* qualitative and quantitative conformity (Loew & Kaszkin, 2002).

A medicinal product is therapeutically equivalent with another product if it contains the same active substance or therapeutic moiety and, clinically, shows the same efficacy and safety as that product, whose efficacy and safety has been established (EMEA, 2001). Depending on the type of herbal drug preparation, this may be demonstrated in terms of bioequivalence in pharmacokinetics, bioassays or pharmacological studies. For equivalence in bioassays and pharmacological studies, essentially similar extracts should possess the same chromatographic profile qualitatively and quantitatively in *in vitro* and *in vivo* studies. However, in practice, indirect evidence or where active components are unknown, the use

of pharmacokinetic surrogates for bioequivalence is generally the most appropriate procedure to substantiate the therapeutic equivalence between medicinal products (Loew & Kaszkin, 2002). Two (herbal) medicinal products are said to be bioequivalent if they are pharmaceutically equivalent as well as show comparable bioavailability (rate and extent) after administration in the same molar dose are similar to such degree that their effects, with respect to both efficacy and safety, will be essentially the same. Thus, bioequivalence is assumed if the 90% confidence interval of the ratio (test/reference) for the parameters the area under the concentration versus time curves (AUC) and maximal concentration (Cmax) lies within the range of 0.85–1.25 (log transformed data). Statistical evaluation of tmax only makes sense if there is a clinically relevant claim for rapid release or action in relation to adverse effects. Bioequivalence may also be assumed if the non-parametric 90% confidence interval lies within clinically determined range (EMEA, 2001).

Few studies comparing the bioequivalence of different brands of a particular herbal product have been reported. In a dissolution and bioequivalent study of nine silymarin products (extracted from *Silybum marianum*) from the German market, three yielded 100%, 50% and 0% of silymarin after 1 h under official dissolution study conditions. A bioequivalence study of three of these products showed that the bioavailability of one product was two-fold greater than the other two preparations (Schulz *et al.*, 1997). Examination of several *Ginkgo biloba* L. products available on the United States market showed clear differences in the quantity of constituents and *in vitro* dissolution rates, with respect to pharmaceutical quality (Kressmann *et al.*, 2002b). Another study by this group also compared the bioavailability of two *G. biloba* L. brands, both claiming to contain the same quantity of flavone glycosides and terpene lactones. From the results, the bioavailability of ginkgolide A, ginkgolide B and bilobalide of these two brands were clearly different and did not show bioequivalence of the test and reference products. These results indicate that the pharmaceutical properties of a herbal medicinal product, in fact, have a significant impact on the rate and extent of drug absorption and most likely on efficacy in humans (Kressmann *et al.*, 2002a).

In summary, apart from qualitative and quantitative analysis, biopharmaceutical, pharmacokinetic and pharmacological studies of extracts are also necessary for the development of herbal medicinal products with high quality, clinical effectiveness and safety (Fig 1). *In vitro* bioassays and animal models function as broad spectrum tools for the evaluation of the biological activity. An appropriate

animal model design is one of the most powerful tools in determining the pharmacological profile of particular active chemical component(s) in herbal medicines. The results will help define the treatment parameters of herbal medicines leading towards further clinical research and applications (Teixeira & Fuchs, 2006).

EVALUATION OF ANTI-INFLAMMATORY ACTIVITY OF HERBAL MEDICINES

Anti-inflammatory activity of herbal medicines is one area of popular pharmacological study. Inflammatory responses are considered to be the main mechanism affecting many chronic diseases, including arthritis, cancer, asthma and atherosclerosis-related diseases. In normal physiologic conditions, a regulated response protects against further injury and repairs damaged tissue, while in pathologic situations, inflammation can result in tissue destruction and leads to organ dysfunction.

The inflammatory process begins with the release of mediators such as histamine, prostaglandins and cytokines from the affected tissues, therefore selective inhibition of pro-inflammatory cytokine expression is a major strategy in anti-inflammatory drug development (Hillier & Bunton, 2007). The two types of inflammation, namely acute and chronic inflammation, are categorised by the duration of response and the prominent inflammatory cell type involved. Acute inflammation is generally of short duration and is the result of an initial response by immune cells, primarily neutrophils, to an infectious agent (mainly bacteria). Chronic inflammation is a result of prolonged exposure to viable and inert state of pathogens, and involves lymphocytes, macrophages and plasma cells of immune system (Lydyard *et al.*, 2004). Therefore, the properties of anti-inflammatory agents should be based on the modulatory effects on any mediators, cytokines and/or chemokines involved in inflammatory processes and, thus, will have different biological models of assessment in laboratory studies.

Various types of inflammatory bioassays are currently available as presented in Table 4. Among the methods used for screening of anti-inflammatory drugs, one of the most commonly employed techniques is based upon the ability of such agents to inhibit the oedema produced in the hind paw of the rat after injection of phlogistic agents, such as brewer's yeast, formaldehyde, dextran, egg albumin, kaolin, Aerosil® and sulfated polysaccharides such as carrageenan or naphthoylheparamine. Other irritants such as histamine, xylene, arachidonic acid, phorbol myristate acetate, oxozolone, croton oil and formalin are also used. In general, the most effective and widely

Table 4. Types of inflammatory bioassays

In vitro assays	• TNF-induced adhesion molecules enzyme-linked immunosorbent assay (ELISA) • IL-8 ELISA • Electrophoretic mobility shift assay (EMSA) for NF-κB • PGE2 immunoassay • COX-2/Trypsin/15-Lipoxygenase inhibition assay • Human purple acid phosphatase (PAP) inhibitor assay • Cellular-based cytokines assay (*e.g.* LPS pre-stimulated macrophage, PGE2 released, luciferase-GFP macrophage assay etc.)
In vivo models	**Acute inflammation** • Carageenan-induced paw oedema in rats • Histamine-induced paw oedema in rats • Acetic acid-induced vascular permeability • Xylene-induced ear oedema (thickness and weight parameter) • Arachidonic acid (AA)-induced ear oedema • 12-O-tetradeconylphorbol-13-acetate (TPA)-induced ear oedema in mice • Oxazolone-induced ear oedema in mice **Sub-acute inflammation** • Carrageenan-induced granuloma pouch model • Formalin-induced paw oedema **Chronic inflammation** • Cotton pellet-induced granuloma in rats • The Glass Rod granuloma

used model for inflammation is carrageenan-induced paw oedema (Suralkar *et al.*, 2008).

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used anti-inflammatory medicines. In general, NSAIDs such as ibuprofen, aspirin and naproxen prevent the synthesis of prostaglandins by counteracting the cyclooxygenase (COX) enzyme, thus blocking the inflammatory process and subsequently pain. In addition to conventional synthetic drugs, there are many herbal preparations that have been pharmacologically/clinically proven to possess anti-inflammatory properties (Chrubasik *et al.*, 2007). 'Heat clearing' herbs are commonly used in TCMs for conditions that are associated with inflammation. Previous *in vitro* studies have concluded

that baicalein, a compound isolated from *Scutellaria baicalensis*, dose-dependently inhibited interleukin (IL)-1β and tumour necrosis factor (TNF)-induced endothelial leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) expressions (Kimura *et al.*, 1997; Krakauer *et al.*, 2001). Furthermore, wogonin, another compound extracted from *S. baicalensis*, inhibited the gene expressions of cyclooxygenase (COX)-2 and TNF in *in vivo* inflammatory models (Chi *et al.*, 2003). Yun *et al.* (2008) suggested that the anti-inflammatory properties of asiatic acid, derived from the leaves of *Centella asiatica*, may be as a consequence of its inhibition of iNOS, COX-2, IL-6, IL-1β and TNF expressions through the down-regulation of nuclear factor (NF)-κB activation.

In a human colonic epithelial cells bioassay, luteolin, a major flavonoid of Lonicera japonica, was found to possess inhibitory effects on TNF-induced IL-8 production, a chemokine that plays a central role in the initiation and maintenance of inflammatory responses in inflammatory bowel disease (Kim *et al.*, 2005). In another *in vitro* study, ochnaflavone, a biflavonoid isolated from the same herb, was identified to inhibit lipopolysaccharide (LPS)-induced nitric oxide formation due to its inhibition of NF-κB pathway, which may be the basis for the anti-inflammatory effects of *L. japonica* (Suh *et al.*, 2006). *Belamcanda chinensis*, another commonly used Chinese anti-inflammatory herb, hinders the NF-κB pathway through the action of its flavonoid, irigenin (Ahn *et al.*, 2006). Kim *et al.* (1999) suggests another anti-inflammatory mechanism of *B. chinensis* is the inhibition of prostaglandin E2 (PGE2) production by tectorigenin and tectoridin due to the inhibition of the induction of COX-2 in experimental inflammatory cells. In laboratory studies, *Coptis chinensis* (berberine as active compound) effectively protects against Angiotensin (Ang)-II-induced endothelial inflammation (Ko *et al.*, 2007). In addition, *Gardenia jasminoides*, (with respect to genipin and geniposide as the herb's active compounds), showed acute anti-inflammatory activities in carageenan-induced rat paw oedema, inhibited vascular permeability (induced by acetic acid) and production of exudates and nitric oxide in the rat air pouch oedema model (Koo *et al.*, 2004; Koo *et al.*, 2006) (Refer to Table 5 for a summary of additional herbs with respect to their related active constituent).

Recent observations suggest that regulation of inflammation and other related processes *via* specialised receptors may modulate the reduction of atherosclerosis and the whole metabolic syndrome (Fruchart *et al.*, 2001; Barbier *et al.*, 2002; Francis *et al.*, 2003). Nuclear receptors such as PPAR and LXR and related pathways have been implicated in atherosclerotic inflammatory processes. In a review by Huang *et al.* (2005b), herbal medicines have revealed biologically

Table 5. Recent progress in anti-inflammatory studies of herbal medicines

Methods	Herbs	Active compound(s)	Reference(s)
TNF-induced adhesion molecules ELISA	*Scutellaria baicalensis*	Baicalein	(Kimura *et al.*, 1997)
	Camellia oleifera	Sasanquasaponin	(Huang *et al.*, 2005a)
	Paeonia suffruticosa	1,2,3,4,6-penta-O-galloyl-β-D-glucose	(Kang *et al.*, 2005)
	Paeonia lactiflora	Paeonol	(Nizamutdinova *et al.*, 2007)
TNF/Ang-II-induced monocyte chemoattractant protein (MCP)-1 assay	*Coptis chinensis*	Berberine	(Ko *et al.*, 2007)
	Paeonia suffruticosa	1,2,3,4,6-penta-O-galloyl-β-D-glucose	(Kang *et al.*, 2005)
TPA/AA/Dimethylbenzene/xylene-induced ear oedema	*Scutellaria baicalensis*	Wogonin	(Chi *et al.*, 2003)
	Chrysanthemum indicum	Flavonoids	(Cheng *et al.*, 2005)
	Chrysanthemum morifolium	Heliaol	(Akihisa *et al.*, 1996)
	Houttuynia cordata	Methyl nonyl ketone	(Lua *et al.*, 2006)
PGE_2, IL-6, IL-1β & TNF assay	*Centella asiatica*	Asiatic acid, baicalein	(Krakauer *et al.*, 2001; Yun *et al.*, 2008)
	Sophora japonica	Oxymatrine	(Xua *et al.*, 2005)
	Artemisia annua	Dihydroarteannuin	(Li *et al.*, 2006b)

Table 5. *Contd.*

Methods	Herbs	Active compound(s)	Reference(s)
	Phyllantus urinaria	Trimethyl-3,4-dehydrochebulate, methyl brevifolincarboxylate	(Fang *et al.*, 2008)
IL-8 ELISA	*Lonicera japonica*	Luteolin	(Kim *et al.*, 2005)
iNOS promoter-luciferase construct & NF-κB assay	*Lonicera japonica*	Ochnaflavone	(Suh *et al.*, 2006)
	Paeonia lactiflora	Paeonol	(Nizamutdinova *et al.*, 2007)
	Sedum sarmentosum	Flavonoids	(Jung *et al.*, 2008)
EMSA & PGE_2 immunoassay	*Belamcanda chinensis*	Irigenin, tectorigenin and tectoridin	(Kim *et al.*, 1999; Ahn *et al.*, 2006)
	Artemisia annua	Dihydroarteannuin	(Li *et al.*, 2006b)
	Scutellaria baicalensis	Oroxylin A	(Chen *et al.*, 2000)
Carrageenan-induced rat paw and air pouch oedema, acetic acid-induced vascular permeability and croton oil-induced ear oedema in mice	*Gardenia jasminoides*	Geniposide and genipin	(Koo *et al.*, 2004; Koo *et al.*, 2006)
	Sedum sarmentosum	Flavonoids	(Jung *et al.*, 2008)

active compounds capable of nuclear receptor modulation such as PPAR and LXR. Two gypenoside saponins from *Gynostemma pentaphyllum* revealed PPAR-α and LXR-α activation, respectively (Huang *et al.*, 2005c, 2006).

Due to the adverse reactions of many synthetic anti-inflammatory agents, products derived from natural sources are becoming extremely popular (Agarwal, 2005). It has been demonstrated that the role of pharmacological bioassays to validate the anti-inflammatory activities of various herbal medicine is crucial to reflect the preliminary evidences of efficacy, including safety issues, prior to clinical studies. Thus, it is anticipated that herbal pharmacology assessment, based on bioassay studies, can be a widely acceptable quality assurance method in herbal medicines research.

EVALUATION OF ANTI-DIABETIC ACTIVITY OF HERBAL MEDICINES

Diabetes mellitus is defined as an elevation of glucose caused by a relative or absolute deficiency of insulin (Mycek, 2000). Diabetes mellitus is one of the most predominate chronic diseases in modern society. This serious disease has a high prevalence worldwide and major implications in other vascular-related diseases including stroke and coronary events (Cheung *et al.*, 2007). Significant amount of research has been carried out to develop conventional medicines for its 'cure'. However, very limited amounts of these medicines are able to be used with great success without serious side effects. This has instigated the development of natural anti-diabetic agents, with a better or at least similar efficacy to the currently used diabetic drugs and relatively fewer side effects.

A significant amount of research has been carried out on herbal plants to analyse the chemical compositions and their relevant biological activities for the treatment of diabetes (Zareba *et al.*, 2005). For example, there has been much progress on the anti-diabetic effects of berberine, a natural alkaloid isolated from *Coptis chinensis* (Huang lian). The hypoglycaemic effect of berberine was discovered when it was used to treat diarrhoea in diabetic patients in China (Ni, 1988). Since then, berberine has been used as an anti-hyperglycaemic agent by many physicians in China (Yin *et al.*, 2008). Berberine was reported to reduce body weight and cause a significant improvement in glucose tolerance in animal models of metabolic syndrome. 5'AMP-activated protein kinase (AMPK) was proposed to mediate the metabolic activities of berberine (Lee *et al.*, 2006). Further studies suggested that berberine enhanced glucose metabolism by stimulation of glycolysis, which is related to inhibition of glucose oxidation in

mitochondria. Thus, berberine-induced AMPK activation is a likely consequence of mitochondria inhibition that increases the AMP/ATP ratio (Yin *et al.*, 2008).

Currently, research on anti-diabetic activities in herbal medicines not only targets the control of diabetes, but also includes the study on the pathophysiology and complications of diabetes. Lipid abnormalities are found to be predominantly related to the diabetes disease cycle (O'Brien *et al.*, 1998) and an accumulation of low-density lipoprotein and triglycerides levels are the main cause of serious cardiovascular disease and stroke (Cho *et al.*, 2006). A recent study showed that a novel berberine derivative, dihydroberberine, was able to improve *in vivo* efficacy in terms of counteracting increased adiposity, tissue triglyceride accumulation and insulin resistance in high-fat-fed rodents (Yin *et al.*, 2008). Berberine may also ameliorate retinopathy complication by modulating PPAR α/β/γ protein levels in type 2 diabetic rat retinae (Zhou *et al.*, 2007). Furthermore, to prevent and delay the diabetic complications such as diabetic nephropathy, vasculopathy and retinopathy, flavonoids and their derivatives from silymarin and berberine were found to be the active components for aldose reductase inhibitor activity (Feng *et al.*, 2005).

Currently, many methodologies in herbal medicine diabetes research are being vigorously reviewed. This is very crucial to reinforce the current research progress and also for future research planning. Antioxidant activity assays, α-amylase activity assay, bioassay on HepG2 for lipid lowering properties are among them. Classical anti-diabetic methodologies include α-glucosidase inhibition activity assay, cellular assay on glucose metabolism and the design of animal models in various aspects of diabetes complications.

α-Glucosidase is one of the glucosidase enzymes located in the brush-border surface membrane of the intestinal cell (Andrade-Cetto *et al.*, 2008). This is one of the key enzymes involved in the digestion of carbohydrates. α-Glucosidase inhibitor acts by blocking the activity of α-glucosidase, leading to the reduction of glucose level in the blood just after food intake (postprandial effect). It has been demonstrated that α-glucosidase inhibitor is beneficial for the treatment of postprandial hyperglycaemia in type 2 diabetes (Andrade-Cetto *et al.*, 2008). Flavonoids and polyphenols are the main active components of herbal plants which possess α-glucosidase inhibitor activities (Mai *et al.*, 2007; Andrade-Cetto *et al.*, 2008). Several studies have been conducted on various herbs with significant success in its α-glucosidase inhibitory action, including *Salacia oblonga, Punica granatum*, pine bark, green teas and *Curcuma longa* (Kim *et al.*, 2002; Li *et al.*, 2005; Du *et al.*, 2006).

Another approach in the treatment of diabetes mellitus is through the regulation of glucose metabolism in the liver. The liver is one of the most important organs for glucose metabolism whereby it appears to synthesise more glucose than any other organ in the body (Yin *et al.*, 2002). HepG2, human hepatocellular carcinoma line, is one of the most common experimental cell lines employed in determining the therapeutic effects of herbal medicines. The regulatory effect of glucose metabolism of berberine was examined in HepG2 cell lines and results showed that there was a defined glucose-lowering activity in a dose-dependent manner (Yin *et al.*, 2002). In HepG2 cell lines, a natural steroidal hormone, ecdysterone (indicated in anabolic and tonic action) was also evaluated. The result showed that ecdysterone exhibited glucose metabolism modulating action (Chen *et al.*, 2006a).

Animal models are extensively employed in the study of diabetes and include mice, rats, pigs and rhesus monkeys. Many references are available to help researchers select the appropriate animal model (Shafrir, 2007; Srinivasan & Ramarao, 2007). Zucker diabetic fatty (ZDF) rats have been frequently used as a genetic model for obesity and type 2 diabetes (Skalkos *et al.*, 2005). Regulation in cardiac fibrosis and lipid metabolism activities in *Salacia oblonga* and the enhancement of PPAR-γ, mRNA and protein expressions activity in *Punica granatum* are examples of recent animal studies (Li *et al.*, 2004a; Huang *et al.*, 2005d).

Keeping quality control and assurance of herbal medicines with respect to efficacy and safety in mind, it is hoped that the development of these scientific approach in herbal medicines will further strengthen the justification of anti-diabetic herbs in clinical use. Table 6 presents a summary of preclinical studies available for the study of the effects of anti-diabetic herbal medicines.

DEVELOPMENT OF NEW *IN VITRO* ASSAYS

There are many technical difficulties facing *in vitro* and *in vivo* biological studies. An *in vitro* cellular bioassay may not reflect the *in vivo* dosage, bioavailability and safety. Another issue is the need for large amounts of extract or isolated compounds for animal studies. The challenge is to correlate these parameters in a simplified model. One critical technique is to evaluate *in vitro* cellular toxicity caused by crude herbal extracts using the trypan blue exclusion method before assessing other *in vitro* biological effects. This was shown in an evaluation of the anti-inflammatory effect of heat clearing herbs (Omar *et al.*, 2008). Recent progress in the development of high-throughput, molecular biology techniques and their application in

Table 6. Recent progress in anti-diabetic studies on herbal medicines

Methods	Herbs	Active compound(s)	Reference(s)
α-Glucosidase inhibitory assay	*Punica granatum*	Oleanolic, ursolic, gallic acids	(Skalkos *et al.*, 2005; Katz *et al.*, 2007)
Protein tyrosine phosphatase 1B (PTP1B) inhibitor colorimetric assay	*Cornus officinalis*	Ursolic acid	(Zhang *et al.*, 2006b)
	Lagerstroemia speciosa, *Tiarella polyphylla*	Corosolic acid	(Shi *et al.*, 2008)
Intestinal brush border membrane vesicles (BBMV); human skin fibroblasts cell line Hs68; and mouse adipocytes 3T3-L1 glucose uptake assays; rat hepatoma cell line H4IIE glucose production assays	*Poeonia suffruticosa*	Poeonol	(Lau *et al.*, 2007)
	Pueraria lobata	Puerarin	(Xu *et al.*, 2005)
Nicotinamide and streptozotocin-induced diabetic rats/mice	*Cordyceps* sp.	Polysaccharides	(Lo *et al.*, 2004)
	Stephania tetrandra	Fangchinoline, tetrandrine	(Tsutsumi *et al.*, 2003; Ma *et al.*, 2007)
	Pueraria thunbergiana	Tectorigenin, kaikasaponin III	Lee et al., (2000)
	Panax ginseng	Ginsenoside Re	
Zucker diabetic fatty (ZDF) rats	*Panax quinquefolius*	Ginsenosides	(Banz *et al.*, 2007)
	Platycodon grandıflorum	Saponins	(Kim *et al.*, 2000)

Table 6. *Contd.*

Methods	Herbs	Active compound(s)	Reference(s)
	Punica granatum	Oleanolic, ursolic, gallic acids	(Skalkos *et al.*, 2005; Katz *et al.*, 2007)
Alloxan-induced diabetic rabbits/rats	*Lycium barbarum*	Polysaccharides	(Luo *et al.*, 2004)
	Rehmannia glutinosa	Oligosaccharides	(Zhang *et al.*, 2004)
	Momordica charantia	Polypeptide (p-insulin), sterol glucoside (charantin, pyrimidine nucleoside (vicine)	(Li *et al.*, 2004b; Reyes *et al.*, 2006)
	Andrographis paniculata	Andrographolide	(Reyes *et al.*, 2006)
	Punica granatum	Oleanolic, ursolic, gallic acids	(Jafri *et al.*, 2000; Katz *et al.*, 2007)
KK-Ay mice (animal model of genetic type II diabetes)	*Anemarrhena asphodeloides*	Mangiferin	(Miura *et al.*, 2001)

system-wide experimental approaches allows an alternative methodology to address the above problem in regard to toxicity dose for biological evaluation. The cytokinesis-block micronucleus cytome assay (CBMN) is a comprehensive system for measuring DNA damage, cytostasis and cytotoxicity (Fenech, 2007). The CBMN assay offers improved sensitivity as the results are not confounded by altered cell division kinetics caused by cytotoxicity of agents tested or suboptimal cell culture conditions. In its current basic form, the CBMN assay can be used to measure genotoxicity and cytotoxicity using simple morphological criteria, such as chromosome breakage, chromosome loss, chromosome rearrangement, cell division inhibition, necrosis and apoptosis. Moreover, the CBMN assay can evaluate both genotoxic and cytotoxic potential of not only crude extracts but also fractionated extracts which will give a more precise toxicity dose for evaluation of the safety of medicinal plants. From this, the antigenotoxic properties found within a number of plant derived compounds can be interpreted as an indication of inflammation protective properties. Thus, the CBMN assay of the effects of herbal extracts offers a new insight into diseases in which inflammatory processes are at work such as cardiovascular disease, asthma, neurologic disease, inflammatory bowel disease and rheumatoid arthritis.

Reporter gene assays are highly versatile and reliable bioassays increasingly used as high-throughput drug screening platforms. Reporter genes are frequently used as indicators of transcriptional activity or activation of particular signalling pathways within the cell. New *et al.* (2003) reviews applications of reporter gene assays in natural products. Biological activities examined *via* the reporter gene assays include inflammation and diabetes (PPAR, LXR), postmenopausal disorders (ERE, Gal4), drug metabolism (CYP3A4) and antimicrobial effects (SecA). One particular advantage for herbal medicine development is its ability to screen extracts, fractions and purified compounds and potentially identify their mechanism of action through signalling pathways.

Bioassay-guided fractionation of herbal plants can be a lengthy process and hinder drug research. Crude fractions are fractionated using chromatographic means such as partitioning or HPLC. The fractions are subsequently tested in a biological assay. Several cycles of fractionation and testing may be required to obtain the pure active compound. LC-MS and NMR is required to determine the final structure. An innovative approach suggested by Koehn and Carter (2005) is to combine a real time read out bioassay with an analytical instrument, thus creating an 'on-line' bioassay. For example, the sample of interest is injected through a HPLC and then split for the

bioassay (such as an enzyme assay) and the detector (such as the mass spectrometer). The peak data obtained from the assay is correlated to the MS data and thus provides information on the bioactive compound (Schenk *et al.*, 2003; Cummins *et al.*, 2003).

Computers can also provide an *in vitro* platform. Molecular modelling has given insight into receptor interaction with molecules. Main banks of the coordinates of receptors can be found on the RCB Protein Data bank on the worldwide web. Coordinates of structures can be found in crystallography libraries or evaluated using various modelling programs. The results can then be correlated with biological data, eliminating the great time and effort required for biological testing. In a paper by Huang *et al.* (2005c), Gypenoside TR1 from *Gynostemma pentaphyllum* activated LXR-α in a receptor-based assay and molecular modelling predicted its orientation in the LXR-α receptor protein. In a recent study by Salam *et al.* (2008), structure-based virtual screening of the PPAR-γ ligand binding domain against a natural product library revealed 29 potential agonists. *In vitro* testing of this list identified six flavonoids which stimulated PPAR-γ transcriptional activity in a transcriptional factor assay.

Thus, new technologies are helping to eliminate many of the complexities associated with the biological assessment and standardisation of herbal preparations and are providing new information on their safety, efficacy and potential modes of action.

CONCLUSIONS

It is estimated that there are at least 250,000 higher plants on earth of which between 25,000 and 75,000 plant species are used for traditional medicine (Schultes, 1972). Many phytochemical and biological studies focused on isolation and identification of bioactive compounds (Balandrin *et al.*, 1985; Verpoorte, 2000), the evaluation of biological activities of herbal medicines supported by quality standardisation has only gained increasing attention in recent times.

Since herbal medicines consist of a mixture of multiple bioactive components, they provide synergistic actions through multiple modes of action and can also provide combination therapies which can simultaneously target various elements of human diseases, providing a better efficacy and safety compared to single chemical entities. However, due to the complex nature and the large number of herbal materials and products world wide, the quality standardisation of herbal medicines is still an arduous task for researchers, industry and regulatory organisations. Thus, the development of herbal medicines from the rich traditional source requires an integrated

and multidisciplinary approach including pharmacognosy, analytical and pharmaceutical chemistry, pre-clinical and clinical pharmacology.

At present, most research involving herbal medicines concentrates on establishing biological activities of purified single compounds, or crude extracts without a defined fingerprint. The proposed quality and biological activity assessment platform (Fig 2) may have the effect of shifting the emphasis of herbal medicines research from single activity to multiple biological activities linking to various standardised extracts. Despite a long history of herbal use in a variety of pathological conditions, comprehensive scientific validation is lacking and methodologies are not vigorous. Media coverage can be very non-supportive and at times distorted, especially when adverse effects are reported due to the lack of credible scientific data entailing the specific bioactive ingredients and mechanisms of action.

In order for herbal medicines to achieve a sustained growth, an integrative quality standardisation and biological testing is required in terms of raw material authentication, process development, chemical characterisation, safety assessment and efficacy evaluation to qualify many herbal products as drug substances for registration or prescription. Solid scientific evidence to the functional claims would therefore be essential for herbal medicines to be accepted by the mainstream pharmaceutical market and to further contribute to the health and wellness of the mankind.

A significant amount of research in herbal medicines has been conducted in recent years to define the efficacy and safety of particular herbs, based upon their use in traditional medicines. Currently, various appropriate assay methods have been established and continuously being developed to identify the active components and

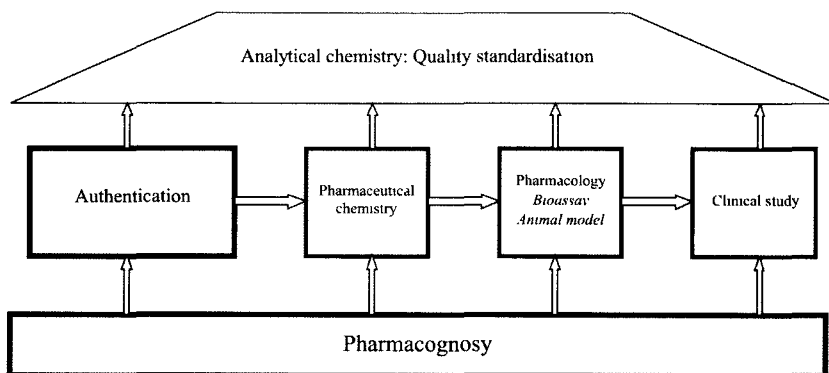

Fig 2. Quality control and biological activity assessment platform for herbal medicines

understand the mechanism of action of herbal plants in relation to their anti-inflammatory and anti-diabetic activities. Eventually, these developments will be integrated into the systematic biological evaluation and quality standardisation research platform, which will define the active components, their modes of action, quality standards, clinical safety and efficacy profiles of herbal medicine products.

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Chemical Analysis and Quality Control of Essential Oils

LAHLOU M.[1,*]

ABSTRACT

Essential oils are valuable natural products used as raw materials in many fields. They are products, generally of rather complex composition comprising the volatile principles contained in the plants, and more or less modified during the preparation process. Nowadays, the development and application of methodologies for the determination of the chemical composition of aromas and similar mixtures is a challenging task. Furthermore, the scientific and industrial value and applicability of an essential oil are strictly and strongly related to its quality. This latter is depending on different factors; so, the final product may range anywhere from excellent to mediocre. Therefore, the concept of "natural" is clearly not enough to guarantee best clinical results. Good or high quality oils are crucial to the success of aromatherapy treatments, including environmental ones. This paper is aimed at elucidating the most developed and sophisticated analytical techniques to study the chemical composition of essential oils. It is important to use good quality essential oils because their therapeutic benefits derive from their origins, their purity and chemical make-up. For this reason, essential oils should be subjected to rigorous quality control standards. Various steps for these standards are also given and discussed.

Key words : Aromatherapy, chemical analysis techniques, essential oils, purity, quality control, quality standards

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INTRODUCTION

Essential oils, also known as volatile oils or essences, are volatile secondary metabolites derived from various plants parts. They are 'products, generally of rather complex composition comprising the volatile principles contained in the plants, and more or less modified during the preparation process' (Lahlou, 2004a). They are used as raw materials in many fields, including perfumes, cosmetics, aromatherapy, phytotherapy, spices and nutrition (Buchbauer, 2000; Lahlou, 2004b). These oils are readily absorbed through the skin, and have been shown to have various therapeutic uses (Lahlou, 2005).

Essential oils are formed in special cells or groups of cells (glands) and glandular hairs (*e.g.* the Lamiaceae – Thyme, Peppermint, Sage); oil cells (*e.g.* the Lauraceae – Cinnamon, Cassia); oil and resin ducts (*e.g.* Aiaceae – Cumin, Fennel, Celery and Gymnospermae – Pine, Fir); oil reservoirs (*e.g.* Citrus – orange, lemon, lime) (Cabrera, 2001). They are generally found to predominate in one particular part or organ, such as leaves, flower calyces, fruit, roots, etc.

Volatile oils that occur in plants give them their characteristic odours, flavours, or other such properties. Their chemical composition differs from various species and different chemotypes. There are estimated to be about 17,500 aromatic species. Among the plants notable for their essential oils are members of the following plant families: Anacardiaceae, Apiaceae, Asteraceae, Chenopodiaceae, Cupressaceae, Gentianaceae, Lamiaceae, Lauraceae, Myrtaceae, Pinaceae, Piperaceae, Poaceae, Rutaceae, Verbenaceae and Zingiberaceae (Lahlou, 2004a).

According to Tadmor *et al.* (2002), the demand for high quality, safe, effective, and clean natural plant products and their formulations with various substances have been growing significantly in the industrialized world. In the past, herbs and essential oil bearing plants were largely harvested from the wild and brought to the market without many questions asked about their origin, methods of cultivation, botanical identity, purity, safety, and efficacy. However, with further improvements in communication and education, there has been a growing consciousness in industrialized countries about personal health, environmental safety, sustainable harvesting, and loss of genetic diversity resulting from extensive wild harvesting of the medicinal species. This increased awareness has led to a wider use of alternative medicine practices, which include the use of botanicals as medicines though purchased as dietary supplements, by the consuming public. This phenomenon has been largely media and market driven rather than a scientific/medically driven movement. Yet, with increased consumer usage, and increased

advertisement, the expectations by the public have also increased leading to a more stringent demand for quality and traceability.

Producers must understand consumer demands and develop methods of production that meet those demands of product quality and efficacy, which exceeds or corresponds to international standards. Producers, marketers and scientists must work together to develop and introduce a product that will bring a reasonable return to the producer, while the consumer will receive a high quality, healthy, and safe product.

The development and application of methodologies for the determination of the chemical composition of aromas and similar mixtures is a challenging task. By means of developed and sophisticated analytical techniques, reproducible quantitative and qualitative results can be arrived.

The goal of this paper is to elucidate the most developed and sophisticated analytical techniques to study the chemical composition of essential oils. Their purity and quality control standards are also given and discussed.

CHEMICAL ANALYSIS TECHNIQUES

The analytical methods applied to the analysis of essential oils can be classified into two different groups:

- Chromatographic methods like gas chromatography (GC), high-performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC), including multidimensional and chromatographic coupling techniques resulting in the separation to individual components;
- Hyphenated techniques that means instrumental on-line coupling of chromatographic separation devices to spectrometers like coupling of GC with mass spectrometry (MS), Fourier transform infrared spectrometry (FT-IR), UV or atomic emission spectroscopy (AES), GC/NMR as well as coupling of HPLC with MS. The advantage of those techniques is that more information about the structure of the separated components is available and often identification is possible.

Chromatographic Methods

Gas Chromatography (GC)

Gas chromatography finds its main application with volatile compounds, fatty acids, mono- and sesquiterpenes, and sulphur

compounds. However, the volatility of higher boiling plant constituents (alcohols, acids) can be enhanced by conversion to esters and/or trimethylsilyl ethers so that there are only few classes which are completely unsuitable for GC separation.

GC is the most efficient chromatographic technique for separating volatile mixtures, because of its high resolving power and the availability of universal detection using flame ionization detection (FID). Mostly capillary columns with dimethyl polysiloxane (methyl silicone) non-polar and Carbowax 20 M polar phases are used. These latter include DB-Wax, BP-20, PEG 20 M and HP 20, while methyl silicone phases include SE-30, SF-96, OV-1, Ovlytical 101, BP 1, CPSIL 5CB, SP 2100, DB 1, DB 5 and HP 1. Among these fused-silica capillary GC columns DB 1 or DB 5 and CPSIL 5 are mostly preferred. Identification is based on direct comparison of retention times with standards or precise knowledge of retention indices, *e.g.* Kovats' retention indices.

Gas chromatographic retention indices (Kovats indices) are a valuable aid in the identification of monoterpenes and sesquiterpenes in essential oils and related natural and synthetic products. Some 900 Kovat's indices of 400 individual compounds on methyl silicone (dimethyl polysiloxane) and/or Carbowax 20 M liquid phases are summarized from the general literature (Davies, 1990). The dependence of retention index on temperature has been extensively described, with specific reference to terpenes. Temperature has a relatively small effect on Kovat's indices of terpenes on methyl silicone phases, but can have quite marked effects on the indices on CW 20 M. GC is certainly a very rapid method of separation, since no preliminary operations are required. It is also a method of choice when only a very small quantity of oil is available.

When greater sample amounts are available, it is undoubtly an advantage to perform some prefractionations of the essential oil before any GC separation. Generally, the purpose of a prefractionation is to separate the oil into constituent groups such as terpenoid hydrocarbons and oxygenated derivatives (Dung & Thang, 2005).

Furthermore, according to Singh and Marimuthu (2005), GC has a molecular mass operating range from 2 to about 1500 (in molecular hydrogen scale mass units). Within this mass range, suitable compounds which can be chromatographed will be discussed as permanent gases (highly volatile), volatile compounds (those of higher mass).

There are two primary considerations which must be taken when discussing the analytical separation of essential oils. Firstly,

recognizing that chromatographic methods-primarily gas chromatography- will be the most appropriate analytical instrumental approach, one must decide whether the performance of the separation will be adequate for the problem. Secondly, once the method has been chosen, the technique used for characterization of the separated components need to be considered. This will largely be the realm of mass spectrometry.

The development of stationary phase can generally be seen as a search for specific improved properties conferred by the phase on the chromatographic separation. Thus, the development seek to produce more thermally and chemically stable phases that give greater selectivity in the separation of components by different phase chemistry. However, improved stability does also mean that a given column should be more reliable over a greater period, and this translates to improved long term reproducibility of analysis; which should make analytical characterization more secure.

Comprehensive Two-dimensional Gas Chromatography (GCxGC)

The recently developed technique of GCxGC described by Dung and Thang (2005) addresses a number of shortcomings of conventional multidimensional gas chromatography when analysing very complex samples, or simple where the occurrence of overlapping peaks of different polarity arises. GCxGC is a true multidimensional GC-MS (MDGC) method since it combines two directly coupled columns, which provide orthogonal separation of compounds on the two columns, and importantly is able to subject the total sample to simultaneous two-column separation. Since components now have retention in two separate columns, and since the second column brings its own separation power to the analysis, the net capacity is the product of the capacities of the two participating columns. This certainly expands the separation space, and even on a statistical basis should lead to a considerable increase in resolvable components. A single column cannot achieve anywhere near this capacity, and so must be considered of limited separation performance. Thus a combination of non-polar column with a polar polyethylene glycol phase column may a good first choice for a suitable dual column set for essential oils.

The complexity of these compounds is ideally suited to two-dimensional analysis, which is both why MDGC is applied in this area, and also why GCxGC should be a fertile analysis area.

Multidimensional GC-MS (MDGC)

The complexity of essential oils is ideally suited to two dimensional analysis which is both why MDGC is applied in this area. The

typical approach of this method consists to isolate a specific region or regions of components that elute from one column and direct these zones or heart cuts to a second column. This involves separation of the components contained in a small segment or segments of a complex chromatogram obtained by first stage gas chromatographic columns, in an intermediate cryogenic trap (Singh & Marimuthu, 2005).

The virtues of MDGC are to increase the separation space of gas chromatography analysis, and to provide enhanced solution for given regions of a chromatographic analysis

Chiral Gas Chromatography

Recent advances into the stationary phases has resulted in the separation of even enantiomer. Many chiral monoterpene alcohols, monoterpene ketones and pheromones could be separated in enantiomers on chiral stationary phases.

Enantiomer separation and enantiomer excess or ratio determination is of great importance in fields of flavours and essential oils, not only to characterise a vegetable matrix or extract or evaluate the biosynthetic pathway of one or more of its components, but also to establish its origin and/or to identify possible adulterations (Dung & Thang, 2005).

The development of stable chiral phases for gas chromatography has allowed the enantiomeric compositions of terpenoids and a host of other compounds to be staffed in remarkable detail. Such analysis are becoming vital in the industrial setting for the detection of adulterants, characterization of oil profiles for quality control and when coupled with the development of improved biochemical techniques, has facilitated investigations into the underlying mechanisms associated with the biosynthesis of the terpenoids (Singh & Marimuthu, 2005).

Preparative Gas Chromatography (PGC)

This technique involves the use of columns with grater capacity and injection of samples for the isolation purposes. Columns used PGC range from wide bore glass capillaries to larger packed columns. Such columns require samples from few µl to few mL. PGC columns are heavily loaded to increase their sample capacity. Large quantities of essential oil components could be handled by the use of adsorption column chromatography (Dung & Thang, 2005).

Gas Liquid Chromatography (GLC)

Gas liquid chromatography is the most important chromatographic technique used for the analysis of essential oils. GLC has its own importance not only by the research chemist but also by the perfumer for quality control of the product. The constituents of essential oils are so complex such as hydrocarbons, aldehydes, alcohols, ketones, acids and esters. Due to this fact, gas chromatographic investigations are conducted under unfavourable conditions and more complications may arise both in quantitative and qualitative process on account of the fact that the number of components is usually large.

The perfumer and essential oil industries were among the pioneers in applying the techniques of gas chromatography which has undergone a lot of change since its inception. The components of essential oils and aromas belong to a variety of chemical substances. The choice of two stationary phases is quite important in separating given mixtures in its simplest form (Singh & Marimuthu, 2005).

Thin Layer Chromatography (TLC)

Thin Layer Chromatography is one of the most important methods for analyzing essential oils apart from gas chromatography (GC). The ratio of fronts (Rf) value is dependent upon many variables which must be watched during the preparation and evaluation of the chromatogram if reproducible results are to be obtained: Quality of the layer material, activation grade of the layer, layer thickness, chamber saturation, quality of the solvent, development distance, and distance of the starting point from the surface of the solvent. Sometimes a better separation is achieved by the stepwise elution technique, in which different solvents in succession are made to pass over the chromatogram in the same or rectangular direction.

The effectiveness of TLC had also been improved by various modification techniques involving impregnation techniques using paraffin- and $AgNO_3$. The $AgNO_3$-impregnated- TLC allows the separation of terpenes according to the number of double bonds (Aitzetmüllet & Goncalves, 1990).

According to Singh and Marimuthu (2005), TLC is a conventional technique which has the advantage of simplicity and rapidity of not giving artefact formations. It has an added advantage of colour reaction of essential oil components with different spray reagents which can be utilized for the detection of adulterants in essential oils.

High-performance Liquid Chromatographic Analysis (HPLC)

The relatively good separation obtained by GC has delayed the application of HPLC to the analysis of volatile compounds, such as sesquiterpenoids. However, this analysis technique offers some advantages when compared to GC.

The applications of HPLC in the analysis of volatile compounds can be placed in three major groups (Dung & Thang, 2005):

- Prefractionation of complex volatile mixtures prior to GC or GC-MS analysis;
- Qualitative and quantitative detection of specific volatile constituents;
- Semipreparative or preparative isolation of volatile compounds.

HPLC is often used as prefractionation of complex mixtures into groups of components in order to simplify GC and GC-MS analysis. Using reversed-phase materials, fractionation is carried out according to the polarity and the chain length of essential oil constituents. This technique is also used to isolate pure compounds.

Hyphenated Methods

Hyphenation refers to the coupling of spectroscopic detection methods or other specific types of detection techniques, or using different analysis approaches in unrelated dimensions of an analysis to improve the separation performance or quality of data from an analysis. These can be collectively termed multidimensional methods. Thus in this case of analysis can be included systems which incorporate separations prior GC, multicolumn separations and specific identification methods following the GC separation.

Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography coupled to mass spectrometry (GC-MS) and other similar detection schemes are the techniques normally employed for chemical analyses. It is considered as one of the most widely and powerfully utilized analytical techniques.

The great majority of GC-MS applications utilize capillary GC with quadrupole MS detection and electron ionization. Nevertheless, there are substantial numbers of applications utilizing different types of mass spectrometers and ionization techniques coupled with multidimensional high-speed and pyrolysis-gas chromatographic methods (Dung & Thang, 2005).

A very important aspect in the application of any GC-MS method involves its desired ability to provide almost unequivocal confirmation of compound identity.

Gas Chromatography-Fourier Transform IR Spectroscopy (GC-FT-IR)

GC-MS is considered to be the method of choice in the identification of volatile compounds including sesquiterpenoids. However, in distinguishing isomers, which often occur in the terpene group, capillary GC-FT-IR coupling offers a useful supplementation. Advantages of FT-IR spectroscopy are high resolution and sensitivity. The more time-consuming interpretation and the absence of a database of reference vapour phase spectra may be reasons why this technique is not generally accepted for the analysis of volatile compounds (Dung & Thang, 2005). However, a GC-FT-IR-MS instrument is available, whereby simultaneously IR and mass spectra can be obtained. Thus, the unambiguous identification of critical isomeric sesquiterpenes is possible.

Linked GC-FT-IR-MS

It has been demonstrated that GC-FT-IR-MS is a powerful technique for analysis of complex mixtures. Because IR and MS yield complementary information, a combined GC-FT-IR-MS instrument is an extremely versatile tool for many types of analyses (Dung & Thang, 2005).

It is clear that the recent developments in multispectral detection systems for gas chromatographic effluents have provided unrivalled analytical capabilities to analytical chemists. The impressive developments in both GC-MS and GC-FT-IR promise to yield improved analytical power for combined GC-FT-IR-MS in the near future.

High-Performance Liquid Chromatography-Capillary Gas Chromatography (HPLC-GC)

HPLC and GC constitute orthogonal separations. They offer different mechanisms of separation, and as a generality HPLC will effect a broad class separation of a sample, and following this the separated fractions may be introduced into a GC system for further high resolution separation based on boiling point and/or polarity.

Large volume injection or complete fraction injection allows conservation of mass of sample analysed, and will be important for low-abundance constituents. The HPLC step achieves isolation of

components of similar chemical composition, primarily based on polarity, and hence will separate oxygenates, from saturates, from unsaturated/aromatic hydrocarbons. The HPLC technique determines the particular class separation achieved. So each transferred fraction is analysed separately before a subsequent fraction can be introduced into the GC system (Dung & Thang, 2005).

Gas Chromatography-Carbon-13- Nuclear Magnetic Resonance (GC-^{13}C-NMR)

Sometimes it may be useful to confirm by an analytical method the results of GC and GC-MS. Here ^{13}C-NMR followed the GC-MS analysis to confirm the structure assignments proposed by retention data and mass spectra. Structurally, closely related molecules such as stereoisomers, which exhibit insufficiently resolved mass spectral patterns, and compounds inseparable by GC. However, this analysis has limitations, too. Spectra containing an immense density of individual lines, especially in the aliphatic region, are difficult to elucidate. Here the ambiguous assignment of signals may be very difficult. To make specific assignments more certain, distorsionsless enhancement by polarization transfer (DEPT) experiments can be carried out. By this primary, secondary, tertiary or quaternary carbon atoms can be distinguished. In both cases identification is based on comparison of the sample spectrum with spectra of pure compounds. Qualitative as well as quantitative analysis is possible (Dung & Thang, 2005).

Further Combined Techniques: GC-UV, GC-AES, SFE-GC

- ***GC-UV, GC-AES***

Both techniques can give additional information being to some extend complementary to MS and/or IR data (Dung & Thang, 2005).

- ***SFE-GC***

The silica-gel phase used SFE elution gave separation based on solute polarity, with three fractions (hydrocarbons, aldehydes and esters, and alcohols) introduced separately into the GC system. Clearly the primary driving force for developing such hyphenated systems is the lack of resolution of the (single column) capillary GC method. The prior separation step will essentially introduce to simplify the subsequent GC presentation. If the GC analysis could be significantly to give much better resolution, then the rationale for hyphenated HPLC-GC or SFE-GC may be lost (Dung & Thang, 2005).

MAIN COMPONENTS IDENTIFIED

Essential oils are naturally occurring mixtures of many volatile compounds. Each constituent possesses a distinct molecular formula. Structures of some of these identified and analysed components using the analytical techniques previously mentioned are summarised in Fig 1.

FACTORS INFLUENCING THE CHEMICAL COMPOSITION OF ESSENTIAL OILS

An intimate knowledge of essential oil composition allows for a better and specially directed application (Buchbauer, 2000). To add to the complexity of volatile oils, there is evidence that the time of harvest influences the oil composition and consequently the potency of their biological activity (Lahlou, 2001, 2004a, b). Other factors such as genotype, chemotype, geographical origin, environmental and agronomic conditions, seasonal variations and climate, production technique and purity, the effect of plant maturity at the time of oil production and the existence of chemotypic differences can also influence the composition of the oil (Lahlou, 2005).

ESSENTIAL OILS QUALITY AND PURITY

The quality and purity of essential oils are important to aromatherapists, who want a wholly natural product that duplicates the aromatic profile of the living plant source. Many lovers of essential oils are intimidated by the marketing hype, aggressive advertising and slick packaging that too often accompany the oils to the marketplace. Using both good quality and purity of essential oils are absolutely vital in aromatherapy. This because the quality of the essential oils used in products or in a treatment/practice will determine how successful the results will be. Furthermore, for clinical aromatherapy, the volatile oils should be produced in this way sufficiently pure and of higher quality, because cheaper, inferior quality essential oils deliver poor results in this way: an allergic reaction to the oil and skin irritation, sensitization, or other undesired side effects could happen (Roberta Lee, 2004).

Unwanted side effects are usually caused when an essential oil has been adulterated with a synthetic chemical component, or perhaps because it is entirely synthetic. Unscrupulous traders and dealers will often 'stretch' expensive oils by adding cheap synthetics to them to make the product more profitable for themselves.

This type of adulteration can cause problems for unsuspecting customers who then attempt to use these oils in aromatherapy -

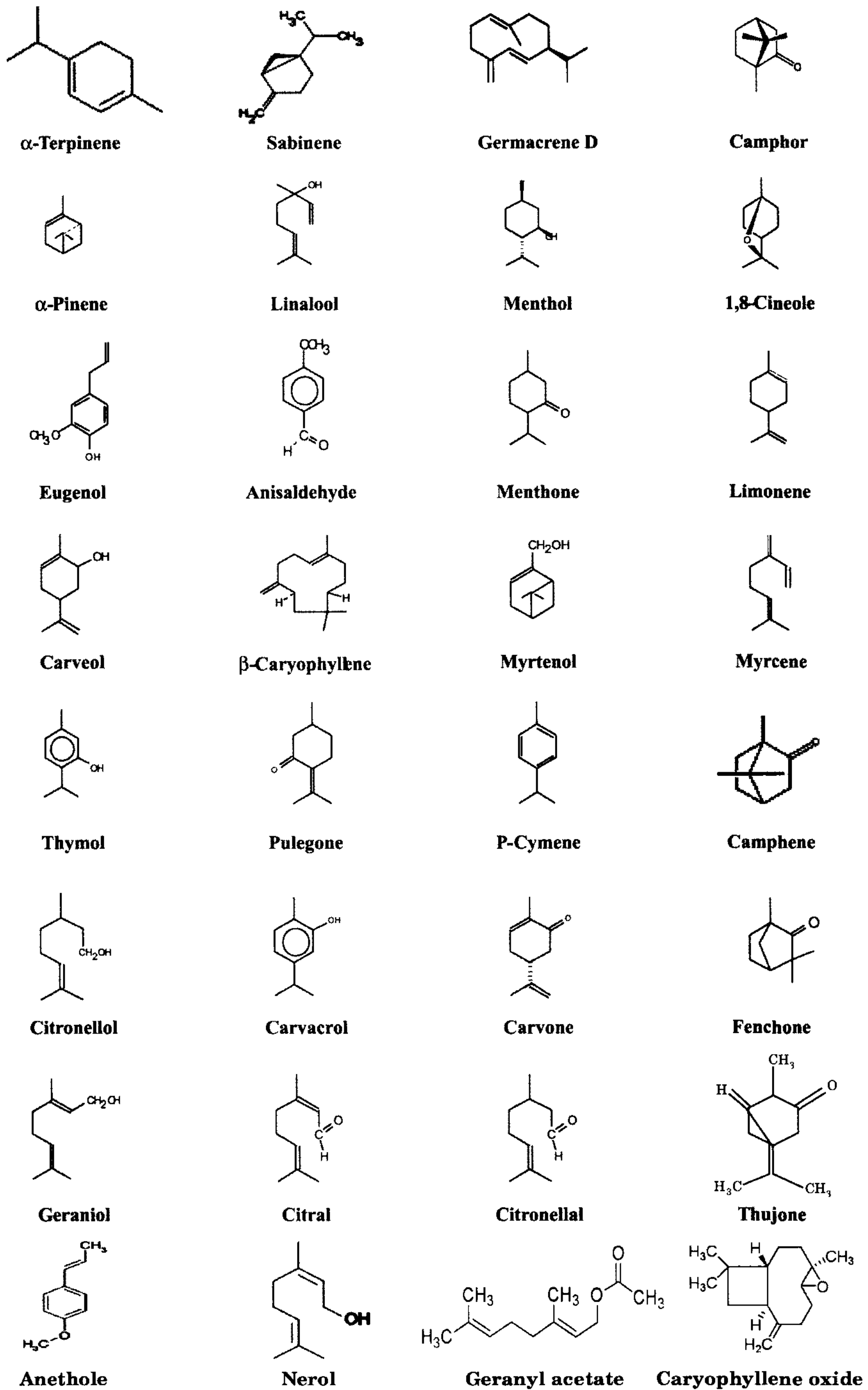
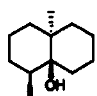
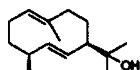


Fig 1. Some components found in essential oils (After chemical analysis and identification)

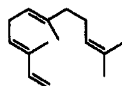
Fig 1. *Contd.*



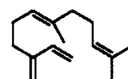
Geosmin



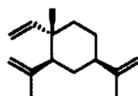
Germacradienol



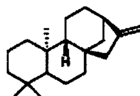
α-Farnesene



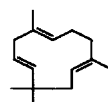
β-Farnesene



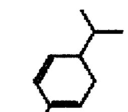
β-Elemene



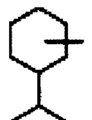
Kaurene



α-Humulene

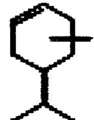


α-Phellandrene

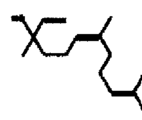


Menthane

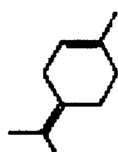
Menthene



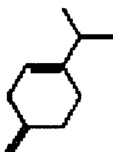
Menthadiene



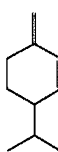
Nerolidol



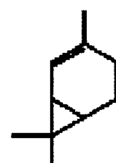
Terpinolene



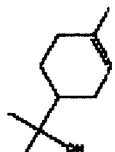
β-terpinene



β-phellandrene

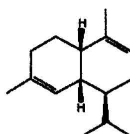


2-Carene

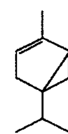


α-Terpineol

Neral

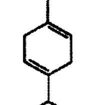


α-Muurolene

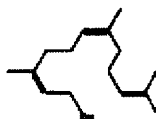


α-Thujene

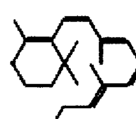
β-pinene



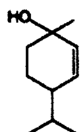
γ-Terpinene



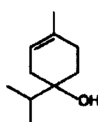
Farnesol



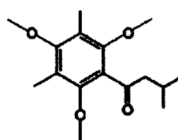
Retinol



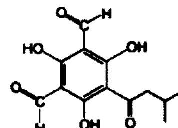
δ-2-p-menthen-ol-(1)



Terpinen-4-ol



Torquatone



Jensenone

sometimes with disastrous results. Many people are allergic to synthetic compounds, hence the recent move to 'fragrance-free' cosmetics.

Of course not everyone is sensitive to synthetic or adulterated oils so the problem should not be exaggerated, but it is still unethical and dishonest for a supplier to sell adulterated or synthetic oil under the pretence that it is a natural, pure essential oil. Synthetic or adulterated oils contain little or no therapeutic qualities and will not produce the results that we are looking for.

The ultimate test for the purity and high quality of essential oils is the human nose. Chemical tests such as liquid gas chromatography, the standard chemical assay for essential oils, although helpful, can also be duplicated or adjusted using other natural or synthetic oils. Only the nose is able to distinguish a natural from a synthetic fragrance source, which again is based on training and experience.

Essential oils have tiny molecules and are thus easily absorbed. It is important to use good quality essential oils because their therapeutic benefits derive from their origins and chemical make-up.

Quality Control of Essential Oils

Quality Standards

The quality of an essential oil is a second important consideration. Depending on the type of equipment, process, care and time taken to extract an essential oil, the final product may range anywhere from excellent to mediocre. Therefore, the concept of "natural" is clearly not enough to guarantee best clinical results. An essential oil may be natural but only contain the most basic spectrum of volatile components, missing the fragrance depth of some base notes, perhaps, or the brilliance of some top notes. Good or high quality oils are crucial to the success of aromatherapy treatments, including environmental ones (Lahlou, 2005).

Otherwise, the price of special essential oils that can be purchased on the market strongly depends on the yield and the quality of the product. The quality, which depends on the quantitative and qualitative variation of different monoterpenes, varies with respect of the origin and the harvesting period. The composition is predominantly related to the genetic background (genotype), the origin, the growing conditions (*e.g.* optimal harvesting time), and the applied technological process for isolation (Strehle *et al.*, 2005).

Despite attempts by leading aromatherapists and their organizations, no clearly defined and universally accepted and applied

standard for the quality and purity of essential oils exists. Unless a company is distilling its own essential oils or working directly with growers and distillers, its oils probably come from one or more importers of essential oils. These companies are reputable and provide good quality products, but most of their oils are sold to large fragrance and perfume manufactures, who demand oils that are consistent and unchanging from lot to lot (industrial quality). If the manufacturer of a popular cologne or perfume needs to make more products, the ingredients for the newest batch need to smell exactly the same as those used for the batch produced before. Suppliers are adept at "enhancing" the chemical makeup of essential oils with other natural and synthesized fragrances to create a consistent product. Such manufacturers can supply the essential oils preferred by discriminating aromatherapists, but the buyer needs to communicate a realistic quality standard to the supplier and confirm that quality standard upon receipt.

The evaluator should be aware of the acceptable colour and viscosity range of the oil as well the characteristics and duration of the top note, middle note and dry-out aroma as it volatilizes over a fairly standard period of time. These standards are intuitive, unique and highly specialized. When used along with the technical, scientific approach, they make possible an effective and reliable quality assessment of essential oils.

Vigorous quality checks are made throughout the entire process of sourcing and authenticating the provenance of pure essential oils. Failure to meet any one of our quality standards will result in an essential oil or other raw material being firmly rejected.

Quality Control Standards

The quality control of an essential oil includes:

- Verification of the plants botanical species;
- Crops were not subjected to agrochemicals;
- Low pressure distillation techniques employed;
- Visually inspecting the oil;
- Odor evaluation of the oil;
- Measuring the oils physical parameters;
- Testing the oils purity using GC or GC/MS technology.

We believe that essential oil quality control should begin at the earliest stage of production, preferably with inspection of the crop at the growing phase. This important aspect is often overlooked when considering quality issues.

The importance of purity with essential oils can not be overstated, if for no other reason than maintaining the good reputation of aromatherapy. Cheaper, inferior quality essential oils deliver poor results and it is a waste of money.

An essential oil may be natural but only contain the most basic spectrum of volatile components, missing the fragrance depth of some base notes, perhaps, or the brilliance of some top notes.

What Affects the Quality of an Essential Oil?

Everything that happens to a plant will be transferred into its essential oil. Economic and therapeutic valour of essential oil strongly depend on the quality of the product which depends on the quantitative and qualitative variation of different monoterpenes, varies with respect of the origin and the harvesting period. This quality is also intimately related to the following factors:

- Growing location, altitude and season of plants;
- Fertility of the soil;
- Weather conditions;
- Methods of cultivation;
- Use of pesticides or herbicides (these chemicals will end up directly in the oil);
- Time of year (and time of day) plants were harvested;
- Time between harvesting plants and distilling the oil;
- Type of pressing or distillation process (extraction);
- Pressure level during distillation;
- Temperature of distillation;
- Solvents used in distillation or pressing (which will also end up in the oil).

In distilling pure, therapeutic-grade oils, low pressure and temperature must be maintained to preserve key chemical constituents in the oil that would be damaged by heat. But higher temperature and pressure yield more oil volume – a temptation to unscrupulous essential oil producers. Oils produced at high temperatures don't work as well for healing as *therapeutic-grade oils*.

At the extraction stage, the skill and experience of the distillery becomes the next critical factor in the quest for excellence, since all the expertise and effort of the farmer will have been wasted if the oil is not extracted correctly. This is why an essential oil produced

in one country (or region) can be of a higher quality than precisely the same oil produced in another.

Essential oil Purity

According to Vankar (2004), essential oils should be subjected to both qualitative and quantitative tests to know its purity. Oils are tested in four stages. The first stage is sensory evaluation in which the smell, viscosity, color and clarity of the oil are assessed. The second stage is an odor/smell test, which helps to determine if oil is really what it is claimed to be, since certain adulterants can be identified by this test. In the next stage, physical parameters such as specific gravity, optical rotation and refractive index are determined. If these test results are satisfactory, the oil is then subjected to chemical evaluation.

Using the finest quality essential oils is absolutely vital in aromatherapy. Because the purity of the essential oils will determine how successful the treatment or products will be. In aromatherapy, we use the very purest, highest quality, pure essential oils.

At best, essential oils that have been adulterated will simply not deliver the health benefits that are expected. At worst, there is the risk of serious skin irritation or sensitization due to an adverse reaction to the synthetic chemicals that have been used to adulterate, extend or 'standardize' the essential oil.

An essential oil that is guaranteed by the manufacturer to be 100% pure and natural would seem ideal. But unless this guarantee is defined and put into contract form, it doesn't assure anything. The 100% pure and natural designation should be accompanied by the standard Latin and common name for the plant, indicating that the product was distilled exclusively from the plant source and not cut or altered by the addition of other natural or synthetic ingredients. Other factors to check for are acceptable extraction techniques, and storage conditions. The supplier should be aware of the standard proposed by the buyer and be held to them.

DISCUSSION

Several methods and techniques are used to the control of quality/ purity of the essential oils. These compounds pose their own challenges, and in particular the role of chiral analysis by using GC x GC has a potential for significantly simplifying the approach to chiral analysis. The choice of column sets is limited to a small number of recommended dual columns, since this allow advanced pattern recognition or retention correlations to be used to compare and

contrast a variety of samples. This is similar to the retention time (index)/mass spectral correlations in single column studies of essential oils.

One of the major expectations of analysis of essential oils must be the availability of mass spectral data for separated components, and similar to validate that the incredible range of separated peaks are meaningful compounds, GC × GC-TOF-MS is a further important technology by providing MS data. Presently TOF-MS is the only viable technology for fast mass spectral data acquisition for peaks that may have base widths of as little as 100 ms. Whatever the future holds, it can be reasonably assured that GC × GC will hold many surprises and much value in respect of new information derived from GC analysis.

Therefore, GC-MS is considered one of the most widely and powerfully utilized analytical techniques. This is probably related to the explosion of application stems from the excellent qualitative information obtained the high sensitivity inherent this impact on the practice of GC.

HPLC and GC constitute orthogonal separations. They offer different mechanisms of separation. HPLC will effect a broad class separation of sample and following this the separated fractions may be introduced into a GC system for further high resolution separation based on boiling point or polarity. This system was fully automated but off-line sampling of HPLC fractions may also be introduced by the use of large volume injection or may be subsampled to introduce a representative part of the fraction. The HPLC step achieves isolation of components of similar chemical composition primarily based on polarity and hence will separate oxygenate from unsaturated/ aromatic hydrocarbons.

Gas chromatography (GC) finds its main application with volatile compounds, fatty acids, mono- and sesquiterpenes, and sulphur compounds. However, the volatility of higher boiling plant constituents (alcohols, acids) can be enhanced by conversion to esters and/or trimethylsilyl ethers so that there are only few classes which are completely unsuitable for GC separation. Alternatively, the less volatile constituents can be separated by HPLC, a method which combines column efficiency with speed of analysis. A major difference between HPLC and GLC is that the former procedure normally operates at ambient temperature, so that the compounds are not subjected to the possibility of thermal rearrangement during separation. HPLC is mainly used for those compounds which are non-volatile, *e.g.* higher terpenoids, phenolics of all types, alkaloids, lipids, and sugars. HPLC works best for compounds which can be detected in the ultraviolet

or visible regions of the spectrum and provides a most important and versatile method of quantitative plant analysis.

CONCLUSIONS

Essential oils are complex mixtures of numerous components. Their use in pharmacy is due to the physiological effect of a single component of the essential oil, or - more often - of a group of components. The evaluation of an essential oil basing only on the percentage of the main component and physicochemical constants gives the risk of non-detecting lower qualities and falsifications.

Because of their scarcity, volatile oils may be extremely expensive and adulteration is common. A good quality/purity of essential oils is always and really needed.

The very best oils are labelled *genuine* and *authentic* meaning that they are pure, natural and complete, containing absolutely no fixed vegetable oils or synthetic substances. They should be distilled at a reduced pressure to assure the highest quality extraction. They should not been redistilled.

Considering the explosion of prices of the essential oils during the last seven years, the detection of the increasing number of falsifications is not only of scientific interest, but also of great economic importance.

Possibilities for an adequate screening of essential oils are introduced and their affectivity/efficacy is compared with established tests. By means of these methods, especially by temperature programmed GLC with packed columns, the quality of an oil can be checked in a short time and falsifications can clearly be identified considering not only the main components of an essential oil, but also characteristic minor compounds.

One of the many factors that determine the purity of essential oil is the chemical constituents. These constituents can be affected by a vast number of variables. The key to producing a therapeutic-grade essential oil is to preserve as many of the delicate aromatic compounds within the essential oils as possible.

However, for an essential to produce its purported therapeutic results, it must be a pure and unadulterated plant extract. The best way to assure the purity and quality of each batch of pure essential oil is by knowing the growing and distillation source, and testing each essential oil with advanced modern technology.

In most cases, quality control of natural products is important to guarantee that the received delivery meets specification ranges

defined by international organizations or between the individual suppliers and customers. Various analytical methods are described in this case for reliable quality and purity control of essential oils for any health benefit, uses and further applications.

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11

Development and Validation of Ultraviolet Spectrophotometric Assay Method for the Powdered Leaves, Extracts and Formulations of *Loranthus micranthus*

UZOCHUKWU I.C.[1] AND OSADEBE P.O.[2,*]

ABSTRACT

A simple, precise and reproducible ultraviolet spectrophotometric method for the assay of powdered leaves, extracts and formulations of Loranthus micranthus was developed and validated. The complexation of the flavonoids of L. micranthus with methanolic aluminum nitrate solution was employed for the assay. The absorbance of the formed complex was determined spectrophotometrically at the λ_{max} *of 300 nm. The time-absorbance relationship, optical characteristics, linearity, accuracy, precision and repeatability of the complexation method were studied. A 1:2 (aluminum:flavonoids) stoichiometry was obtained for the complex. Beers law was obeyed in the concentration range of 0.4 to 3.6 mg%. The calibration curve had a regression coefficient of 0.9913 ± 0.0010. The limit of detection and the limit of quantitation of the complexation method were found to be 0.04 and 0.12 mg% respectively. Percentage recoveries between 92 and 113% were obtained. The precision and repeatability were within acceptable limits. The complexation spectrophotometric method proved to be robust and therefore recommended for the assay of powdered leaves, extracts and formulations of Loranthus micranthus.*

Key words : Assay, complexation, *Loranthus micranthus*, phytomedicine, spectrophotometry, validation

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INTRODUCTION

Loranthus micranthus (African mistletoe) is a semi-parasitic plant traditionally employed in Nigeria for the management of diabetes mellitus and respiratory infections. Its antidiabetic, antimotility and antimicrobial activities have been scientifically validated (Osadebe *et al.*, 2004; Osadebe *et al.*, 2006; Osadebe & Uzochukwu, 2006; Osadebe & Ukwueze, 2004; Osadebe & Akabogu, 2005). The safety of the leaves of *L. micranthus* and another species of African mistletoe, *Loranthus bengwensis,* has also been reported (Osadebe *et al.*, 2004; Obatomi *et al.*, 1994). It is expected that the formulation and commercialisation of the leaves of *L. micranthus,* its extracts and fractions will contribute to better management of diabetes mellitus and respiratory diseases, create employment as well as generate income for the indigenous communities.

Although the WHO adopted a resolution (WHA40.33) urging member states to ensure quality control of drugs derived from traditional plant remedies by using modern techniques and applying suitable standards (WHO, 1996), this is yet to be actualized in most developing nations. In such cases where the isolation of the active constituents have not been done or characterized, the whole plant extract may be considered as one active constituent and the assay of marker substances may be adopted for its assay (EMEA, 2001). The whole plant extract might reflect true activity of a phytomedicine and some persons have argued strongly on the effect of a complete extract being the most desirable (Onunkwo, 2005). If a reproducible ultraviolet (UV) spectrophotometric fingerprint of the authentic herbal plant can be determined, such fingerprint can serve as a good identification and quantitation basis. The assay developed from such fingerprint may be considered suitable as an overall method of assay (EMEA, 2001).

The growing use of medicinal plant materials in the management of diseases has created a corresponding need for reliable quality control methods for medicinal plant materials. The absence of validated chemical assay methods for many phytomedicines constitutes a limiting factor to their standardization, formulation, commercialisation and acceptance by healthcare providers and consumers.

Ultraviolet-visible spectrophotometry is about the most versatile modern analytical instrument and spectral methods are especially important for the identification and quantitative analysis of phenols (Harbourne, 1984). A previous study reported that the antidiabetic activity of *L. micranthus* leaves may be attributed to the weakly acidic flavonoids content (Osadebe *et al.*, 2006).

The study therefore employed the complexation of the flavonoids (polyphenols) of *L. micranthus* with methanolic aluminum nitrate solution in the assay of its powdered leaves, extracts and formulations.

MATERIALS AND METHODS

Plant Material

Loranthus micranthus Linn (Loranthaceae) leaves parasitic on *Kola acuminata* were collected from Akwaeze, Eastern Nigeria in January 2006. Mr Ekekwe, J.M.C., a plant kingdom scientific analyst, formerly at the Botany Department of the University of Nigeria, Nsukka, identified the plants. The leaves were dried under the shade to a constant weight and pulverized with a Corona® grinder. The powder was sieved with a 1 mm sieve.

Preparation of Reagent

Preparation of 0.05 M methanolic aluminum nitrate solution

Aluminum nitrate (3.7513 g) was weighed and dissolved with about 80 mL of deionized water in a volumetric flask. The resulting solution was made up to 100 mL mark with deionized water (0.1 M aluminum nitrate solution). Ten milliliter of the prepared 0.1 M aluminum nitrate solution was added to 10 mL of absolute methanol.

Preparation of Crude Methanol Extract and Flavonoids-Rich Extract of *L. micranthus* Leaves

The dried powdered plant leaves (80 g) of *L. micranthus* was extracted with 90% aqueous methanol for 5 h using a Soxhlet apparatus (Osadebe *et al.*, 2004). The resulting methanol extract was concentrated and dried for 48 h in a hot air oven at 60°C.

Another 80 g of dried powdered plant leaves of *L. micranthus* was first extracted with n-hexane for 5 h and the resulting marc further extracted with absolute methanol for another 5 h using a Soxhlet apparatus (Antri *et al.*, 2004). The resulting methanol extract was concentrated and dried for 48 h in a hot air oven at 60°C. The resulting methanol extract, designated as flavonoids-rich extract (FRE), was confirmed to be majorly flavonoids using aluminum chloride solution.

UV-Vis Spectrophotometric Scan of CME, the Complexes of CME and FRE

A stock solution of the crude methanol extract (CME) of *L. micranthus* was prepared by dissolving 124 mg of the CME in sufficient volume

of 90% aqueous methanol to obtain 100 mL of the solution. Dilute CME solution (1.24 mg%) was subsequently prepared by appropriate dilution of the stock solution with 90% aqueous methanol. The resulting diluted solution of CME (1 mL) was mixed with 9 mL of methanolic aluminum nitrate solution. Similarly, 1 mL of 5 mg% of FRE was mixed with 9 mL of methanolic aluminum nitrate solution. The diluted CME solution (1.24 mg%) and the resulting complexes of CME and FRE were each scanned using a UNICO® 2102 UV-Vis PC spectrophotometer between the wavelength of 200 and 700 nm at 1 nm interval against the blank. The acquired absorbance data were transferred to Microsoft Excel® toolpack and the data used to plot the graphs of absorbance against the wavelength (absorbance spectra). The wavelength of maximum absorption for the extract and the complexes were determined from the graphical plots.

Determination of Stoichiometry of Complex Formation by Methanolic Aluminum Nitrate

Flavonoids-rich extract (50 mg%) was dissolved in sufficient quantity of 90% aqueous methanol and the volume made up to the 100 mL mark with 90% aqueous methanol. A series of the complexes formed between the FRE of varying volume and 1 mL of methanolic aluminum nitrate solution were prepared to afford mole ratios between 0.091 and 0.947. The absorbance values of the resulting complexes were determined using a UNICO® UV-Vis 2102 PC spectrophotometer at 300 nm wavelength. A graphical plot of the absorbance values versus mole ratio afforded a curve with two straight lines of different slopes at whose intersection the mole ratio of the formed complex was obtained.

Determination of Time-Absorbance Relationship of the Methanolic Aluminum Nitrate Complex of FRE

A 1 mL portion of the FRE (50 mg%) was mixed with 7 mL of 90% aqueous methanol in a test tube and complexed with 2 mL each of methanolic aluminum nitrate solution (0.05 M) and acetate buffer (pH, 6.00). The absorbance of the formed complex was determined in duplicate every five minutes for forty minutes at 300 nm using UNICO® 2102 UV-Vis PC spectrophotometer.

Determination of the Calibration Curve of *L. micranthus* Complexed with Methanolic Aluminum Nitrate Mixture Using FRE as Reference Sample

Fifty milligrams of the FRE was accurately weighed out and dissolved in sufficient aqueous methanol (90%) to make 100 mL in a volumetric

flask (50 mg%). Complexes (0.5 to 3.0 mg%) of the FRE were appropriately prepared. Two other set of dilutions were similarly prepared. The absorbance values of the resulting complexes were determined spectrophotometrically at 300 nm. Graphs of absorbance versus concentration were plotted using Microsoft Excel® tool pack to afford the three calibration curves of the FRE complex.

Determination of Linearity and Optical Characteristics of the Complexation Method

The mean regression coefficient of the linear plots of the calibration curves of *L. micranthus* complexed with methanolic aluminum nitrate solution (FRE as reference) was used as a measure of linearity of the assay method. Regression coefficient greater than 0.95 was considered acceptable. Limit of detection and limit of quantitation of the complexation method were calculated based on the standard deviation and slope of the calibration curves according to the ICH harmonized tripartite guideline (EMEA, 2006).

Determination of Accuracy of the Complexation Method

Flavonoids-rich extract (50 mg) was dissolved in sufficient quantity of 90% aqueous methanol and the volume made up to the 100 mL mark with 90% aqueous methanol. Five mg% of FRE was appropriately prepared from the stock solution by dilution with 90% aqueous methanol. 2 mL, 1.8 mL and 2.2 mL of the 5 mg% FRE were mixed with 4.0, 4.2 and 3.8 mL of 90% aqueous methanol respectively in a test-tube. 2 mL each of methanolic aluminum nitrate and acetate buffer (pH, 6.0) were added to each of the test-tubes. The absorbance values of the resulting complexes were determined spectrophotometrically at 300 nm. Percentage recoveries for the three samples were calculated from the calibration curve of FRE complexed with methanolic aluminum nitrate.

Determination of Precision of the Complexation Method

Ten aliquots (1 mL each) of the FRE (50 mg%) were obtained, mixed with 7 mL each of 90% aqueous methanol and complexed with 2 mL each of methanolic aluminum nitrate (0.05 M) and acetate buffer (pH, 6.0). The absorbance values of the formed complexes were determined spectrophotometrically in duplicate at 300 nm. The mean, standard deviation, variance and coefficient of variation were determined. Also, a duplicate analysis was done using the duplicate data obtained. The relative range value, R, was calculated using the equation

$$R = \frac{X_1 - X_2}{(X_1 + X_2)/2} \quad \text{...... Equation 1}$$

Where X_1 and X_2 are the duplicate results from the individual sample. X_1 - X_2 = the absolute difference between X_1 and X_2. Relative range equal to or less than 0.02 was considered acceptable.

Determination of Repeatability of the Complexation Method

One aliquot (1 mL) of the FRE (50 mg%) was mixed with 2 mL each of acetate buffer (pH, 6.0), 7 mL each of 90% aqueous methanol and complexed with 2 mL of methanolic aluminum nitrate (0.05 M). The absorbance of the resulting complex was determined spectrophotometrically in duplicate at 300 nm. This procedure was done once daily for 5 days. The mean, standard deviation, variance and coefficient of variation were determined. Also, a duplicate analysis was done using the duplicate data obtained. The relative range value was calculated using the equation 1 above. Relative range equal to or less than 0.02 was considered acceptable.

Assay of Powdered Leaves, Crude Ethanol and Crude Aqueous Extracts, Ethanolic and Aqueous Formulations of *L. micranthus*

Powdered leaves of *L. micranthus* (500 mg) were macerated in a test-tube with 10 mL of 90% aqueous methanol for exactly 1 h. The extract (E_1) was filtered through a Whatmann filter paper No 1. One mL of the extract (E_1) was diluted with 49 mL of 90% aqueous methanol (E_2). 1 mL of E_2 was mixed with 2 mL methanolic aluminum nitrate and 2 mL of acetate buffer (pH, 6.0) and the absorbance of the resulting complex determined spectrophotometrically at 300 nm.

Similarly, 1 mL each of crude ethanol and crude aqueous extracts of *L. micranthus* were each diluted with 49 mL of 90% aqueous methanol. The resulting diluted samples were handled in the same way as with the powdered leaves extract.

Ethanol and aqueous formulations of *L. micranthus* (2 mL each) were each mixed with 2 mL of methanolic aluminum nitrate and 2 mL of acetate buffer (pH, 6.0) and the absorbance of the resulting complex determined spectrophotometrically at 300 nm. The concentrations of the tested samples were calculated from the calibration curve of FRE complexed with methanolic aluminum nitrate.

RESULTS AND DISCUSSION

The results of the spectrophotometric scan of CME and the complexes of CME and FRE are shown in Fig 1. While the CME absorbed maximally at 275 nm, the formed complexes showed strong absorption maxima at 300 nm. The 275 nm absorption band is characteristically a benzenoid band, which generally occurs between 250 and 280 nm (Finar, 1986). The benzenoid band represents the Ð to Ð* transition in the absorbing group of molecules upon irradiation with ultraviolet radiation. Flavonoids are polyphenols and contain conjugated systems and thus show intense absorption in the ultraviolet region of the electromagnetic spectrum (Harbourne, 1984). The bathochromic shift of this band to 300 nm (on complexation with methanolic aluminum nitrate) is highly characteristic and lends itself to the UV-Vis spectrophotometric identification and assay of *L. micranthus* leaves, extracts and formulations.

Plant flavonoids such as quercetin, myricetin, morin, kaempferol and isorhamnetin have been chelated with methanolic aluminum nitrate and the resulting complexes subsequently detected fluorimetrically (Crozier *et al.*, 2000). Quercetin and quecertin alkyl derivatives and or its glycosides have been detected in some mistletoes species (Fernandez *et al.*, 1998; Wagner *et al.*, 1996). The flavonoids of *L. micranthus* leaves therefore serve as good biomarker in the identification and assay of *L. micranthus* leaves, extracts and formulations.

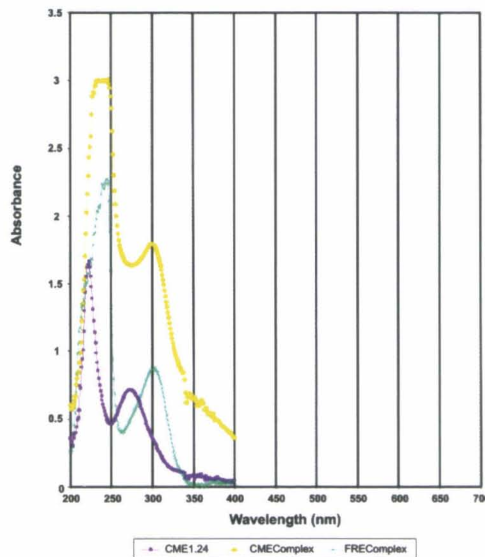

Fig 1. Spectrophotometric scan of CME, FRE and their complexes with methanolic aluminum nitrate solution

The graphical plot for the determination of the stoichiometry of complex formation by methanolic aluminum nitrate solution is shown in Fig 2. The stoichiometry of the complex formation between the methanolic aluminum nitrate solution and FRE of *L. micranthus* leaves was ascertained by the mole ratio method (Skoog & West, 1986). The concentration of aluminum nitrate (complexing agent) was held constant while the concentration of the extract was varied. The graphical plot of the absorbance against the mole ratio afforded a graph, whose intersection corresponds to the optimum combining ratio of the complex. Spectrophotometry is one of the most useful tools for elucidating the composition of complex ions in solution (Skoog & West, 1986).

We obtained a complex formation mole ratio of 0.33 suggesting the formation of a 1:2 stoichiometry between methanolic aluminum nitrate and FRE of *L. micranthus*. This finding suggests that methanolic aluminum nitrate solution acts as a bidentate ligand when complexing with the FRE of *L. micranthus* leaves. The possession of secondary valency by aluminum is consistent with this bidentate activity. Aluminum nitrate can accept electrons from the flavonoids of *L. micranthus*.

Our result is consistent with studies by Zang *et al.* (2005), which showed that the dominant species observed from the aluminum complexation reaction with some flavonoids has a 1:2 (aluminum (III): flavonoid) stoichiometry. The group generated aluminum

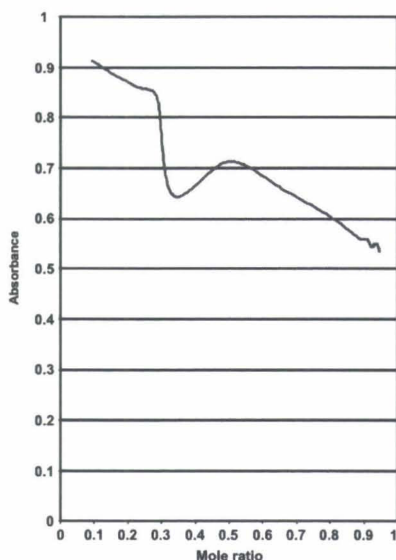

Fig 2. Plot of determination of stoichiometry of the complexation between FRE and methanolic aluminum nitrate solution

complexes of the type $[Al^{III}$ (flavonoid $-H)_2]^+$ by electrospray ionization in order to allow differentiation of isomeric flavonoids by tandem mass spectrometry. Phenolic compounds have been shown to chelate with metal ions at the 3', 4' orthopositioned hydroxyl groups (Mayer, 1998; VanAcker *et al.,* 1996; Miller *et al.,* 1996). Free hydroxyls of flavonoids offer possible sites for chelation with metal ions.

Information on the stoichiometry of the complex ions in solution can find usefulness in the estimation of the molecular weight of the complexing flavonoids. This can be the subject of further studies on the plant flavonoids.

The result of determination of time-absorbance relationship of the formed methanolic aluminum nitrate complex is shown in Table 1. The result indicates that complexation of methanolic aluminum nitrate with the FRE of *L. micranthus* leaves was instantaneous at room temperature and that the stability of the formed complex was unaffected by storage for 40 min. This implies that the formed complex can be handled routinely at room temperature without undue degradation. The developed method therefore appears robust.

The linearity of the complexation method was tested by determining the regression coefficient of calibration plots of appropriately complexed FRE of *L. micranthus* leaves. The obtained linear calibration curves had correlation coefficients (r^2) in the range of 0.9911 and 0.9949. Regression coefficient of the calibration plot up to 0.95 was considered acceptable. The obtained regression coefficients are high and show that Beer's law was obeyed within the tested concentration range. Relative concentration of unknown samples can therefore be derived from the calibration curves once the absorbance reading is known from spectrophotometric measurements. Obtained result will reflect the relative amount of the plant flavonoids.

Table 1. Result of time absorbance relationship of complex formed between FRE and methanolic aluminum nitrate

Time (min)	Absorbance ± SEM
5	0.5460 ± 0.0000
10	0.5470 ± 0.0020
15	0.5465 ± 0.0005
20	0.5455 ± 0.0005
25	0.5455 ± 0.0005
30	0.5455 ± 0.0005
35	0.5460 ± 0.0010
40	0.5465 ± 0.0005

A reference flavonoid compound could be used for the calibration curve determination. However, no compound has yet been isolated from the leaves of *L. micranthus*. Moreover, our study focuses on the whole plant as one active ingredient, affording a wholistic assay and comparison of *L. micranthus* products. The result of determination of the concentration of unknown samples of *L. micranthus* product by the complexation method will reflect the amount of flavonoids present in the sample.

The results of optical characteristics of the complexation method are shown in Table 2. The developed complexation method was sensitive with overall limit of detection (LOD) and limit of quantitation (LOQ) of 0.04 and 0.12 mg% respectively. The Beer's law limits of 0.4 to 3.6 mg% were considered acceptable for routine assay. Little ligand consumption and easy sample preparation confer additional advantage on the aluminum nitrate ultraviolet complexation assay of *L. micranthus* products.

Table 2. Result of optical characteristics of the complexation method

Parameter	Value
Wavelength	300 nm
Slope	0.0738 ± 0.0042
Beer's law limits	0.4 – 3.6 mg%
Intercept	0.2340 ± 0.0108
Correlation coefficient	0.9913 ± 0.0010
Limit of detection	0.04 mg%
Limit of quantitation	0.12 mg%

The accuracy of the developed complexation method was tested by determining the percentage recoveries of three different samples of FRE complexed appropriately. Percentage recoveries between 92 and 113% were obtained (Table 3). Even though the recoveries may appear poor in comparison to what may be expected, similar recoveries have been recorded (Jun *et al.*, 2005) for some plant materials. Further improvement in the quantitative recoveries of the developed method is however recommended.

Table 3. Result of determination of accuracy of the complexation method

FRE Sample	Mean Absorbance ± SEM	Percentage recovery (%)
0.9 mg%	0.3155 ± 0.0021	94.0
1.0 mg%	0.3210 ± 0.0000	92.0
1.1 mg%	0.3445 ± 0.0007	113.0

The precision of the developed method was tested by determining the absorbance values in duplicates of ten different aliquots of appropriately complexed FRE of *L. micranthus* leaves. The result of determination of precision and repeatability of the developed method are shown in Table 4. The obtained relative range for the precision test varied from 0.0000 to 0.0117. Relative range equal to or less than 0.02 was considered acceptable.

Table 4. Result of determination of precision and repeatability of the complexation method

Validation Test	Relative range	Decision
Precision	0.0000 – 0.0117	Acceptable
Repeatability	0.00175 – 0.0149	Acceptable

The repeatability of the method was tested by determining the absorbance values of appropriately complexed aliquots of FRE daily for five days. The obtained relative range varied from 0.0017 to 0.0149. Relative range equal to or less than 0.02 was considered acceptable.

The results of assay of powdered leaves, crude ethanol and crude aqueous extracts, ethanolic and aqueous formulations of *L. micranthus* based on the developed complexation method are shown in Table 5. The obtained results show that within the limits of a developing economy, samples of *L. micranthus* products can be assayed. Commercial samples of the leaves of *L. micranthus* can thus be tested before their being used for formulation. Extracts of the plant leaves can also be standardized prior to formulation. In-process testing of batches of products can also be done. In all, *L. micranthus* phytomedicines will be consistently manufactured with reproducible quality. The developed complexation method may serve

Table 5. Result of assay of powdered leaves, crude ethanol and crude aqueous extracts, ethanolic and aqueous formulations of *L. micranthus*

Sample	Mean absorbance at 300 nm	Percentage of FRE (%)
Powdered leaves	0.4620	14.55000
Crude ethanol extract	0.4500	2.28000
Crude aqueous extract	0.4370	2.13000
Ethanolic formulation	1.0843	0.01172
Aqueous formulation	0.9933	0.01043

Dilutions were made as appropriate.

both as an assay of the plant and as a specific assay of the plants antidibetic activity, since the antidiabetic activity of the plants has been demonstrated to be due to the flavonoids content of the leaves. The process is simple and does not require prior separation and extraction or very costly instrumentation.

A simple, precise and reproducible ultraviolet spectrophotometric method for the assay of powdered leaves, extracts and formulations of *Loranthus micranthus* has been developed and validated.

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12

Exploring Pharmacovigilance for Traditional Herbal Medicines

PULOK K. MUKHERJEE[1,*] AND S. PONNUSANKAR[1]

ABSTRACT

From the early stage of human civilizations plant and plant-derived products are part of the healthcare systems. Their development was supported by the diverse biodiversity in flora and fauna due to variations in geographical landscaping. Evolution of plant-derived remedies for healthcare through day-to-day life experiences is a part of cultural heritage in India, China and several other countries. In all the traditional systems of medicine, medicinal plants and plant-based formulations are employed for healthcare and disease treatment. Most of the herbals used in all these systems are believed to be safe, and many have beneficial role in therapy. Due to variations in herbal products including the content of the constituents, lack of quality control in manufacturing, standardization of doses and active ingredients etc, these drugs may produce adverse effects. Several drug-interactions associated with their use have also been reported. Further, approaches to assess clinical trials data, the dose response curve data, and the high-quality clinical trials are also lacking. There is an increasing public awareness because of the extensive use of herbal medicines, and high profile of safety concerns on public health at several levels increased the need to develop pharmacovigilance practices for herbal medicines. Existing surveillance system at present situation is inadequate, and cases of herbal toxicity are likely to be significantly under-reported. Hence, phytosurveillance on these herbal

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products is need of the day, which will allow us to develop quality, safety and efficacious herbal products.

Key words : Adverse drug reaction, efficacy, herbals, herbal pharmacovigilance, phytosurveillance, pharmacovigilance, quality control, safety

INTRODUCTION

In the developing world, herbs and plants are essential part of medicine and therapy. People in different parts of the developing world use herbal remedies for various ailments and they believe that herbal remedies were able to restore an individual's physical, emotional and mental health and offer a natural approach to maintaining the overall wellbeing of the body (Cammie Lai, 2004). Today herbal supplements have a major industry oriented business. In many developed countries, the herbal remedies business gives sales growth which is very much comparable to that of the cosmetics and pharmaceutical industries. Eisenberg *et al.* (1998) in their study found high prevalence of the concomitant use of alternative medications and prescription medicines in USA. From the study, they also revealed that, majority of the patients did not realize that combining these products with their prescription medications can result in drug-herb interactions and physicians were also not aware of patients taking such products. Reasons for the increase in consumer use of herbal medications include perception of efficacy and safety, non-prescription accessibility, sense of using a "natural" product, desperation and dissatisfaction with use of prescription drugs and lesser cost of herbal medicine etc (Bressler, 2005). The number of incidents/reports of clinically important interactions between herbals and prescription medications is increasing (Cranwell-Bruce, 2008; Gardiner *et al.*, 2008; Skalli, 2007). However, an insufficient quantity or number of information exists on how common this problem is in clinical practice.

Many herbs are used together with other herbs and formed as formulation. The adverse effects of cumulative or synergistic activities of different combinations of herbs and other herbs, or drugs and herbs are poorly studied. Thus, the added potential for adverse effects of herb-drug interactions is often unknown. However, the risk of potentially harmful herb-drug interactions increases with the popular use of herbal medicinal products among general population (Fugh Berman & Ernst, 2003). In addition, lack of quantitative data on herbal medications, makes it difficult to predict the potential for interactions with prescription and over-the-counter medicines. Various

findings from the research studies suggest that there is widespread lack of interest in herb-drug interactions within the pharmaceutical and herbal industries and highlight the lack of research currently being undertaken. It was well understood that, many patients take herbal and conventional medicines concomitantly, often without the knowledge of their physicians, and considering lack of understanding of herb-drug interactions, more systematic research and surveillance in this area is need of the day.

Pharmacovigilance

The World Health Organization (WHO) defines pharmacovigilance as "the science and activities relating to the detecting, assessment, understanding and prevention of adverse effects or any other possible drug related problems". The central theme of pharmacovigilance should be demonstration of safety rather than identification of risks.

The Erice declaration (1997) asserted that, "Monitoring, evaluating and communicating drug safety is a public-health activity with profound implications that depend on the integrity and collective responsibility of all parties such as consumers, health professionals, researchers, academic, media, pharmaceutical industry, drug regulators, governments, and international organizations- working together". This declaration still has relevance today because it emphasizes that safety information on medicines (now include herbals also) must serve the health of the public. Sir Alasdair Breckenridge, The Chairman of the Medicines and Healthcare Products Regulatory Agency (MHRA) said there are problems in the regulation of herbal medicines in the UK (Kayne, 2006). These include:

- lack of knowledge about the products being used,
- limited use of yellow card adverse drug reporting- scheme, this represent under-reporting rather than an indicating an absence of adverse reactions
- variable manufacturing standards (particularly of unlicensed products) due to lack of knowledge, error or deliberate intent
- ambiguity over nomenclature due to incomplete description of source materials or errors in translation from other languages
- drug interactions with herbal medicines

Why Pharmacovigilance for Herbals?

Pharmacovigilance is an increasingly important discipline in present day situation. Existing surveillance system for monitoring the adverse reactions to herbal products is inadequate, and cases of herbal toxicity are likely to be significantly under-reported. As such there is no

differentiation between chemically defined drugs and herbal medicine in the filling of procedure of adverse events. When safety issues are raised, there is no debate that adverse effects of herbal medicines must also be closely monitored and evaluated as those of single entity molecules. Are they really treated equally? The recent experience with "phytovigilance" (another term for pharmacovigilance with herbal medicinal products) raises the suspicion that there is a tendency to a disproportionate treatment of herbal remedies in the discussion of rare but nonetheless serious adverse events.

In many developing countries, regulation does not require manufacturers to demonstrate the safety or efficacy of natural products in human trials before marketing and there are no specific warnings about known or potential drug interactions on labels (Mukherjee *et al.,* 2007). Botanicals have special features that in most cases have the potential either to hamper or to have an adverse impact on the evaluation of herb-drug interactions. Unlike conventional medicines, botanicals are complex mixtures with multiple or unknown active ingredients. It may enable to alter the human physiologic characteristics through more than one mechanism of action, making it difficult to relegate these products to any one drug class. Because, process defines the product, extrapolation of scientific data across products from different manufacturers or sources is not possible. Defining the herb-drug interaction is lies in proper identification of plant, which includes Latin binominal and authority, identification of the plant and part(s) used in the preparation of herbal product, and the processes used to extract and isolate the desired active from plant resources (Huang *et al.,* 2004). So, ineffective product labeling leads to lot of confusion in identifying the product.

Similarly, concomitant use of herbals with approved prescription medications can result in therapeutic failures or adverse events and can produce variable outcomes of clinical trials if the concomitant use is not controlled (Huang *et al.,* 2004). Research publications have shown that co-administration of St John's Wort (SJW) decreases plasma levels of various drugs (Markowitz *et al.,* 2000; Johne *et al.,* 2002; Bauer *et al.,* 2003; Yue *et al.,* 2000). Existing surveillance on monitoring the adverse reactions is inadequate; hence cases of herbal toxicity are likely to be significantly under-reported. Adverse effects of herbals may be related to inherent toxicity of the herbal substance itself, individual susceptibilities such as hypersensitivity and idiosyncratic reactions or interactions between herbs and medications (Haller, 2006).

There are gaps in the inefficient regulatory processes that have allowed entry of unhealthy product in the market (Mukherjee *et al.,*

1998). Self-medication with prescription medicines, a long-time practice that is unsafe yet difficult to control, public consider herbals are generally safe because of the long tradition of usage and the concept of being natural and organic, and importantly practitioners and users of herbal medicines are likely to believe in unscientific, yet anecdotal claims etc are some of the reasons for not receiving the ADRs of herbal remedies. It is surprised that these are not recognized, and if ever observed, are attributed to the remedy's beneficial healing effect rather than harm. Because of the scarcity of local data and lack of rigorous investigations about herbal traditional remedies, the promotion of the use of such products focused on claims of good effects and neglected the possibilities of ADRs. There may be a need to re-examine the registration procedure of herbal products. Since the thalidomide disaster in 1960s, globally pharmacovigilance have taken some new directions. Emphasis on knowledge, information and education has been the guiding principle for the promotion of ADR monitoring programme. In developing countries where more than 60% of the population relies on alternative medicine, herbal pharmacovigilance can promote safer medicines in the market and provide better communication of harm-benefit of drugs and their use. It can also lead to rational drug use and prevention of drug related injuries (Ponnusankar *et al.,* 2007).

CHALLENGES IN MONITORING THE HERBAL MEDICINAL PRODUCTS

Quality Control and Efficacy of Herbal Medicinal Products

Herbal medicines are promoted in the market as natural, and therefore safe and harmless. However, no regulation controls the manufacturing of such products, consequently, quality control issues such as misidentification of herbs, method of processing, product uniformity, batch-to-batch variation, standardization of dose, contamination, mislabeling, and toxicity may be a problem (Peng, 2004) in herbal drugs. Quality control in the manufacture of many herbal products is far below the standards generally recognized as necessary for therapeutic drugs (Snodgrass, 2001). Similarly, consistency of many herbal products prevents extrapolating results of any high-quality clinical trial to other products or even to other batches of the same product from the same manufacturer is lacking.

Standardization has been strongly recommended as the answer to the quality question (Mukherjee *et al.,* 2006a). This is complicated by the fact that the concept is evolving around the world, when performed properly; it is a "seed-to-shelf" process that ensures lot-to-lot

consistency of botanical products. The purpose of the process is to minimize batch-to-batch variation caused by seasonal conditions and chemo type. For certain ingredients and products, monitoring marker compounds provides a positive control for production and confirmation that the product contains the correct amount of extract. Manipulation of marker compounds (one or more constituents that occur naturally in the plant) ensures batch-to-batch consistency but not necessarily quality of a finished product. Hence, standardization means not more than simple measurement and manipulation of marker compounds and can best be described as the continuum of steps necessary for production of a consistent product. Manufacturing botanicals to meet analytical standards for marker compounds does not necessarily ensure product efficacy or generic equivalence with products that have shown efficacy. Compounds other than the marker compound may also contribute the pharmacologic response, and there may be differences in bioavailability (Scott, 2002).

Herbal medicines are complex mixtures of more than one active ingredient. Many times, it is unclear that which or how many constituents are pharmacologically important. This multitude of active ingredients increases the possibilities of interactions between conventional medicines and herbals, herb-herb interactions. The interaction of drugs with herbal medicine is a significant safety concern, especially for drugs with narrow therapeutic index such as warfarin and digoxin (Zhou *et al.,* 2007). This interaction occurs when one compound affects the metabolism of another compound. This may occur during absorption, distribution, metabolism, excretion or at the site of drug action. Herb-drug interaction report should be interpreted with caution, however, since definitive evidence of pharmacological or toxicological interactions cannot be derived from individual case events (Haller, 2006). Reports of potential interaction derive from clinical trials, trials designed to monitor adverse effects, post-licensing drug monitoring, controlled trials on health volunteers required for a licensing application, findings from animal studies or *in vitro* on human or animal tissues and spontaneous adverse event reports from doctors, pharmacists or other health care professionals (Broughton & Denham, 2000). Table 1 represents some of the important herb-drug interactions in humans reported in various literatures.

Herb-drug interactions can broadly be divided into pharmacokinetic (including effects on drug transporters such as p-glycoprotein and induction/inhibition of CYP450 enzymes) and pharmacodynamic (additive or synergistic effects on effector organ) interactions. A significant number of herb-interactions are linked to alterations in CYP450 enzyme activity by herbs. *In vitro* and *in vivo* studies that

have been published (Evans, 2000; Ioannides, 2002; Wilkinson, 1997) on effects of herbs on metabolizing activity. However, the following factors might explain the discrepancies in the study results include (Haller, 2006):

1. Differences in phytochemical concentrations (higher doses used *in vitro* studies)
2. Variability in product content and doses of active constituents
3. Low bioavailability of herbal product, *i.e.* poor dissolution and gastrointestinal absorption of active substances
4. Inter-individual differences in drug metabolism due to genetics, dietary, alcohol, and smoking habits.
5. Inadequate sample size in clinical studies to detect clinically significant changes in drug metabolizing enzyme activity

The strength and potency of these products are not easily quantified, and impurity and stability are often difficult to monitor. Hence, botanicals must be regulated as in western countries and the requirements include Good Manufacturing Practices (GMP), labeling, packing, marketing and reporting requirements etc. (Huang *et al.*, 2004). Researchers, manufacturers, and the regulatory agencies must apply rigorous scientific methodologies and clinical trials to ensure the quality and consistency of the traditional herbal products, to gain public trust and confidence and to bring herbal product into mainstream of today health care system. Thousands of years of traditional use of herb(s) can provide us with valuable guidelines to the selection, preparation and application of herbal formulations (Mukherjee *et al.,* 2006b). To be accepted as a viable alternative to the conventional medicine, the same rigorous methods of scientific and clinical validations applied to single component chemical component, must be applied to herbals too.

Drug control authority is responsible for ensuring the quality, efficacy, effectiveness and safety of all marketed medicinal products. Quality, efficacy and effectiveness are usually established through data obtained from animal studies, preclinical and clinical trials involving humans, and *in vitro* testing to ensure compliance with acceptable standards (Haq, 2003). It is a well established fact that, pre marketing clinical trials do not have the statistical power to detect rare adverse reactions, nor they have sufficient follow-up to identify delayed adverse effects or effects from long-term exposure. In view of establishing the safety of herbs, initiating the pharmacovigilance program will assist in understanding and prevention of adverse effects or any other possible drug related problems.

Safety Concerns with Herbal Medicinal Products

Herbal products impose a number of challenges to qualify control, regulatory and quality assurance. Most herbal formulations available in the market have not been subjected to drug approval process to demonstrate their safety and effectiveness and majority of them were prepared based on traditional approach. Some of them contain heavy metals, spurious, adulterated or misbranded drugs, poisonous organic substances, undeclared drugs etc. (Pal & Shukla, 2003). Without quality control, there is no assurance that the herb contained in the bottle is the same as what is stated outside on the label. There are case reports of serious adverse events after administration of herbal products. In most cases, the herbs involved were self prescribed and bought over-the-counter or obtained from a source other than a registered practitioner. Most of the commercially available Ayurvedic preparation does not even conform to ancient Ayurvedic text. The widespread disregard for quality control in the herbal industry has tarnished the reputation of many important medicinal plants.

Botanicals have certain peculiar features and quality concerns that require special considerations in the regulatory approaches for further product development. Many herbal products are available in the market and have had a long history of human use and accumulated data on safety experiences but lack of rigorous proof of efficacy and safety. 'Rational phytotherapy' is based on a reproducibility of effects. As the raw material for herbal medicines is of biological origin, the phytochemical composition must necessarily change according to growth conditions, origin and processing. Thus, herbal products must be standardized in order to achieve a safe and reliable effect. As the quality of a product cannot be defined retrospectively, the key steps in the production of herbal remedies must be examined for quality and safety assessment (Mukherjee *et al.,* 2006c).

Phytochemicals are similar to therapeutic drugs, used by the general population. It has the potential to cause interactions with various classes of drugs. Such interactions include the induction or inhibition of drug metabolizing enzymes and drug efflux proteins. Ever increasing use of herbs with western medicines raises the potential for drug-herbal interactions, which may alter the bioavailability through altered absorption, metabolism and distribution. Any inhibitory effect of herbs on modulating enzymes may result in enhanced plasma and tissue concentrations leading to toxicity, while in any inductive effect may cause reduced drug concentrations leading to decreased drug efficacy and treatment failure. Hence, measuring the safety evaluation of herbs through cytochrome inhibition assay is need of the day.

Approaches to Study CYP Enzymes, Drug Metabolism and Herb-Drug Interactions

In the last 20 years, great progress has been made in the characterization of human CYPs, as a result it is now possible to gain information on the human drug metabolism of drug candidates and drug-drug, herb-drug interactions. Considerable knowledge on use of purified enzymes, identification of substrates and inhibitors, specificity of CYP enzymes has gained its momentum in the study of human metabolism. Thus, characterized microsomes from human livers became a suitable tool to anticipate human metabolism and herb-drug interactions. Several issues need to be addressed in the role of CYPs in the metabolism of drug are; the comparative metabolic profile of a drug (identification of stable/reactive metabolite), knowledge of major metabolic routes involved in metabolite formation and the human enzymes involved, and potential enzyme-inhibiting or enzyme inducing properties of herb/drug (Donatao & Castell, 2003). Similarly, CYP 'phenotyping' is also gaining its importance because of its clinical implications of the high variability of CYP isoforms in humans. Xenobiotics tend to be metabolized by several isoenzymes, and only a few compounds are exclusively metabolized by one enzyme. Due to the importance that CYP phenotype has for the pharmacokinetics, pharmacodynamics and potential toxicity of a herb, the identification of CYP isoforms involved in its metabolism is also of relevance (Rodrigues, 1999).

Several *in vitro, in vivo* and *in silico* approaches were available for the estimation of CYP inhibition. *In vivo* interaction studies of CYP are usually necessary to provide evidence of their clinical importance. Because of the problems in extrapolating the results of these animal studies to humans, various *in vitro* methods have been developed by employing human tissue-derived systems. The recent development of knowledge in the area of informatics several *in silico* methods are also used to study the CYPs and their interactions with xenobiotics. A detailed view on various aspects of these studies was discussed below.

In vitro Techniques

Drug-drug, herb-drug, drug-xenobiotics interactions occurring during metabolism can be studied using *in vitro* approaches. One drug or xenobiotics may modify the metabolism of another one by various mechanisms: enhancing the metabolism or reducing the metabolism by acting on the expression or on the activity of the responsible enzyme. Experimental tools are available to study the mechanism of action via *in vitro* approaches. *In vitro* approaches offer several

decisive advantages: they allow us to study a large number of products simultaneously in well-defined and reproducible conditions, they are not too expensive, and they are very rapid compared to clinical or animal experimentations.

In vitro drug-herb interactions data are necessary for devising mechanism based (clinical) herb-drug interaction study strategies. The effects of herbs on well characterized drug metabolism reactions known to be specific for various human drug-metabolizing enzymes are routinely examined using *in vitro* approaches. Frequently, human liver microsomes, a rich source of human drug metabolizing enzymes such as cytochrome P450s, are used as an *in vitro* system. As far as interactions at the level of xenobiotics-metabolizing enzymes are concerned, the approach used depends on the mechanism of interaction.

Experimental hepatic models used for these studies include:

- Microsomes, a subcellular preparation of endoplasmic membranes, the most universally used *in vitro* model
- Hepatocytes in culture or liver slices
- Recombinant enzymes resulting from genetically transformed organisms in order to express (or over express) a specific enzyme, namely human liver cytochromes

Microsomes (sub cellular fractions)

Microsomes are often the first hepatic model used in metabolism studies. The metabolism of chemical entity can be easily investigated by incubating the drug with hepatic microsomes, followed by analysis of incubation mixtures by various techniques such as spectrophotometry, fluorimetry, HPLC, LC-MS/MS, LC-MS/ESI etc. The above techniques considerably simplified and speeded up the identification of metabolites as well the identification of CYP involved in the metabolism. Microsomes are prepared from liver tissue by homogenization, and can be stored at -80°C for years with little or no loss of CYP enzyme activity (Pearce *et al.,* 1996). The major limitation of hepatic microsomes model are that they have very low phase II activities, less incubation time (<1 h), and poorly metabolized drugs and secondary metabolism are hardly detected. Hence, the results obtained *in vitro* are markedly different from those of *in vivo* results (Donatao & Castell, 2003).

Hepatocytes – liver slices

Liver slices are relatively simple to use and not require sophisticated instrument. The drawback is the survival of cells within the sliced

tissue, however several authors have claimed that liver tissue slices can metabolize drugs for about 24 h, which is considerably longer than by microsomes incubations (Berthou *et al.*, 1989; Beamand *et al.*, 1993; Vickers *et al.*, 1993).

Hepatocytes – Cultured cells

Cultured hepatocytes represent a complex, more predictable model of metabolism of *in vivo.* Human hepatocytes from liver transplant programmes and surgical waste are making the prediction of human metabolism with more accuracy (Ponsoda *et al.*, 2001). Human hepatocytes have some advantages that make them the closest model for *in vivo* studies (Sun *et al.*, 1996; Placidi *et al.*, 1997; Olinga *et al.*, 1998; Donato *et al.*, 1999). As intact cells are used, plasma membrane, metabolic pathways, levels of physiological cofactors and coenzymes and active gene expression are reasonably well maintained for several h/days in culture. A major drawback is the inability of differentiated hepatocytes to grow efficiently *in vitro*, cell cultures need to be prepared each time from liver tissue and fully differentiated cells need to be used. The viability of thawed cells is satisfactorily achieved, by cryopreservation entrapped in polysaccharide matrixes, is about 70–80% and their metabolic activity of phase I and II enzymes is 60% of that of freshly isolated cells (Guillouzo *et al.*, 1999; Madan *et al.*, 1999) and it is quite acceptable for short-term assays. Hence, cultured human hepatocytes can be safely used in drug metabolism studies for up to 2–3 days, but isolated human hepatocytes shows 50–60% reduction of CYP activity during the first 24 h in culture due to adaptation of cells to culture conditions. Primary cultured hepatocytes show a gradual loss of CYP activity (David *et al.*, 1998) that is preceded by a decrease in CYP mRNA expression (Woodcroft & Novak, 1998). Some studies were performed with rat hepatocytes, where CYP activities rapidly decay during the first 48 h in culture (Donatao & Castell, 2003). In many respects, hepatocytes are more appropriate *in vitro* model for the prediction of drug metabolism *in vivo.*

cDNA expressed hepatoma cell lines

Hepatoma cell lines have been investigated as alternatives to primary hepatocytes cultures in drug metabolism studies (Roe *et al.*, 1993). The cell lines shows very limited metabolic capacity due to a very low expression of CYP activities (Enosawa *et al.*, 1996), despite of its unlimited life span and simple to culture (Donatao & Castell, 2003). Hence, hepatoma cell lines do not constitute a real alternative to primary cultured hepatocytes.

But human hepatoma BC2 cell line retains contact growth inhibition *in vitro*, and the activity expressed is much higher than

recorded in HepG2 cells, but still lower than hepatocytes (Gomez-Lechon *et al.,* 2001).

In vivo Enzyme Studies

Animal studies may give important information on herb–CYP interactions. Although *in vitro* models may provide a quick screening method for the herb–CYP interactions, *in vivo* interaction studies are usually necessary to provide evidence of their clinical importance. But interspecies variations in the substrate specificity, catalytic features, and amino acid sequences of CYPs may cause difficulty in extrapolating animal data to humans (Boobis *et al.,* 1990; Lewis *et al.,* 1998; Lin, 1995). For example, chlorzoxazone 6-hydroxylation is extensively catalyzed by CYP2E1 in humans (Halpert *et al.,* 1994; Murray & Reidy, 1990), but by CYP1A2 and CYP3A1 in rats. Therefore, it may be difficult to predict accurately the effects of herbal preparations in humans based on animal studies, and human studies are usually required to confirm herb–CYP interactions.

Single probe substrates/inhibitors can be used to explore the effects of herbs on the activity of specific CYP enzyme *in vivo*, *e.g.*, caffeine or debrisoquin for CYP2D6, (Wieling *et al.,* 2000), midazolam or erythromycin (Rivory *et al.,* 2001) for CYP3A4 (Brockmoller & Roots, 1994) etc. A cocktail of probe drugs has been used to explore the activities of multiple CYPs (Frye *et al.,* 1997). For example, alprazolam and caffeine can be administered simultaneously for the assessment of *in vivo* CYP3A4 and CYP1A2 activity, respectively (Schmider *et al.,* 1999). Similarly, a cocktail containing tolbutamide (CYP2C9), caffeine (CYP1A2), dextrometharphan (CYP2D6), oral midazolam (intestinal wall and hepatic CYP3A), and intravenous midazolam (hepatic CYP3A) have been used to investigate the effects of St. John's Wort on the activities of various CYPs in humans (Wang *et al.,* 2001). The value of the cocktail approach may be limited due to marked intrasubject variability and the possibility of interaction between the co-administered probes.

Computational In silico Methods

The decisive progress yielded nowadays by ongoing research, along with the major new possibilities offered by computer science, allowed rapid development of reliable "*in silico*" approaches to predict drug interactions. The major in silico methods include simple rule-based modeling, structure activity relationship (SAR), three dimensional quantitative structure activity relationship (QSAR) and pharmacophores (Ekins & Wrighton, 2001).

Several expert systems utilizing rule- and knowledge-based databanks have been introduced. These systems are capable of predicting the potential sites of metabolism in the drug molecule and to further provide metabolic trees and pathways with estimations about the likelihood for the production of each metabolite (Greene *et al.*, 1999; Button *et al.*, 2003; Langowski & Long, 2002).

SAR and QSAR studies, elucidation of three-dimensional structure of proteins, receptors, enzymes, etc., will allow an objective representation of the binding of a drug to its biochemical target. This will allow a rapid comparison of different products and the determination of the critical chemical functions, structures, and affinities involved in an enzymatic process. A better knowledge of the kinetic parameters and their relation to the biochemical or pharmacological process is a necessary step to learn this approach. Research going on in different laboratories will certainly contribute very efficiently to this direction (Rodriges *et al.,* 2001; Testa, 2000; Ter Laak & Vermeulen, 2001)

Large databases are becoming available, based on the numerous results obtained by HTS, that allow correlation studies between *in vitro* and *in vivo* data. New software applications are being developed to handle these data and to extrapolate the results to similar situations or products. A dynamic computer based method, called Quantitative Drug Interactions Prediction System (Q-DIPS), has been developed to make both qualitative deductions and quantitative predictions on the basis of databases containing updated information on CYP substrates, inhibitors, inducers, and pharmacokinetic parameters (Bonnabry *et al.,* 2001, 2001a). Three dimensional structure activity relationship studies are improving very rapidly, as is our knowledge of the architecture of the enzyme active site. This, combined with computer modeling, will allow a successful design of pharmacophore models of great help for the prediction of possible herb-drug interactions and their mechanism (Ekins *et al.,* 1999). These tools are useful for understanding the reactions catalyzed by CYPs, predicting possible herb-drug metabolism interactions, and other pharmacokinetic parameters such as clearance (Ekins & Wrighton, 2001). With the isolation and identification of some of the active constituents of herbal preparations, there has been an increasing use of *in silico* models to study their pharmacological effects. These approaches have also been used to study herb-CYP interactions.

A study conducted by Koul *et al.* (2000) using a structure–activity relationship analysis to investigate the effect of structural modifications of piperine (pentadienyl or piperidine, on the inhibition

of the CYP-catalyzed reactions, arylhydrocarbon hydroxylation (CYP1A), and 7-methoxycoumarin-O-demethylation (CYP2) in microsomes prepared from untreated, 3-methylcholanthrene- and phenobarbital- treated rat liver. This study has indicated that saturation of the side chain resulted in a marked increase in the inhibition of CYPs; whereas modifications in the phenyl and basic moieties in a few analogues led to maximum selectivity in inhibiting either constitutive or inducible CYP activities. QSAR studies have been used to analyze the inhibitory effects on caffeine N3-demethylation (a marker activity of CYP1A2) in human liver microsomes of naturally occurring flavonoids that exist in many herbs (Lee *et al.,* 1998). QSAR analysis has indicated that the volume to surface area ratio was the most effective factor for producing the inhibition of caffeine N3-demethylation by these flavonoids, and the electron densities on the C3 and C40 atoms exercised significant influence on the inhibitory effect.

Toxicity Concerned with Herbal Medicinal Products

The use of herbal medicine is ancient, plant chemicals are still backbone of our pharmacopoeia because more than 50% of drugs used in various pharmacopoeia are obtained from herbs or derived from modification of chemicals found in plants (Bagnais *et al.,* 2004). If plants contain pharmacologically active compounds, they also contain toxic substances. Several factors, like active uptake by tubular cells and high concentration in the medullary interstitium, make the kidneys particularly vulnerable to toxic abuses, more specifically risk to renal patients. Moreover, many herbal preparations contain unidentifiable pharmacologically active compounds, undisclosed drugs and heavy metals such as lead, mercury, arsenic etc. (Mukherjee, 2002, 2005).

The origin of herbal poisoning can range into the following; (1) herbal plants properly identified, with unknown or underestimated toxicity; (2) herbal plants contaminated with drugs, hormones, or heavy metals, (3) herbal plants incorrectly identified; and (4) herbal plant interaction with conventional drugs. Besides the large number of clinical drugs reported to have potential hepatotoxicity, renal toxicity, cardio toxicity etc during epidemiological, prospective studies etc, other agents such as excipients present in formulation, herbal medicines which are increasingly consumed and often not disclosed, recreational and illegal compounds (Larrey & Pageaux, 2005) etc. were also be taken into consideration for its toxicity. Among them, herbal and dietary supplements are a significant component of the over-the-counter market. ADRs affecting the liver represent an

important challenge for prescribers and healthcare authorities. Acute liver failure is the most severe clinical expression and represents the first cause of fatalities related to drugs. Now, the particular interest is being paid to the frequency of serum alanine aminotransferase (ALT) elevation over the three times the upper limit of normal, the increase in bilirubin over the two times the upper limit of the normal (called as Hy's rule), and clinical events, in the treated group compared to the placebo/known comparator, were set as the assessment of hepatic adverse events during clinical trials (Larrey & Pageaux, 2005). The incidence of herb-induced adverse effects in the liver, may be assessed through prospective studies, epidemiological studies etc. The course of acute liver injury failure may be modulated by the following parameters such as, the continuation of causative drug administration despite the onset of liver injury, the age with the higher risk of older people, a pre-existing cirrhosis, fasting, denutrition, chronic alcohol abuse, and sometimes the amount of ingested drug (Larry, 2002).

Bagheri *et al.* (2000) performed a prospective study at inpatient department 1 week per month for a period of 5 months. The patients were selected by a computerized process using biochemistry laboratory data, based on serum enzyme values, alanine aminotransferase (over 2 fold normal) and alkaline phosphatase (over 1.5 fold normal). All the cases of liver injuries were identified from the liver function tests performed in the central biochemistry laboratory of the hospital. Following the patient selection, hospital medical records were inspected for additional data. From the study selected population, *i.e.* 1976 ALT and 1814 AP assays were performed during the study period, 7.9% (n=156), 8.8% (n=159) tests, respectively, fell into the selected criteria. Using the hospitalization database, the incidence of drug induced liver injuries was estimated as 6.6 per 1000 patients a week. Further the patients were followed-up for further treatment in collaboration of the patient general practitioner. From the study, it was concluded that computerization of biochemical data would allow the development of systems to improve detection of drug-induced injury. Moreover, underreporting remains important for such potentially serious ADRs, even in university hospital.

This study allowed determining the incidence of drug-induced liver injury in hospitalized patients through data analysis from laboratory recording. The development of computerization of biochemical data could improve the detection of drug-induced injury. The same procedure could also be applied to wide use of herbal remedies as over-the-counter drugs, to know the potentially liver damaging herbals in general practice.

Table 1. Herbs that interact with conventional medicines in humans

Herb	Prescription drug	Results/Comments	Reference(s)
St John's Wort (*Hypericum perforatum)*	Alprazolam	↓ AUC by 41%, t ½ by 24%, ↑ C_{max} by 15% Minor induction of CYP3A4	Markowitz *et al.* (2000)
	Amitriptyline	↓ AUC by 22% and nortriptylline by 41% Induction of CYP3A	Johne *et al.* (2002)
	Cyclosporine	↓ blood concentration and rejection events ↓ AUC by 46%, C_{max} by 42%, C_{trough} by 41%, altered metabolite profiles Induction of enzyme and P-gP	Barone *et al.* (2001); Bauer *et al.* (2003)
	Digoxin	↓ AUC by 25%, C_{max} by 33%, C_{trough} by 26% P-gP induction	Johne *et al.* (1999)
	Warfarin	↓ INR Enzyme induction	Yue *et al.* (2000)
Ginkgo *Ginkgo biloba*	Aspirin	Spontaneous hyphema Additive effect	Rosenblatt, and Mindel (1997)
	Trazodone	Coma Unknown	Galluzzi *et al.* (2000)
	Warfarin	PT 16.9 min, PTT 35.5 min, left parietal haemorrhage Additive effect	Mathews (1998)
Garlic (*Allium sativum)*	Fluindione	↓ INR Additive effect	Pathak *et al.* (2003)
	Saquinavir	↓ AUC by 51%, ↓ C_{8h} by 49%, ↓ C_{max} by 54% Induction of CYP3A4 and Pg-P	Piscitelli *et al.* (2001)
	Warfarin	↓ INR and clotting time Additive effect	Sunter (1991)
Milk thistle (*Silybum marianum)*	Indinavir	↓ AUC by 9%, ↓ trough level (C_{8h}) by 25% Modulation of CYP3A and P-gP	Piscitelli *et al.* (2002)

Table 1. *Contd.*

Herb	Prescription drug	Results/Comments	Reference(s)
Kava Kava (*Piper methysticum*)	Levodopa	↑ 'off' period	Schelosky *et al.* (1995)
Ginseng (*Panax ginseng*)	Phenelzine	Serotonin syndrome Additive effect	Gwilt *et al.* (1994)
	Warfarin	↓ INR to 1.5 Antagonistic effect and/or enzyme induction	Janetzky and Morreale (1997)
Betel nut (*Areca catechu*)	Procyclidine	Several extrapyramidal symptoms Antagonism of procyclidine by arecoline	Deahl (1989)
Chamomile (*Matricaria chamomilla*)	Warfarin	↓ INREnzyme induction	Segal and Pilote (2006)
Green tea (*Camellia sinensis*)	Warfarin	Thickening of blood Antagonistic effect	Taylor and Wilt (1993)
	Warfarin	Decreased INR to 1.37 from 3.79 Antagonistic effect due to presence of vitamin K in herb	Taylor and Wilt (1993)
Ayahusca (*Banisteriopsis caapi*)	Fluoxetine	Tremors, shivering, sweating, severe nausea and vomiting	Callaway and Grob (1998)
Celery (*Apium graveolens*)	Thyroxin	Decreased T4	Moses (2001)
Prickly pea (*Opuntia streptacantha*)	Oral hypoglycemic agent	Mean fasting glucose level increased upto 205 mg/dL	Meckes-Lozyoa and Roman-Ramos (1986)
Ginger (*Zingiber officinale*)	NSAIDS	No symptomatic relief	Srivastava and Mustafa (1989)

Table 1. *Contd.*

Herb	Prescription drug	Results/Comments	Reference(s)
Licorice (*Glycyrrhiza glabra*)	Sennoside	Myoclonus due to metabolic alkalosis	Ishiguchi *et al.* (2004)
Alfalfa (*Medicago sativa*)	Immuno-suppresive agents (azathioprin, cyclosporine)	Rejection of kidney transplantation	Light and Light (2003)
Shankha-pushpi (*Evolvulus alsinoides*)	Phenytoin	Loss of seizure control	Dandekar *et al.* (1992)

AUC – Area Under the Curve; t ½ - the time taken for plasma concentration to reduce by 50%; C_{max} - The maximum or "peak" concentration of a drug observed after its administration; C_{trough} - the minimum or "trough" concentration (C_{min}) of a drug observed after its administration and just prior to the administration of a subsequent dose; P-gP- P-glycoprotein; INR – International Normalized Ratio.

Precise identities of the culprit substances, toxicological characteristics and pathogenetic mechanisms are mainly unknown. Herbal plants reported to cause renal damage include *Securidaca longepedunculata, Euphorbia metabelensis, Crotalaria laburnifolia, Callilepsis laureola, Aristolochia heterophylla, Taxus celebic,* etc. (Bagnais *et al.,* 2004). The Dietary Supplement Health and Education Act (DSHEA) of 1994 states that dietary supplements are not required to undergo premarket safety and efficacy testing. Also, there are no requirements for product labeling to warn of known or potential adverse reactions. As a whole, the lack of enforcement of good manufacturing practices in the dietary supplements industry is evident in reports of impurities and adulteration. Herb induced renal dysfunction, nephrotoxicity and adverse reactions data is limited, because reporting of these events are voluntary. A variety of dietary supplements ingested for medicinal purposes have been associated with renal toxicity such as Cat's claw (*Uncaria tomentosa*), Chaparral (*Larrea tridentate*), Cranberry (*Vaccinium macrocarpon*), Ephedra (*Ephedra sinica)*, Germaninum, Licorice (*Glycyrrhiza glabra)*, Pennyroyal (*Hedeoma pulegioides)*, Thunder god vine (*Tripterygium wilfordii hook F)*, Worm wood oil (*Artemisia asbinthium*), Yellow oleander (*Thevetia peruviana)*, Yohimbe (*Pausinystalia yohimbe*) etc. (Gabardi *et al.,* 2007).

Most data published in rental toxicity of herbs are case reports, with no clear identification of the herbal product involved in renal toxic effect. Various renal syndromes such as acute tubular necrosis, acute interstitial nephritis, hypokalemia, hypertension, papillary necrosis, chronic interstitial nephritis, nephrolithiasis, urinary retention and cancer of the urinary tract were reported (Larrey & Pageaux, 2005). Frequency of medicinal herb administration of chronically ill patients should be taken into account by nephrologists and such information may be lifesaving.

METHODS OF HERBAL PHARMACOVIGILANCE

An adverse drug reaction (ADR) is defined by the World Health Organization as any response to a drug that is noxious and unintended and which occurs at doses normally used in man for the prophylaxis, diagnosis and therapy of disease, or for the modification of physiological function (WHO, 1964). This definition excludes accidental or deliberate excessive dosage or maladministration. Monitoring ADR or adverse event (AE) is a source of new information regarding medicines approved for the market, which is applicable to herbals too. For the practicing doctor, diagnosing a drug induced problem is critically important and identifying the method of reporting such AE as important as identification. Similarly, early detection/recognition of events led to harm/benefit issue to patients and better communication if the causes of such events are identified and managed. Further, detection of AE or ADR of herbals lead to better report analysis that may contribute to safer use and utilization of herbals, hence the need to develop the science of herbal pharmacovigilance or phytosurveillance is the present day need of our country.

RADAR Approach

Clinical management of herbal adverse events follows the same approach as like as the conventional medicines. However, some unique aspects such as lack of consumer and provider awareness, non-specific symptomatology, and insufficient reliable information on herbal products present additional challenges. Christine A Haller (2006) has suggested a systematic approach called RADAR, that involves the elements including recognition (R), assessment (A), definite diagnosis (D), advice (A) and reporting (R). Different aspects of this RADAR approach as may be applicable with natural products have been summarized in Table 2.

Table 2. Different aspects of RADAR approach as may be applicable with natural products

Elements	Parameters
Element-1 Recognition (R)	Health care providers must obtain history of the herbs use through questioning such as - "When you have used the herbals last time?" - "Do you use any herbal therapies for your symptoms?" - "Duration of the herbals use?" etc. Such patient history helps us to recognize the adverse events or the possibility of herbs induced toxicity.
Element-2 Assessment (A)	If the history is elicited the herbs use that could potentially be the etiology of the patient's symptoms, a prompt step-wise assessment is needed. 1. Generate differential diagnosis utilizing the clinical findings, laboratory and diagnostic studies, and results of toxicology testing. 2. Rule out the possible causes also determines the likelihood of the causative exposure. 3. If the event appears to be potentially related to the herbals, obtain the specific product information such as: copy of product label, source including name of the manufacturer, distributor and seller; sample of product for analysis, contact information of the alternative medicine practitioner.
Element-3 Diagnosis (D)	1. Obtain patient urine and plasma specimens from the earliest collection and freeze it in -20°C or until analysis. 2. Obtain the unused herbal products (if available with the patients), unprocessed herbs, decoctions, teas, formulations in their original packing and herbal prescription if available. 3. Try to identify the product (this may be difficult if the product label does not contain sufficient information to identify the active constituents, names may be unfamiliar and dose amounts). 4. Ask assistance for laboratory analysis (the difficult may be identifying the actual content and dose, labeled and actual content, adulterated and contaminated etc)Definitive diagnosis of a herbal adverse events may be delayed for days or weeks for confirmatory laboratory testing results. However, patient and provider contact information must be obtained for later follow-up.
Element-4 Advice (A)	1. Ask the patient to stop taking the herbs; if he/she experiences any ADR. 2. Inform the patient and provider about the possibility of withdrawal symptoms. 3. Provide supportive care and close monitoring for worsening system toxicity such as liver, renal etc.
Element-5 Reporting (R)	Adverse events related to herbals should be reported to peripheral or regional pharmacovigilance centers. It is very

Table 2. *Contd.*

Elements	Parameters
	important that, it should also be reported to regulatory agencies, because of the inadequate surveillance system for tracking ad monitoring post-marketing cases of toxicity and adverse reactions. Consider unique and well-documented cases of herbal toxicity and interactions in scientific journals to inform other health care professionals.

Data Mining in Pharmacovigilance

Data mining is defined as the application of statistical techniques such as predictive modeling, clustering, link analysis, deviation detection and disproportionality measures, to knowledge discovery databases (Wilson, 2003). There is some confusion in the terminology some authors define data mining as the process of acquiring information. And knowledge discovery in databases (KDD) as the process of extracting previously unknown, valid and actionable information from large information sources or databases. This process requires a definition of the project goals, dataset acquisition, data cleaning and reprocessing, data mining, data interpretation and utilization.

Data collection for an individual product is expensive; hence data mining processes are often performed on existing databases, for the purposes of pharmacovigilance. Similarly, for herbal products, databases should be developed through various reporting systems. The necessary size of the dataset required is difficult to determine but will depend on the data quality, the background frequency of the event and the strength of the association of the event with the herb or herbal formulation. To generate a moderately rare event, large databases are required.

Several large databases are used throughout the world and following are the few databases and its characteristics are described.

Spontaneous Reporting Databases

Spontaneous reporting is a passive system, provided by the healthcare professionals to government agencies/drug companies or to the regional or peripheral centers. Its limitation include inconsistent in reporting with more frequent reporting of unusual reactions, reactions for new drugs and serious reactions (Biriell & Edwards, 1997). Further, the accuracy of the data contained the data within the reports is uncertain. However, spontaneous reporting databases

provide more number of collected data these can be mined to obtained details of ADEs. To date, spontaneous ADR reporting is the backbone of most pharmacovigilance centers, medical institution and clinical trials (Koh *et al.,* 2008). These reports will give rise to signals which alert the regulatory authorities or the physicians about the dangers posed by the suspected drug or herb. Voluntary reporting is the cornerstone of signal detection of unexpected results, not only in the world of drug safety but also in respect of herbal safety too (van Puijenbroek *et al.,* 2007).

Prescription Event Monitoring Databases

Prescription event monitoring (PEM) is used to detect ADEs by collecting high-quality data from family doctors, on a select group of patients exposed to a specific (new) drug, for a limited period of time (Mann, 1998). This database contains clusters of patients exposed to certain drugs, hence its lack an adequate control group.

Linked Administrative Databases

Medicaid is the classic example of the large linked health administrative database. Such databases contain data on many subjects and may also be used as a source for data mining. The data are available at relatively small additional costs are not subject to recall or interviewer bias. However, the completeness of detail is questionable, so it may not represent the whole population. The Saskatchewan-linked administrative healthcare utilization database and the Tayside Medicines Monitoring (MEMO) are the classic examples of linked medical health administrative databases (Rawson & Rawson, 1999) used to collect the reports.

Electronic Medical Records

It contain a large number of data fields, including details such as the use of non-prescription drugs, smoking and symptoms and signs, laboratory data etc., on small number of patients and may also be used for data mining. Because of the large number and detail of the variables, which can be combined to generate new diagnoses or adverse events, hypotheses, which are not restricted to existing diagnoses, can be explored (Honigman *et al.,* 2001).

The above mentioned methods were some of the techniques used for signaling the ADR of various medical products. Many other methods were also available and a suitable method should be evaluated, adopted and implemented. Following are the some of the methods, may be used to initiate the herbal pharmacovigilance

system. However, the methods and techniques used in general are inter-linkable hence a method which is adoptable by majority should be identified and introduced.

STEPS TO INITIATE HERBAL PHARMACOVIGILANCE

There is an increasing awareness at several levels of the need to develop pharmacovigilance practices for herbal remedies. Several models of pharmacovigilance and its associated tools have been developed in relation to synthetic drugs, and applying these methods to monitoring the safety of herbal medicines presents unique challenges in addition to those described for conventional medicines. As the interest in phytomedicines and the use of plant-derived therapeutics is increasing there is a need to improve not only quality control but also monitoring of such therapies so as to ensure the public is receiving the safe drug. Fig 1 represents some of the steps to be initiated to introduce the herbal pharmacovigilance to monitor the safety of herbal medicines.

Good Regulatory Practice (GRP)

Good Regulatory Practice should be initiated with herbals and its formulations, like single entity compounds. Although products are being registered at a faster rate, safety has not been compromised at the expense of speed. The Drug Control Authority should provide caution when registering product with limited safety data and herbals formulation containing suspected to be associated with ADR. Inefficiency in providing such information of serious ADRs will lead to major catastrophy in public health.

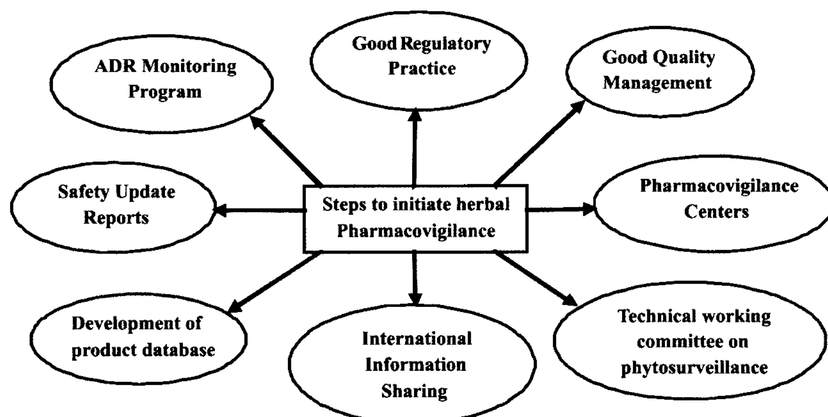

Fig 1. Schematic diagram of Steps to initiate herbal pharmacovigilance

Good Quality Management (GQM)

Compliance with good quality management system, standard and good documentation of product information through computerization will allow efficient and quick retrieval of information when immediate action has to be taken.

ADR Monitoring Program

Adverse Drug Reaction (ADR) monitoring program should be brought under part of the Good Regulatory Practice system; it has been easy for the national center to change the safety issues into policies. ADR reporting program should be initiated in good relations with professionals, industry and local agencies. Information sharing, publication of bulletin and the provision of data on request should also be initiated.

Pharmacovigilance Centers

In different region of the country, Pharmacovigilance centers were initiated by Central Drugs Standard Control Organization (CDSCO). Alternative medicine practitioners should be encouraged to utilize the centers. Feedback from the opinion leaders, practitioners, and medical associations on the safety issues of the registered product should be sought. In order to improve the rationale prescribing and utilization, hospital pharmacists and prescribers should be encouraged to involve in pharmacovigilance activities and information on safety issues are utilized in formulating decisions and policies made by the Drug and Therapeutics Committee (DTC) in hospitals. Data documentation may be initiated through their peripheral centers to the central database. Phytosurveillance will be an new concept to the conventional medical practitioners, hence they should be informed and trained in reporting the ADR through spontaneous reporting system.

Safety Update Reports

Good Regulatory Practice (GRP) should insist the pharmaceutical industry to submit periodic safety update reports, submission of information pertaining to literature reports and outcomes of post-marketing surveillance studies that will enable to understand the recent ADR or AE of the product in various settings.

International Information Sharing (IIS)

Safety issues on marketed products are usually encountered because of underreporting and the inability to generate the signals. For the

newly marketed products, this can be overcome by keeping track of actions taken by other drug regulatory authorities or agencies and WHO. When a local unlabelled or rare ADR is signaled or received, it has been most helpful to be able to obtain information from the WHO database of similar events and through communications with other national centers via WHO's Vigimed e-mail system.

Development of Product Database

During the product evaluation and registration, guidance to the pharmaceutical industry by the regulatory authority will assist to develop product database. A new enhanced computerized system assisted database should be developed, which will enable the ADR reports and information obtained from other sources to be linked with the product data base. Such information on existing product/ newly marketed product, the occurrence of adverse events and drug utilization figures will be more readily available to the hospitals and health care professionals.

Technical Working Committee on Phytosurveillance

A technical working group comprises of regulators, manufacturers, healthcare professionals/practitioners should be framed, to see how herbal industry can be improve pharmacovigilance practices in the country.

Post Marketing Surveillance (PMS)

Conventional medicines are thoroughly tested before they are licensed and access is carefully controlled through the issue of prescriptions and dispensing through pharmacies. Unfortunately, such regulatory control is not favoring the supply herbals in this country. Some adverse reactions may not appear until the herbal formulations are in general use. These may be rare reactions, or those seen only in the very young or the elderly, patients with a specific condition, or ADRs that occur when used in combination with prescription medication. Therefore, it is very important to know the effects of these drugs after the marketing, especially herbals which are prepared not based on traditional approaches, should be closely monitored under their usual conditions of use in daily practice. This process is commonly referred as Post Marketing Surveillance: the systematic and scientific evaluation of all intended and unintended effects of medicines on human health, after their release for marketing.

Spontaneous Reporting Schemes

Spontaneous reporting schemes is the main method of generating and detecting signals of potential safety concerns associated with herbal medicines. It is appear to function effectively as a pharmacovigilance tool for herbal medicines. In Germany, where herbal medicinal products are regulated as medicines, this system is reasonably used by physicians, pharmacists and other healthcare professionals particularly pharmacists (De Smet, 1995). However, in countries such as UK and NZ is likely to be less effective, where herbal medicines are marketed mainly as unlicensed products with no obligations for manufacturers to report suspected ADRs to the competent authority, and herbal medicines were used mostly as self-medication.

ADR Reporting Form

The information required for a report of a suspected ADR is the same for both conventional and herbal medicinal products. It is not desirable to have different reporting forms for different types of preparations. Herbal medicines represent a special case of preparation and a more specialized reporting form may be needed. An ADR reporting form on suspected adverse reactions to medicines, including herbal medicines and vaccines is published by WHO for the use of member countries, who are establishing the national drug monitoring system for the first time to include herbal medicines (WHO, 2004).

CONCLUSIONS

The development of herbal products based on traditional approach is a challenging, also become costlier for a manufacturer if quality and safety is focused. Regulation is in place in most developed countries to ensure that herbal formulation is safer for their consumers. Given the experience with pharmacovigilance systems which exist for many decades, it is likely that procedures may suitably be adopted for safety monitoring of herbals too. Above mentioned initiatives such as Good Regulatory Practice (GRP), Good Quality Management (GQM), ADR monitoring program, co-operation with established Pharmacovigilance centers, submission of safety update reports to the regulatory after the post-marketing surveillance, International Information Sharing, (IIS), Development of product database, technical working committee on Phytosurveillance, Post marketing surveillance approach are some of the well established and practiced methods in developed countries. The implementation of such initiatives is hoped to improve the beginning of herbal pharmacovigilance system. Considering the similarities between the monitoring the safety of

conventional medicines and herbal medicines, there is a case for aligning both methodologies. This refers both to the data model, coding and assessment, terminology used to label the adverse effects etc are same in both systems. Hence, phytosurveillance system may be initiated with the assistance of available infrastructure and model for conventional medicines. Pharmacovigilance system is directly linked with public health issues, it is hoped that implementing such systems will address health issues without comprising the safety through the available safe and effective drugs. Medicinal plants have been accepted as a potential source of alternative medicine across the globe, for various ailments. Thus proper modern monitoring approaches will allow us to use plants as a potential resource for the treatment of various ailments, as well the development of safe and effective therapeutics from these resources.

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13

HPLC as a Tool for Qualitative and Quantitative Evaluation of Herbal Drugs and Formulations

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ABSTRACT

It is now well known that the therapeutic activity of a medicinal plant is due to the presence of certain biologically active chemical constituents, which are either primary or secondary metabolites. The expression of many of these compounds particularly those of the secondary metabolite category are controlled and conditioned by a variety of factors such as its genetic predisposition, habitat of the plant agro climatic conditions, season and also the stage of growth and development of the plant etc. Therefore, it is extremely important to establish the reference samples and to determine the quality parameters of the medicinal plants by undertaking extensive and intensive study of the traditional treatise of the classical medicines or traditional practices, combined with the modern scientific knowledge, methods and using the latest analytical and computational tools. Liquid chromatography with an isocratic/gradient elution remains to be the method of choice in the pharmacopeia and for the analysis of marker compounds that are thermally labile in botanicals and herbal preparations. HPLC is one of the latest analytical techniques, which is very essential for both quantification and quality evaluation of the herbal materials. The technique is based on the same modes of separation as of column chromatograpzhy, i.e. adsorption, partition, ion exchange and gel permeation, but it differs from column chromatography in that the mobile phase in HPLC is

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pumped through the packed column under high pressure. The principle advantages of HPLC when compared to classical column chromatography are improved resolution of the separated substances, faster separation times and increased accuracy, precision and sensitivity with which the separated substances may be quantified. The reversed octadecyl silica (C-18) is most commonly used stationary phase and with smaller inner diameter, such as 1.0 or 2.1 mm i.d. which were well suited to the analysis of components present in botanicals. Most important of all, methods using columns with smaller inner diameter and the right mobile phase can be readily adopted to mass spectrometry. The most common mode of detection remains to be ultraviolet detection using a PDA. HPLC has been the method of choice for the quantification of number of groups of secondary metabolites like phenols especially due to its extremely high versatility, precision and relatively low cost involved in the analysis. Most frequently preferred method is on reversed phase (RP) C18 columns, a binary solvent system containing acidified water, a polar organic solvent (acetonitrile or methanol), and UV–Vis diode array detection (DAD), which constitute a crucial and reliable tool in the routine analysis of plant phenolics. A HPLC method for the separation and quantification of 6 different types of phenolics with a total of 15 different phenolics in a sole analysis, furocoumarins and alkaloids has been developed by us and the method thus developed for phenols has been applied successfully in standardization of individual plants like Heracleum candicans, Ficus carica, Artimesia pallens and Bergenia species. The method has also been applied in standardization of compound herbal formulations like Triphala along with its constituents, Chyavanprash and Ashokarishta. We have also tried to establish the importance for solvent of extraction of furocoumarins and also the importance of column selection in HPLC analysis. We have also demonstrated the importance of sample preparation using solid phase extraction technique with selective elution for phenols and furocoumarins from plant extracts. Thus we have established that HPLC with a proper mobile phase, correct column and proper sample preparation can be an ideal tool for quality evaluation and standardization of herbal drugs/formulations.

Key words : Quality control, standardization, herbal drugs, HPLC

INTRODUCTION

All through the human history, there has been a noticeable concern for health care and the cure of disease, though the concepts themselves took a very long time to develop into a body of knowledge. Nowadays, an increasing amount of insight into the behavior of drugs

at the macromolecular level has been developed and there is a lot of direct and indirect evidence supporting these biochemical postulations of drug action. Perhaps the earliest recorded use of a medicinal plant has been mentioned in 'Rigveda' and one mentioned in the modern texts is that of the herb called "Ma huang," a species of Ephedra used medicinally in China for over 5000 years (Foye *et al.,* 1995). Several years ago the World Health Organization (WHO) made an attempt to identify all medicinal plants that exist in the world. More than 20,000 species were included in the list. NAPRALERT database, documents ethnomedicinal uses alone for 9200 of 33000 species of monocots, dicots, gymnosperms, pteridophytes, bryophytes and lichens, which would suggest that 28% of plants on earth have been used ethnomedicinally (Farnsworth & Soejarto, 1991). Although drugs of plant origin are still employed for some of these uses, synthetic drugs now constitute the major part of the products used. It must be noted that much of the work on synthetic compounds began when scientists and researchers had isolated active natural compounds and had characterized them. Plants that were indigenous to a particular region were not easily available throughout the year as well as in all other regions of the world. Thus the development of synthetic compounds and therefore active ingredients was driven primarily by a need to ensure adequate supply, within standard quality norms. Plant drugs therefore continue to constitute an important part of the medicines used even today especially in the grey areas of modern medicine where there is little or no therapy like the ones used for the immunomodulators used in our traditional systems of medicine *viz.* Ayurveda, Siddha and Unani.

With the introduction of modern medicine in 19th century coupled with fast advances in biological sciences, chemistry and technological tools brought in quick healing devices, fast and powerful diagnostic tools and surgical interventions in the 20th century. Such developments in modern medicine caused a rapid decline in traditional medicine particularly in developed countries, but the plant based remedies continued to meet the health care needs of almost 80% population of the world over today (WHO). Towards the end of 20th century, there began a revival of interest in traditional medicine. Medicinal Plants continued to play a very significant role in the healthcare of humankind. It used to be the main resource base of almost all the traditional healthcare systems. Over seventy thousand angiosperm plants out of the three lakh angiosperm plants recorded so far in the world are used for medicinal purpose by the people of different cultures world over. The resurgence of plant based medicine is mainly due to the increasing evidence/realization of the health hazards associated with the harmful side effects of many synthetic

medicines and also the hazards associated with the indiscriminate use of modern medicine such as antibiotics, steroids and other synthetic drugs. The increasing popularity in plant-based drugs is now felt all over the world leading to a fast growing market for plant based drugs pharmaceuticals, nutraceuticals, functional foods and even cosmaceuticals.

India is sitting on a gold mine of well-recorded and traditionally well-practised knowledge of herbal medicine. This country is perhaps the largest producer of medicinal herbs and is rightly called the botanical garden of the world. There are very few medicinal herbs of commercial importance which are not found in this country. India officially recognizes over 3000 plants for their medicinal value. It is generally estimated that over 6000 plants in India are in use in traditional, folk and herbal medicine, representing about 75% of the medicinal needs of the Third World countries. Three of the ten most widely selling herbal medicines in the developed countries, namely preparations of *Allium sativum*, *Aloe barbedensis* and *Panax* sp. are available in India. There are about 7000 firms manufacturing traditional medicines with or without standardization (Dubey *et al.*, 2004).

During the last decade there has been worldwide revival in use of herbal based products *viz.* herbal drugs, nutraceutical and cosmaceuticals in developing as well as developed countries. Export–Import Bank reports reveal that the global trade of plant-derived and plant originated products is around US $60 billion (with growth of 7% per annum) where India holds stake of US $1 billion which is expected to reach 3 trillion US$ by the end of 2015. In India the annual growth rate in herbal sector is 10–15%. There are more than 5000 small and big pharmacies which required large amount of superior quality raw material. World market for herbal medicines is experiencing an exponential growth rate especially in the developed countries. For example between 1996 and 1998 US demand for herbal medicines increased by 101%. Likewise the demand of herbal medicines in European union is estimated at Rs. 3500 crores. China takes the lead in meeting these requirements. China is estimated to have about 11360 commercial enterprises engaged in production of herbal medicines. China's foreign exchange earnings from export of herbal medicines is estimated at Rs. 29000 crores (1998-1999) as against about Rs. 400 crores for India (2000-2001). This shows scope for additional exports for superior quality raw material/herbal medicines from India. Not only there is a scope for increasing the export there is also scope for increasing domestic use of herbal products.

NEED FOR STANDARDIZATION OF MEDICINAL PLANTS

The EXIM bank report on India's share of global herbal drugs market exemplifies the need for standardization and quality control. Variety of reasons has been cited for the need for scientific evaluation and standardization. Most of the traditional knowledge about medicinal plants was in the form of oral knowledge that had been eroded or distorted due to the persistent invasions and cultural adaptations. There was no uniform or standard procedure for maintaining the inventory of these plants and the knowledge about their medicinal properties. There is a prevalence of using plants and plant based products in various contemporary and traditional systems of medicine, without any written documentation or regulation. Therefore, it is essential that such uses of natural products be documented and studied for systematic regulation and wide-spread application. The leads for a significant number of modern synthetic drugs have originated from isolated plant ingredients, as the search for newer entities begins from either derivatising existing drugs or from traditional or contemporary medicinal systems. Therefore, it is important to undertake phytochemical investigations along with biological screening to understand the therapeutic dynamics of medicinal plant and also to develop quality parameters.

Herbal products may contain a single herb or combinations of several different herbs believed to have complementary and/or synergistic effects. Some herbal products, including many traditional medicine formulations, also include animal products and minerals (Rotblatt & Ziment, 2002). Herbal products are sold as either raw plants or extracts of portions of the plant. Extraction involves boiling or percolating the herb in water, alcohol, or other solvents to release biologically active constituents of the plant. These liquid extracts may then be heated or dried to create more concentrated liquids, pastes, or powders. Both the raw herb and the extract contain complicated mixtures of organic chemicals, which may include fatty acids, sterols, alkaloids, flavonoids, glycosides, saponins, tannins, and terpenes (Rotblatt & Ziment, 2002). It is often difficult to determine which component, if any, of the herb has biological activity in humans. In addition, the processing of herbs, such as heating or boiling, may alter the pharmacological activity of the organic constituents. Similarly, a host of environmental factors, including soil, altitude, seasonal variation in temperature, atmospheric humidity, length of daylight, rainfall pattern, shade, dew, and frost conditions, may affect the levels of components in any given batch of an herb. Other factors, including infections, insects, planting density, competition with other plant species, seeding time, and genetic factors, play an important role in producing uniform herbal products (Wijesekera, 1991).

QUALITY CONTROL AND STANDARDIZATION OF MEDICINAL PLANTS AND HERBAL DRUGS

Most of the herbal drugs produced currently in the developing countries lack proper quality specification and standards and therefore lack consistency in quality in batch to batch products. Most of these drugs do not have well defined and characterized composition. The three pillars of ideal herbal drug and their rational use are quality, safety and efficacy (Fig 1). The traditional medicines used to be an individual based treatment regime wherein the traditional physicians used hand picked plant materials to prepare drugs/formulations to treat their patients. The prescription and preparation of the drugs or remedies were also used to be thus person specific and based on the constitutional nature of the patient called 'Prakrati' as per the 'Tridosha' concept of Ayurveda/Siddha. A well-experienced traditional physician in the past used to have specific knowledge and special ability to collect the right plants having the therapeutically useful agents from certain specific habitats. This transformation of traditional medicine from the individualized system to a commercial manufacturing system resulted in great deterioration in the whole procedure and process of traditional medicine. Indeed, quality of the drugs became the greatest casualty in this transformation.

Over 80 per cent of the raw material required for traditional medicines/herbal medicines used to be collected from wild resources. With the increase in demand of medicinal plants for the commercial herbal medicine sector led to the indiscriminate and unscientific collection without any consideration for the quality of the material collected. Lack of societal support and encouragement the orally transmitted expertise in collecting the quality plant material suffered great setback and even loss of such knowledge system during the course of last 100 years. It has caused extensive erosion and corrosion in the traditional wisdom, knowledge and practice of particularly medicinal plant collection. The increase in commercial demand for medicinal plants from wild resources has also encouraged many to use adulterants or spurious materials, which further complicated the quality standards of plant based drugs and pharmaceuticals.

It is now well known that the therapeutic activity of a medicinal plant is due to the presence of certain biologically active chemical constituents, which are either primary or secondary metabolites. The expression of many of these compounds particularly those of the secondary metabolite category are controlled and conditioned by a variety factors such as its genetic predisposition, habitat of the plant agro climatic conditions, season and also the stage of growth and development of the plant etc. The Traditional Indian System of

Medicines like Ayurveda, Siddha, Unani and Amchi etc. provided specific instructions for collection by indicating location/edaphic conditions, habitat, seasonal and even the stage of the plant growth and developmental stage. Scientific investigations now provide ample evidence to the fact that there is a flux of change in the presence of very many of these chemical constituents, particularly those of the secondary metabolites, in such varied conditions described above. Therefore, it is extremely important to establish the reference samples and to determine the quality parameters of the medicinal plants by undertaking extensive and intensive study of the traditional treatise of the classical medicines or traditional practices, combined with the modern scientific knowledge and methods and using the latest analytical and computational tools (Pushpangadan & Govindarajan, 2005).

CHEMICAL STANDARDIZATION

Chemical standardization included physicochemical evaluation and phytochemical evaluation including chromatographic fingerprinting. It is common to approach problems realizing that there are deeply hidden relationships between variables that can be wrestled from an experiment only by using newer data analysis tools. The information explosion compels the scientists/industries to continuously update their skills and apply new techniques for smooth sailing in herbal drug scenario of the world. Any method developed gains importance only if it is accepted and has a wide applicability. With the increasing globalization and export of the herbal drugs and the stringent rules due to WTO, it necessary that globally acceptable methods are developed so that export of herbal drugs from India increases. The presence of markers does not always guarantee an individual in getting the actual herbal stated by the product label, especially if the product is spiked with the chemical marker. The quantification method for the chemical markers will confirm the compounds presence, but it may not confirm the presence of the claimed plant material. So, to determine the plant material, and to check whether other plant materials have been added with the marker, the fingerprint approach may be useful.

Conventional approaches to metabolite analysis are tedious and time consuming as it involves elaborate sample preparation, multiple procedures, which are limited to analysis of only a few compounds. In addition, since extensive fractionations are often required for these approaches, they can be fraught with artifacts such as metabolite breakdown during fractionation and leading to poor recovery. Therefore, standardization of the crude extract and/or the final product is what is required. It is relatively easy to generate a good deal of

data in a short time by proper use of chromatography and spectroscopy. Identifying the components of a mixture visually can be challenging because of the similarity of many responses (Govindarajan & Vijayakumar, 2005).

CHROMATOGRAPHIC ANALYSIS

Chromatography is a physical method of separation in which the components to be separated are distributed between the two phases; one of these is a stationary phase bed and the other is a mobile phase which percolates through this bed. Basically two chromatographic techniques are used for the standardization *viz.* HPTLC, HPLC with GLC used very rarely. These techniques give out the chromatograms, which serves as the fingerprint. The fingerprint of a particular plant, its extract or its product(s) will be same, if the conditions are maintained. Thus chromatography technique offers the best method for recording the fingerprint which can be reproduced anywhere, provided the conditions are maintained. Chemical standardization of herbal drug is defined as quantification of active components using different chemical techniques. These active components popularly known as marker compounds or reference compounds which, represents the quality and efficacy of the herbal drugs (Rastogi & Govindarajan, 2003).

The information obtained by the chromatographic experiment is called the chromatogram, a record of the concentration or the mass profile of the sample components as a function of the movement of the mobile phase. Information that can be extracted from a chromatogram includes (a) an indication of the sample complexity or the number of components present based on the number of peaks. (b) qualitative identification of the samples based on the accurate measurement of the peak positions, (c) quantitative assessment of the relative concentration or the amount of substance present based on the peak size.

Unlike synthetic organic medicinal compounds that exhibit predictable pharmacological activity at a given dosage, the world of botanicals is quite different in the sense that it is not always known with certainty what constitutes the active ingredient(s). It is generally believed that the reported pharmacological action of a botanical is due to more than one constituent acting synergistically with other constituents present. From the pharmacopoeial perspective, a better quality control of raw material can be achieved by specifying a quantitative test procedure for the determination of the range or a minimum content of the marker substance or the “active” ingredient.

According to the definition of chromatographic fingerprints of a herbal drugs a chromatographic fingerprint is in practice a chromatographic pattern of some common kinds of pharmacologically active and chemically characteristic components in the herbal drugs under study. It suggests that with the help of the chromatographic fingerprints obtained, the authentication and identification of a herbal drugs can be accurately conducted even if the number and/or concentration of chemically characteristic constituents are not very similar in different samples of herbal drugs or, chromatographic fingerprints could successfully demonstrate both the "sameness" and "differences" between various samples. Thus, we should globally (considering multiple constituents) not locally (considering only few marker components) evaluate the quality. In the case of herbal drugs there are always hundreds of components and many of them are in too low concentrations. On the other hand, there usually exists variability within the different and even the same herbal materials. As a result, to obtain reliable chromatographic fingerprints chemically representing pharmacologically active and characteristic components is not a trivial work. The performance of a chromatographic fingerprint obtained is closely dependent on the chromatographic separation degrees and concentration distribution of all chemical components (Gong *et al.*, 2003).

It is well known that chromatography has very powerful separation ability, suggesting the separation of complex systems into many relatively simple sub-systems. Furthermore, hyphenated chromatographic and spectrometric approaches such as high-performance liquid chromatography-diode array detection (HPLC-DAD), gas chromatography- mass spectrometry (GC-MS), capillary electrophoresis (CE)-DAD and HPLC-MS, could show greatly improved performances in terms of the elimination of instrumental interference, retention time shift correction, selectivity, chromatographic separation abilities and measurement precision

Liquid Chromatography (LC)

Liquid chromatography with a isocratic/gradient elution remains to be the method of choice in the pharmacopeia and for the analysis of marker compounds that are thermally labile in botanicals and herbal preparations.

HPLC is one of the latest analytical techniques, which is very essential for both quantification and standardization of the herbal materials. The technique is based on the same modes of separation as of column chromatography, *i.e.*, adsorption, partition, ion exchange and gel permeation, but it differs from column chromatography in

that the mobile phase in HPLC is pumped through the packed column under high pressure. The principle advantages of HPLC when compared to classical column chromatography are improved resolution of the separated substances, faster separation times and the increased accuracy, precision and sensitivity with which the separated substances may be quantified.

The reversed octadecyl silica (C-18) is most commonly used. In the course of our experiments, we found that columns with smaller inner diameter, such as 1.0 or 2.1 mm *i.d.* were well suited to the analysis of components present in botanicals. For columns with smaller inner diameter, it was observed that the system precision for the retention time and peak area/height were comparable to analytical columns with 4.6 mm *i.d.* Most important of all, methods using columns with smaller inner diameter and the right mobile phase can be readily adopted to mass spectrometry. The most common mode of detection remains to be ultraviolet detection.

Methods using gradient elution HPLC with reversed phase columns had been applied for the analysis of multiple constituents present in medicinal plants and herbal preparations (Li *et al.*, 2003; Zhang *et al.*, 2003). Gradient elution HPLC with ultraviolet detection, using a C18 reversed phase column had been used to profile components present in *C. rhizoma*, Radix *aristolochiae*, ginseng, R. *glycyrrhizae* (liquorice), *S. radix, R. codonopsis pilosula* and *S. miltiorrhiza* (Lee *et al.,* 2002). The advantages of liquid chromatography include its high reproducibility, good linear range, ease of automation and its ability to analyze the number of constituents in botanicals and herbal preparation. However, for the analysis of marker compounds in herbal preparations with two or more medicinal plants, co-eluting peaks were often observed in the chromatograms obtained due to the complexity of the matrix (Kim *et al.,* 2005). The complexity of matrix may be reduced with additional sample preparation steps, such as liquid-liquid partitioning, solid phase extraction, preparative LC and TLC fractionation.

HPLC of Polyphenols

HPLC has been the method of choice for the quantification of number of groups of secondary metabolites like phenols. Polyphenols are an important class of drugs that are present in abundance in a number of plant materials and are even considered to be micronutrients (Halliwell & Gutteridge). Most of the herbal medicines and food items like grapes and wines contain a range of antioxidant phenolics with

HPLC being the most preferred method for their analysis and standardization (Revilla & Ryan, 2000). HPLC is the method of choice for the analysis of phenolic compounds especially due to its extremely high versatility, precision and relatively low cost involved in the analysis (Govindarajan *et al.*, 2007; Escarpa & González, 2001). Most frequently preferred method is on reversed phase (RP) C18 columns, a binary solvent system containing acidified water, a polar organic solvent (acetonitrile or methanol), and UV–Vis diode array detection (DAD), which constitute a crucial and reliable tool in the routine analysis of plant phenolics (Robards, 2003). Obtaining good resolution is considered the prerequisite for a method targeted for the separation of multiple phenolic groups (Tsao & Yang, 2003). According to the most relevant bibliography, the HPLC-DAD chromatographic method seems to be a suitable tool for the separation and quantification of phenolic compounds in plant extracts (Robards, 2003). A HPLC method for the separation and quantification of 6 different types of phenolics with a total of 15 different phenolics in a sole analysis (Singh *et al.*, 2007) has been developed (Fig 1). The method thus developed for phenols has been applied successfully in standardization of individual plants like *Bergenia species* (Fig 2) (Singh *et al.*, 2007) and *F. carica* (Fig 3). The method has also been applied in standardization of compound herbal formulations in Chyavanprash (Fig 4) (Govindarajan *et al.*, 2007) and Triphala (Fig 5) (Singh *et al.*, 2008).

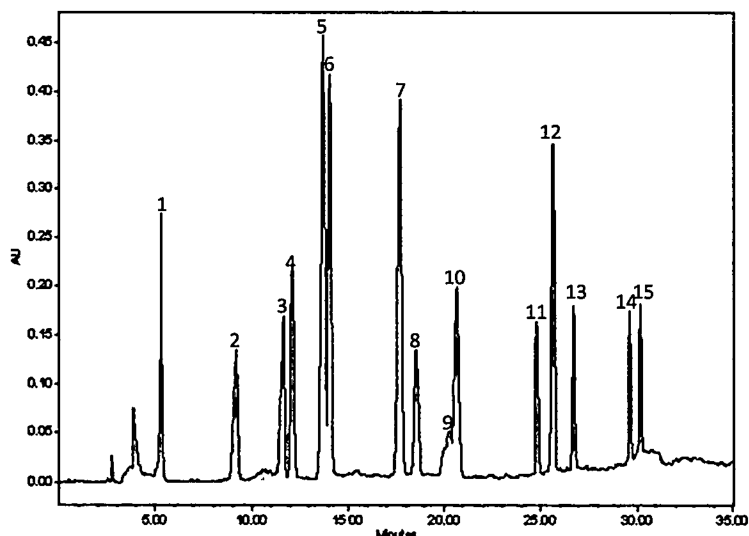

Fig 1. HPLC chromatogram of the phenolic in a sole analysis. 1. Gallic acid; 2. Protocatechuic acid; 3. Chlorogenic acid; 4. Catechol; 5. Vanillic acid; 6. Syringic acid; 7. Vanillin; 8. Rutin; 9. Epicatechin; 10. Ferulic acid; 11. Naringin; 12. Benzoic acid; 13. Myricetin; 14. Quercitin; 15. Apigenine

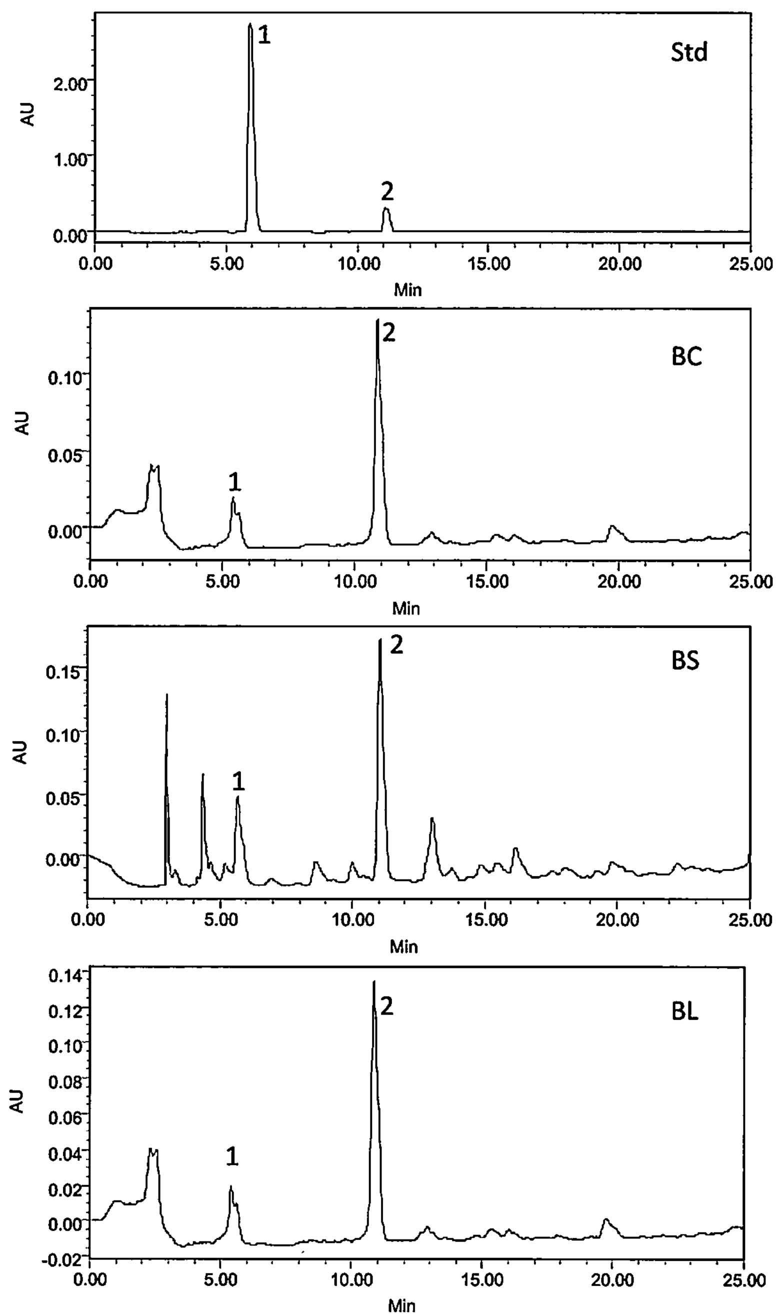

Fig 2. Chromatograms registered for *Bergenia* species (BC: *B. ciliate*; BS: *B. stracheyi;* BL: *B. ligulata*) Bergenia at 272 showing. 1. Gallic acid; 2. Bergenin

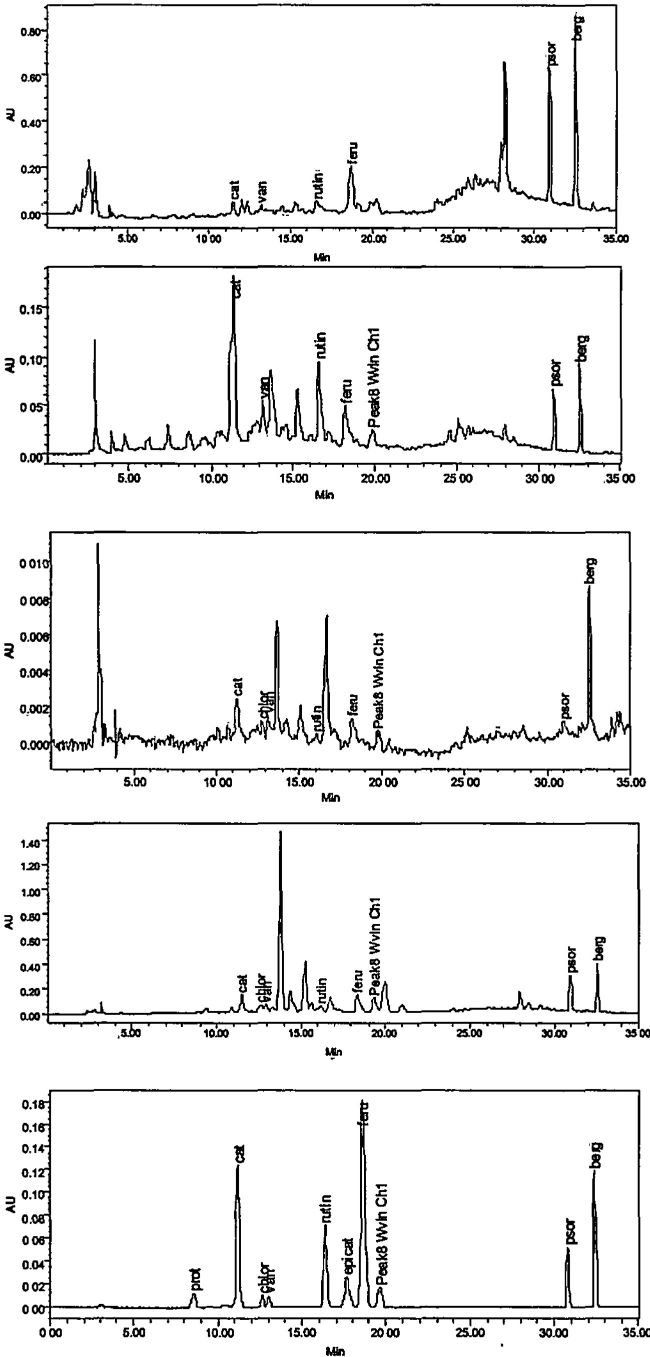

Fig 3. HPLC chromatogram of different parts of *F. carica* along with that of standard

HPLC of Furocoumarins

HPLC-PDA method has been for the estimation of furocoumarins and the same has been applied for quality control and standardization of *H. candicans* (Fig 6) (Govindarajan *et al.,* 2007). Solvent for extraction also plays an important role in bringing out the active secondary metabolites based on the pharmacological profile. It was interesting to note that the concentration of heraclenol was found to be maximum in 30% aqueous methanolic extract, while the anti-inflammatory principle heraclenin was found to be maximum in pure methanolic extract. Similarly the concentration of bergapten, the component reported for melanogenesis stimulation activity (Matsuda *et al.,* 2005) was found to be maximum in pure methanolic extract

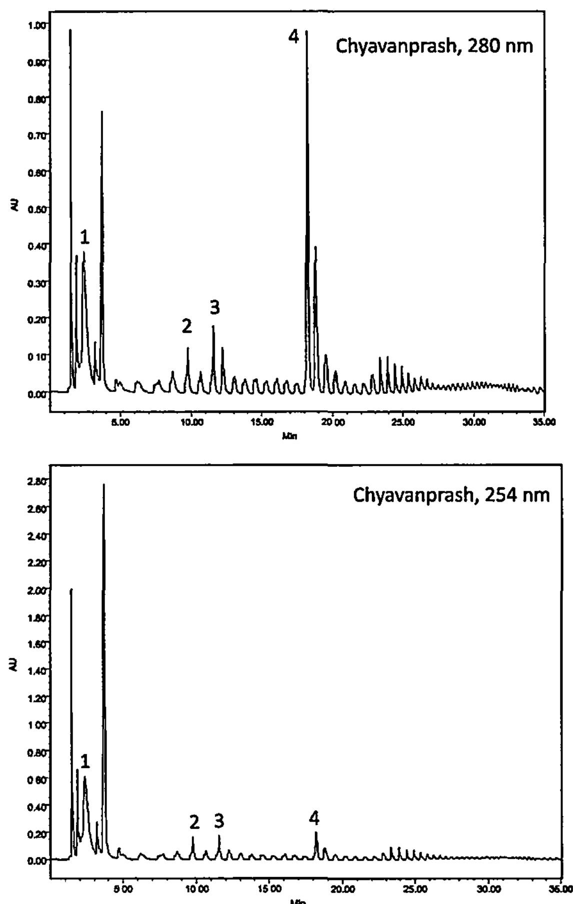

Fig 4. Chromatograms registered for Chyavanprash at 280 and 254 nm, showing the phenolics; 1. Gallic acid; 2. Catechin; 3. Syringic acid; 4. Rutin

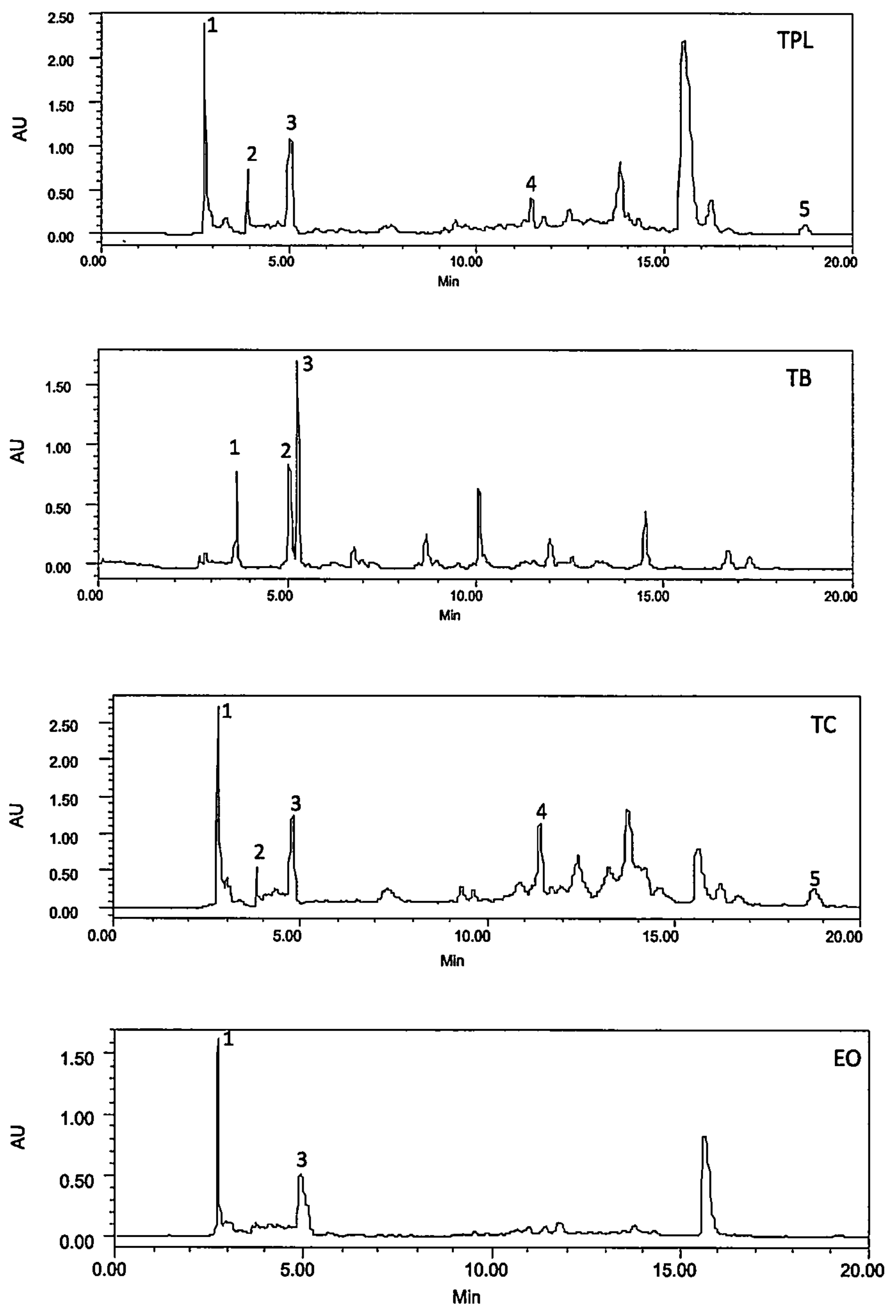

Fig 5. HPLC chromatograms of Triphala and its ingredients. 1. Ascorbic acid; 2. Tannic acid; 3. Gallic acid; 4. Syringic acid; 5. Epicatechin.

TPL: Triphala; TC: *Terminalia chebula*; TB: *Terminalia bellerica*; EO: *Emblica officinale*

(0.281%) while the concentration of anti-psoriatric principle psoralen being maximum in 30% aqueous methanolic extract. Thus showing that the solvent chosen for extraction is one of the important factors in the pharmacological activity elicited by the plant.

Selection of Column

Columns used in HPLC plays a vital role as the efficiency of separation and resolution determines the quantification of the markers. *Heracleum candicans* Wall. ex DC (Apiaceae) is used extensively in Indian system of medicines being a rich source of furocoumarins, extensively used in pharmaceutical industry for their

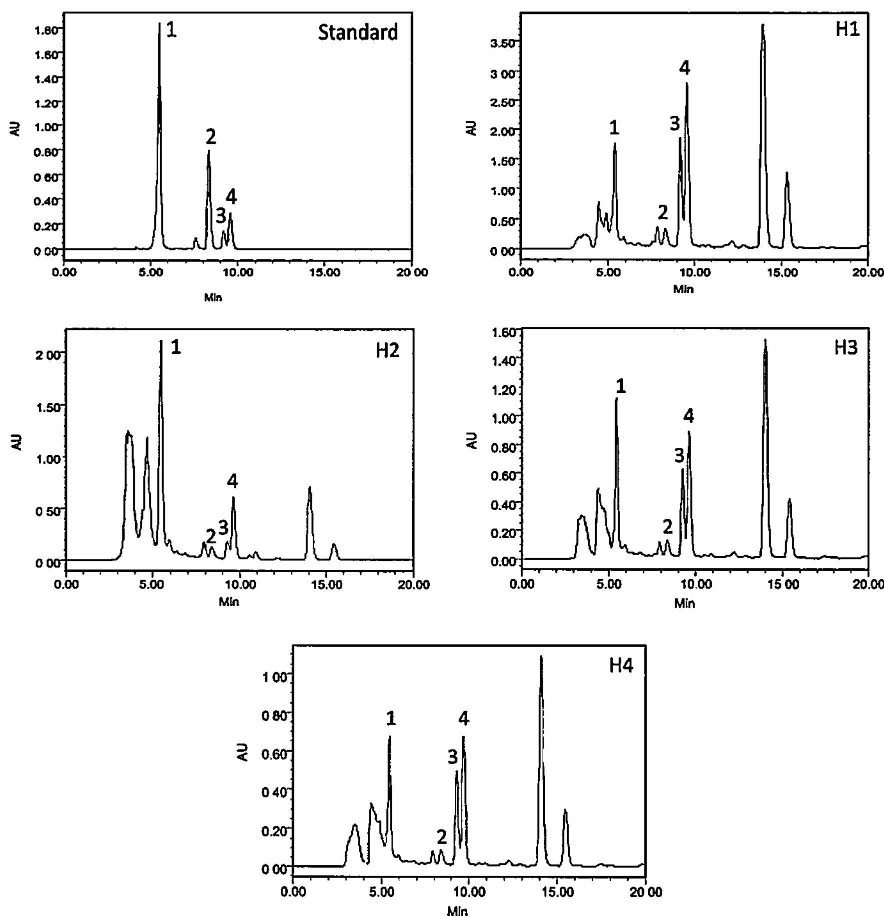

Fig 6. HPLC profiles of the different extracts of *H. candicans* and standard. H1, H2, H2, H3 and H4 are Methanol, 30%, 20% and 10% aqueous methanolic extract respectively, 1. Heraclenol, 2. Psoralen, 3. Heraclinin, 4. Bergapten

photosensitizing property. Thus keeping in view the importance of the plant, a comparison of the analytical HPLC columns has been carried out for the quality evaluation of *H. candicans* using furocoumarins *viz.* heraclenol and bergapten as markers. Four analytical columns (2 RP-8 and 2 RP-18) with different ligand chemisty were used for the analysis. The results indicate that monolithic column RP18e has a better efficiency in terms of time and cost whereas the classical RP18 column has better separation efficiency of the markers used though there are some merged peaks. Also by altering the flow rate and gradient separation better resolution was achieved in monolithic columns (Fig 7). Thus monolithic columns can be used for the quality control as it reduces the time and cost and are specific (Singh *et al.,* 2008).

RECENT DEVELOPMENTS

In recent years, liquid chromatography-mass spectrometry (LC-MS) technique has found increasing application in the analysis of medicinal plant material. It is an expensive technique but the cost may be justified by the wealth of information it provides. The information generated by LC-MS technique can be productively used in the development of public standards for plant drugs. An example of this can be found in the U.S.P. draft monographs on "ginger" and "powder ginger" that were published in Pharmacopoeial Forum (2.3, 3742-3747, 1997). The method submitted to the USP utilized the LC-MS in the identification of peaks due to various isomers of gingerols and shogaols and the retention times information gained thus is used in the liquid chromatographic method for the quantitative determination of gingerols and shogaols.

The latest technique in the liquid chromatography is ultra performance liquid chromatography. In UPLC, the trade mark of Waters Acquity systems, introduced in 2004, particle sizes of around 1.7 lm are used, at a pressure of 15,000 psi (1000 bar). Very few applications to plants have as yet been published, but as an example, the constituents of raw and steamed ginseng root, *Panax notoginseng*, Araliaceae, have been investigated by this method. UPLC was performed on a Waters Acquity system with a 100 · 2.1 mm C18 1.7 lm column and a mobile phase gradient consisting of (A) 0.1% formic acid in water and (B) acetonitrile containing 0.1% formic acid. The UPLC system was connected to a orthogonal acceleration TOF mass spectrometer and was compared to a classical LC/UV analysis. Identification of peaks was achieved by comparison with an in-house database containing 96 protopanaxadiol- type ginsenosides. The faster chromatography displayed higher reproducibility when compared with traditional HPLC (Fig 8) (Chan *et al.,* 2007).

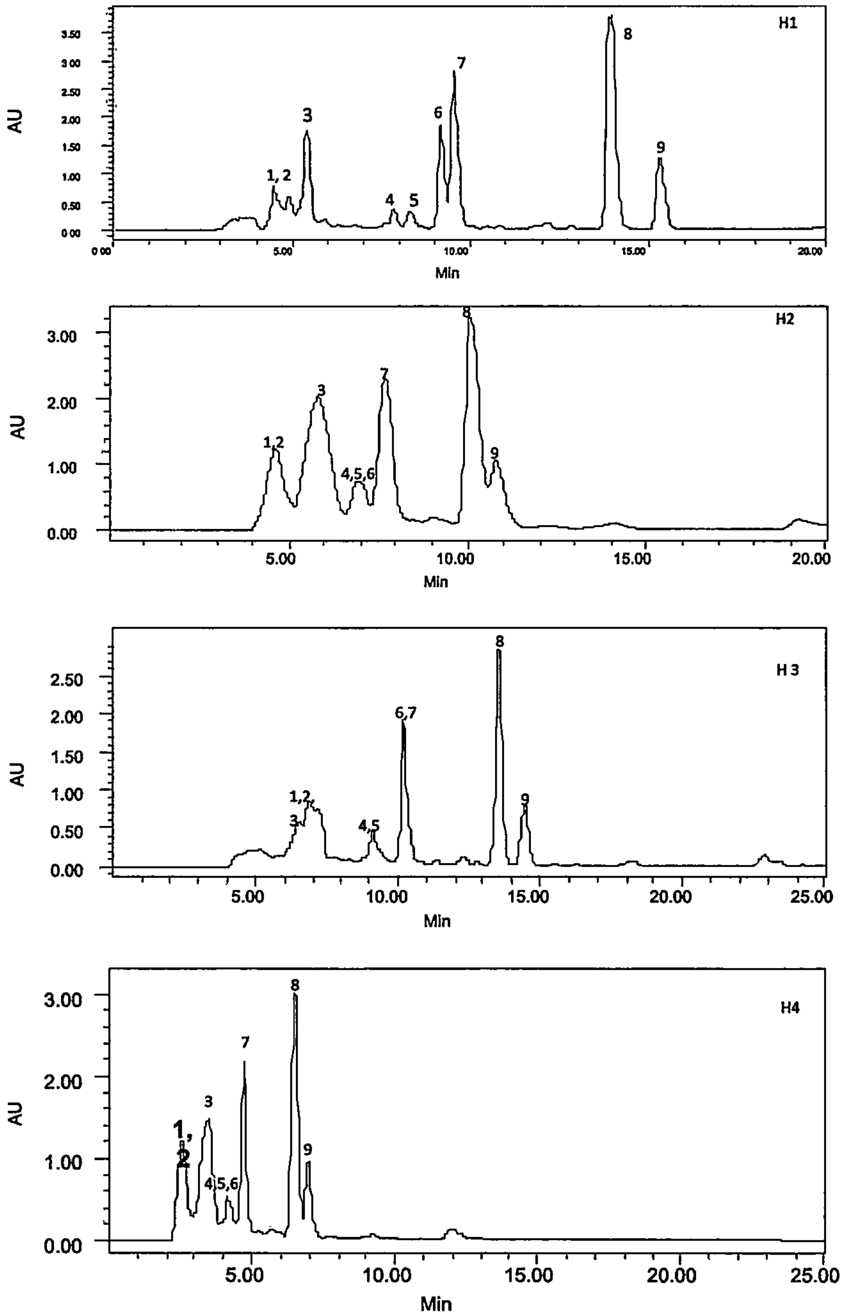

Fig 7. Chromatograms of the methanolic extract of *H. candicans* in various analytical columns

H1: RP-18, H2: Chromolith RP-18e; H3: RP-8; H4: Chromolith RP-8e. 3: Heraclenol; 7: Bergapten

Among the many different HPLC column packings available, monolithic columns have been recently introduced. Traditional HPLC columns are packed with tiny silica particles. The difference with monolithic columns is that they contain a single, solid compound as the stationary phase – usually consisting of a network of polymethacrylate or polystyrene copolymers or bonded silica. In particulate columns, the mobile phase can diffuse between the particles, whereas in monolithic columns it flows through the solid stationary phase, which is usually porous. Compounds in the mobile phase are retained to a greater or lesser extent within the pores of the stationary phase. The advantage of monolithic columns is their faster flow rates and quicker separations.

CONCLUSIONS

Thus a scientifically sound protocol for the procurement of raw drugs from wild sources or protocol for cultivation, post harvesting of medicinal plants etc. have to be worked out for each medicinal plant species in a scientifically sound manner. A well defined protocol and standard operation procedure (SOP) from cultivation methods, harvesting, post harvest handling, preprocessing, storage and upto manufacturing need to be worked out and meticulously followed for herbal drugs (Fig 2). This include correct taxonomic identification & authentication, study on the medicinal part: root, stem, bark, leaves,

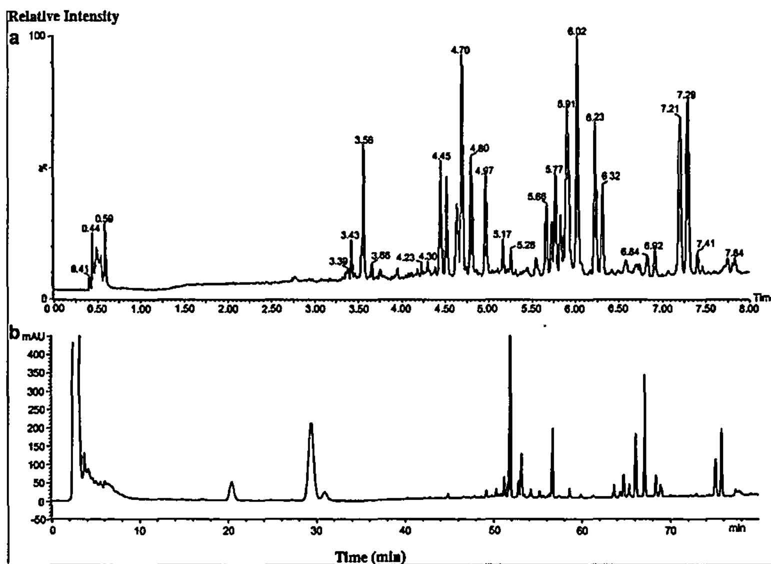

Fig 8. (a) UPLC/TOFMS electrospray total ion current chromatogram (TIC) and (b) HPLC/UV chromatogram (203 nm) of steamed *Panax notoginseng* (Araliaceae) (reproduced from Chan *et al.*, 2007)

flowers, fruits, nuts, gum, resins etc., collection details: Location, stage & developmental stage or growth of the plants for collection methods, pre-processing if any, storage etc. This is followed by the organoleptic examination of raw drug *i.e.* evaluation by means of sensory organs: touch, odour taste, microscopic & molecular examination, chemical composition (TLC, GLC, HPLC, DNA fingerprinting), biological activity of the whole plant, and shelf life of raw drugs. Chromatography especially HPTLC and HPLC (& UPLC) play a crucial role in quantification of the marker components and thus ensuring the desired therapeutic effect.

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14

Consumer Protection and Regulatory Requirements for Herbal Drugs

N. SREEVIDYA[1,2,*] AND S. MEHROTRA[1]

ABSTRACT

The 'back-to-nature' is getting momentum not only in the developing countries, but in the developed countries too. Unfortunately, there is no clear cut law for the quality of raw drugs and consistency in the finished products, hence the efficacy or effectiveness of herbal products are on stake and thus pockets of excellence is the present day requirement. Laws are necessary for consumer protection because of some confusion and willful distortion of claims for the health-giving properties of some food and herbal drug products. In the present communication efforts have been made to compile some case studies regarding challenges and opportunities of law and regulations in herbal products and formulations.

Key words : Herbal drugs, consumer laws, need for study, legal loop holes, case study, suggestions

INTRODUCTION

The last few decades has seen the resurgence in the demand of herbal medicine both as alternative remedies and as inputs to industry. The 'back-to-nature' is getting momentum not only in the developing countries, but in the developed countries too. This reflects in the increasing demand for medicinal plants or herbal products not only

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for the medicine but also for cosmetics, health foods and supplements, fragrance, colouring agents and exotic cuisines. But unfortunately, there is no clear cut law for the quality of raw drugs and consistency in the finished products, hence the efficacy or effectiveness of herbal products are on stake. In the present communication efforts have been made to compile some case studies regarding challenges and opportunities of law and regulations in herbal products and formulations.

Laws and regulations regarding food and herbal drug products are enacted to protect the health, to prevent economic fraud and to ensure the essential quality. Laws also ensure standardized methods of production and processing, distribution of them and regulate health claims. It is necessary to operate within these legal definitions and demarcations of terms for food and herbal drug products. Laws are necessary for consumer protection because of some confusion and willful distortion of claims for the health-giving properties of some food and herbal drug products.

"Consumer" means any person who buys any goods which has been paid and includes any user of such goods other than the person who buys such goods when such use is made with the approval of such person, but does not include a person who obtains such goods for resale or for any commercial purpose. It also includes any services hired or availed of for consideration paid.

Existing Laws for Consumer Protection in India

Article 47 in part IV of the constitution and Article 21 has been interpreted as the guarantee to cover a life with normal amenities ensuring good living which include medical attention, life free from disease and longevity up to normal expectations and the state shall endeavor to bring about prohibition of the consumption except for medical purpose of intoxicating drinks and of drugs which are injurious to health.

In India, traditional medicines are governed by the Drugs and Cosmetics Act of 1940 and the Drugs and Cosmetics Rules of 1945, Drug (prices control) order 1995. They regulate the import, manufacture, distribution and sale of drugs and cosmetics. Drugs and Magic remedies (objectionable advertisements) Act, 1954 deals with the issues related to advertisements.

The Act was amended in 1982 and the definition of 'drug' was amended and Sections 10-A and 26-A were inserted into the Act conferring power on the Central Government to prohibit import of drugs and cosmetics in public interest as also to prohibit manufacture,

sale or distribution thereof. The amended Act came into force with effect from February 1, 1983.

With the amendment of the Act in 1982, the Central Government has now been armed with power to prohibit, in public interest, the import, manufacture, sale and distribution of any drug or cosmetic which is likely to involve any risk to human beings or it would not have the therapeutic value claimed in respect of such preparation.

In 1993, an expert committee appointed by the Indian government developed guidelines for the safety and efficacy of herbal medicines which were intended to be incorporated into the Drugs and Cosmetics Act and rules. It was proposed that no new herbal medicines other than those authorized by the licensing authorities be allowed to be manufactured or marketed, except for those mentioned in and manufactured in compliance with the formulae given in the "authoritative" books for Ayurveda, Siddha and Unani herbal medicines. A manufacturer of a new herbal medicine must include safety data and appropriate efficacy data in the marketing authorization application.

The Bureau of Indian Standards (BIS), a statutory, autonomous body, set up on April 1987, under the Bureau of Indian Standards Act, 1986 governs and sets standards and provides certification to almost all the industrial products to ensure standardisation and quality control. To ensure quality, GMP has been made mandatory for all the drug companies and the Central Excise Rules, 1944 and the relevant entry introduced by amendment controls the payment of taxes to the products of herbal origin. Patents & Intellectual Property Rights in India at present is based on the Patent Act of 1970.

As of now, at the government of India level, the drug policy is handled by the ministry of chemical and fertilizers, the health policy and price control by the Ministry of Health and Family Welfare and IPR/competition by the commerce ministry.

Consumer Protection Act (CPA), 1986 provide protection of the interests of consumers and for that purpose to make provision for the establishment of consumer councils and other authorities for the settlement of consumers' disputes and for matters connected therewith. The CPA sought to provide better protection of consumer interests through speedy and inexpensive redress of consumer grievances by a three-tier adjudicative machinery, consisting of consumer tribunals, set up at district and state levels as well as the national level. The consumer courts are empowered to pass an appropriate order, after adjudication, providing necessary relief, which

include (a) removal of products, (b) replacement of the defective product, (c) refund of the price paid and (d) awarding of a reasonable compensation for any loss or damage suffered by the consumer.

Apart from this a separate department of consumer affairs was set up by the central government, as part of the Ministry of civil supplies, consumer affairs and public distribution, under a cabinet minister. The Ministry has since been renamed as the ministry of consumer affairs, food and public distribution.

The Consumer Protection Council

The Government has established council such as the Central Consumer Protection Council, State Consumer Protection Council, and District Forum. The objects of these Council is to promote and protect the rights of the consumers such as (a) the right to be protected against the marketing of goods and services which are hazardous to life and property; (b) the right to be informed about the quality, quantity, potency, purity, standard and price of goods, so as to protect the consumer against unfair trade practices; (c) the right to be assured, wherever possible, access to a variety of goods and services at competitive prices; (d) the right to be heard and to be assured that consumers interests will receive due consideration at appropriate forums; (e) the right to seek redressal against unfair trade practices or restrictive trade practices or unscrupulous exploitation of consumers; and (f) the right to consumer education.

On receipt of a complaint, if it relates to any goods these Council can, where the complaint alleges a defect in the goods which cannot be determined without proper analysis or test of the goods, shall obtain a sample of the goods from the complainant, seal it and authenticate it in the manner prescribed and refer the sample so sealed to the appropriate laboratory along with a direction that such laboratory make an analysis with a view to finding out whether such goods suffer from any defect alleged in the complaint or suffer from any other defect.

If, after the proceeding conducted under section 13, the Council is satisfied that the goods complained against suffer from any of the defects specified in the complaint or that any of the allegations contained in the complaint about the services are proved, it shall issue an directing to do one or more of the following things, namely, (a) to remove the defect pointed out by the appropriate laboratory from the goods in question; (b) to replace the goods with new goods of similar description which shall be free from any defect; (c) to return to the complainant the price, or, as the case may be, the charges paid by the complainant; (d) to pay such amount as may be

awarded by it as compensation to the consumer for any loss or injury suffered by the consumer due to the negligence of the opposite party; (e) to remove the defects or deficiencies in the services in question; (f) to discontinue the unfair trade practice or the restrictive trade practice or not to repeat them; (g) not to offer the hazardous goods for sale; (h) to withdraw the hazardous goods from being offered for sale.

Need for New Regulations

Use of herbal drugs at the household level has lead the consumer to a highly risk situation because the availability of information regarding safety, efficacy herbal products is minimal, which is available to them through the advertisements or such mass medium, pharmacist, and friends. But the quality and value of much of the information may be doubtful.

In India, there is no statutory organisation such as FDA in US that regulates the accuracy of advertisements for prescription drugs or no effective watch dog groups such as pharma advertising board in Canada or medical lobby for appropriate marketing in Australia. In India no legal constraints to medical advertising is there and the scope of misuse extends beyond journals to package inserts, pamphlets handed by medical representatives, scientific news letters published by drug companies, promotional videos, exhibits at conference and complimentary items.

Screening of advertisements by editorial of magazines or journals is not present. Vague or excessive claims made omission of side effects, of herbal products advertisements continue as financial stability is provided by such advertisements which lack scientific accuracy to those magazines or journals.

The media and the public see publication in peer reviewed journals as validation of the research. According to Richard Horton, Editor of Lancet oral evidence taken in public and reported to the House is available in www.publications.parliment.uk/pa/cmcmhealth.htm provided before the House of Commons select committee on health, there is a whole bunch of promotional journals that sit at the bottom rung. These journals are often very good places to get papers published that are clearly promotional. With diminishing returns from research and development investment, the focus has shifted to marketing. The great tools for marketing are the papers the journals publish. This has led to the swathe of ghost writing using the research that are published as a marketing tool and not as an educational tool.

Herbal drugs containing commonly abused street drugs amphetamine derivative MDMA and mescaline containing cacti & psilocybine containing mushrooms are also available. So, post marketing surveillance studies with herbal drugs, which should allow statements of quality, efficacy, and safety are necessary. Until now neither laws nor concrete normative guidelines for methodology and evaluation of postmarketing surveillance do exist for pharmacovigilance.

GM genetically modified plants/genetically engineered herbal drugs for active constituents to be used in herbal drugs is another issue to be addressed, for which no regulations are available even for food crops.

Exclusive marketing rights (EMR) law is weak. Novartis was granted the EMR for blood cancer drug glivee by the Indian controller General of patent, Designs, trademarks and geographical indications on November 10, 2003. The court of Chennai also granted it interim injuction against the Indian manufacturer in April 2004. So, a patent granted to a specific company may jeopardize the beneficial effects of the herbal products which aim at the local poor people, because of the weak EMR laws (The Hindu Business line, 2004).

Challenges and Opportunities to the Existing Laws

Even though there are stringent laws, herbal drugs which lack a clear boundary between the food and the medicinal components of herbal products can lead to bend and interpret the law in ones own favor. The following cases, for which judgment has been given makes us give a second thought about how safe the laws are and where the lacuna is.

Food or Drug: Case 1

There can be confusion regarding the use of some herbs like ginger, turmeric, pepper etc. in herbal drug formulations to be considered as species/food stuff or herbal drugs in case of dispute, and as to the application of the prevention of food adulteration act 1954 to them.

In the Patna high court ruling, in a sample of turmeric found to be adulterated the accused respondent pleaded that it is meant for pooja and rangoli. This plea is of no avail since any food mentioned in appendix B shall be deemed as edible stuff fit for human consumption otherwise adulteration of any food stuff would become permissible by merely stating that the same was meant for a different use other than human consumption (FAC (a), 1987).

A Novel Combination of Herbal Drug Not in Common Parlance: Case 2

The 1979(1), FAC, 325, (Bombay) state of Maharastra v/s Vilas Dashrath Sethe case was a challenge to the Rule 29 is subordinate to rule 5 of PFA of appendix B relating to definition of standard quality. In this case, it is stated that, if any article of food lies beyond the name under common parlance, *viz.* dhania dal, which is specifically not mentioned in rule 29, then under section 105 of the evidence act, the burden of proving existence of circumstances of bringing the case within an exception in any law defined in the act is upon the PFA act authorities. The court shall presume the absence of an article like any of the article specifically mentioned in clauses (a) to (o) or a product similar to them, it will have to be presumed that dhania dal does not fall under rule 29 test. This may be the case for herbal drugs for which proper tests and terminology not being present in the laws. In such case the consumer is at loss and the court and laws may not be helpful.

Quality Regulations: Case 3

In FAC (1987II), 77–80—Annapurna stores and another v/s Corporation of Calcutta and another Calcutta high court states that a person concerned in anyway with the manufacture, sale or storage of an article of food to which the PFA act applies has the right to know the standards prescribed by the central govt. in regard to such food article. Therefore the standards are laid down in rules which are required to be notified in the official gazette under section 23 of this act. Court in determining whether an article of food is adultered or not cannot take into account the standards of quality or purity laid down in any administrative instruction in this behalf. In the case of herbal drugs also the standards for all the marketed products should be published along with the in house standards provided/used by the manufacturer.

Authority for Quality Testing: Case 4

In the judgment delivered by the Patna High court, Ranchi Bench and the other from National consumers disputes redressal commission under the CPA, it is given that the Government analysts report is the final and deciding one in the cases of adulteration of a drug. In these cases, two reports from different laboratories were produced and no specimen of the drug was there for the public analyst and no report was obtained in respect thereof (Deshmukh, 2005). Further a delay in the public analyst report can cause the delay in the justice

and short of the drugs as evinced in the latest dropsy cases in Lucknow (Times of India, 2005).

So time limit 40 days as in the case of food products should reduced for the analysis and also there must be some recognised laboratories from the private sector which can conduct such test and aid in legal disputes.

What Product Qualifies Drug?: Case 5

The judgment by Supreme Court in November 2003, it has been given that cough drops, Sloan's balm Sloan's rub as Ayurvedic medicament under central excise tariff for the brand VICKS by Proctor & Gamble Ltd. goods classifiable under heading 3003.30 for duty as appleants contended that as the classification of goods in question has to be determined by component authority under drugs act as patent or proprietary Ayurvedic medicament fall under section 3(h) under drugs act.

Circular no. 25/91 dated 3.10.1991, Ayurvedic medicine classification (chapter 30) the government has accepted two tests for determination of classification of products to be the Ayurvedic medicine.

1. The word Ayurvedic medicine not having been defined in the central excise and salt act 1944 or central excise tariff act 1985, the common parlance test should have to be resorted to find out whether a medicine is treated as an Ayurvedic medicine by the public.
2. That it is necessary that the ingredients of Ayurvedic medicine should be mentioned in authoritative books or Ayurvedic medicine.

If the customers and practioners in Ayurvedic medicine dealers and the licensing officials treat the product in question as Ayurvedic medicine and not as allopathic medicine, it gives an indication that they are exclusively Ayurvedic medicine or that they are used in Ayurvedic system of medicine though it is a patented medicine (Deshmukh, 2004).

Tax Evasion or a True Case of Herbal Drug: Case 6

The judgment of Civil Appeals by Shree Baidyanath Ayurved Bhavan Ltd., Dabur, the short question arising for consideration whether Lal Dant Manjan manufactured by the appellant-Company falls within the meaning of an Ayurvedic Medicine to qualify for exemption from payment of excise duty under Notification No. 62/78-CE dated 1-3-1978 issued in exercise of power conferred by Rule 8(1) of the Central

Excise Rules, 1944, is another example of how companies go in for exempted excise duty.

They claimed that the product in question is a scientific medicine which would attract the aforesaid entry and would, therefore, be exempted from excise duty. In common parlance the product in question could not be described as a medicinal preparation and that it could trightly be described as a toilet preparation (Civil Appeals, 1985, 1991).

This leads to the some problems like adulteration of the herbal product, wholly or partly, with synthetic chemicals as it would be easier and cost effective to bulk synthesize them than extracting it from its natural source. As it is well known that herbal drugs have synergy effects and that the isolated/purified single chemical entities lack the same efficiency as that of the herbal product as such, which is some kind of unfair trade practice. Also, there is the possibility of the companies to advertise their products as herbal products but contain synthetic chemical entities and claim the product to be herbal just for tax purposes.

Patents: Case 7

Pratap pharma (pvt.) Ltd. and another, petitioners in their Writ Petitions (C) Nos. 3530, 3559 and 4572 of 1983 (Under Article 32 of the Constitution of India), which was decided on April 1, 1997, raised a common question of law, challenging Section 3(h) of the Drugs and Cosmetics Act, 1940, as amended by Act 68 of 1982 (for short, "the Act") with effect from 1-2-1983 as unconstitutional, being arbitrary and violative of Article 14 and Article 19(1)(g) of the Constitution. The grievance of the petitioners is that while the Act amends the definition of "Patent and Proprietary Medicine" under Section 3(h) of the Act, the definition of "drug" under Section 3(b) read with the definition of "Ayurvedic drug" under Section 3(a) has not been changed; as a consequence, there is no prohibition for patenting the Ayurvedic drugs manufactured by the petitioners whereas under the impugned order of the Drug Controller dated 16-2-1983 it is so construed and manufacture of those drugs is prohibited.

The order passed by the court says that 'the Amendment Act 68 of 1983 and the order passed by the Drug Controller, Government of India, are ultra vires the legislative power. The primary question, therefore, is whether such an amendment is ultra vires the provisions of the Constitution. Under Entry 19 of List III read with Entry 49 of List I of the Seventh Schedule, Parliament is competent to enact and to amend the Act. The regulation of manufacture of drug and patenting it are necessary and are in public interest as the evil is

sought to be remedied by legislative measures. When drugs are administered to human beings/animals, they are required to be regulated as adumbrated under the Act. As a consequence, though by implication the right to practice of medicine or manufacture of the drugs has been guaranteed under Article 19(1)(g), it is a regulation within the meaning of Article 19(6) of the Constitution. As a consequence, it is a reasonable restriction on the right to carry on the trade or business of manufacture of the Ayurvedic drugs by the petitioners (Writ Petitions, 1983).

The licences of some of the traditional Ayurvedic medicine manufacturers had been cancelled accordingly, as per the union government that only qualified persons could manufacture Ayurveda medicines. It is suggested that centre amend the act concerned if necessary, to enable practitioners to manufacture medicines as internalising the rich traditional wisdom of country in this field is very important. Patenting indigenous medicine should be to protect rights but not for raise in price or for any corporate interest.

Case of Spirituous Preparations: Case 8

From the Judgment and Order dated 17-4-1989 of the Madras High Court, decided on March 13, 1996, the appellants who manufacture and deal in Ayurvedic and Unani medicines questioned the validity of the Tamil Nadu Spirituous Preparations (Control) Rules, 1984 framed before the High Court which has upheld the validity of the said Rules.

Rule 3(b), Rule 3(i) defines "restricted preparations" "spirituous preparations that are intended for internal consumption and containing more than 18% v/v of alcohol and medicinal preparations containing intoxicating drugs. Provided that all Ayurvedic preparations containing self-generated alcohol and classified as 'restricted preparations' under the Medicinal and Toilet Preparations (Excise Duties), Rules, 1956 shall be treated as restricted preparations for the purpose of these rules. "any other substance containing alcohol or intoxicating drug, whether self-generated or otherwise, notified under Rule 5 to be a spirituous preparation" Rule 11 provides for grant of wholesale and retail sale licences in different forms prescribed therein for sale of any Homoeopathic medicinal preparation or "any preparation coming under the indigenous system of medicine to a registered medical practitioner or to those holding licence in Forms L-1 and L-2 under the Medicinal and Toilet Preparations (Excise Duties), Rules, 1956 or a licence under the Drugs and Cosmetics Act, 1940 (Central Act 23 of 1940)."

The primary object is to regulate the sale of medicinal or toilet preparations containing alcohol and/or intoxicating drugs, which is consistent with the scheme and provisions of the Act *i.e.* prohibition of the manufacture, sale and consumption of intoxicating liquors and drugs in the State of Tamil Nadu (Civil Appeals 1993). Now-a-days there are several herbal beers that are formulated by DRDO and other institutes/companies coming up with health promoting claims. Such herbal products will also be considered by these rules. So caution has to be exercised in making such preparations.

Narcotic Herbal Drugs: Case 9

Chemists are governed by the drugs and cosmetics act, but the Narcotic act (NDPS) is applicable to them in the case of narcotics being used for medicinal purpose. The recent issue related to the denying of stocking of anti-depressants by the chemists is an example how the industry is negligent of customers care. This denial is because NDPS act calls for manufacture and stockists to fill detailed form regarding bill details, name and quantity of the drug and store the information for minimum two years. Even the retailer has to keep a record of patient's prescriptions. There are many plants which contain such chemical components and such products which contain them will be ruled by those laws. So a proper law which controls these products is necessary.

Export and Import: Case 10

The Judgment of the Court was delivered by Ranganath Misra, for Writ Petition No. 3492 of 1983, decided on March 3, 1987, has mentioned his concern about banning import of certain drugs into India that are banned in the developed countries but which are sought to be dumped on Third World Countries by the Multi Nationals. In this judgment he has expressed that the judicial community expected a statutory body like the Indian Medical Council to immediately come forward to assist the court in the task in view of its expertise on the subject and has regretted that the Indian Medical council should have failed to do so.

He in another judgment, has also directed the Central Government to constitute a high-powered authority to go into the hazards suffered by people of the country on account of such drugs being in circulation and suggest remedial measures including award of compensation. He has further prayed that direction should be given for framing of strict regulations to ensure the quality and standard of approved drugs and to ensure weeding out of some, harmful as also injurious

drugs from the market. The control exercised by the government in this country on such corporations is minimal and inadequate.

Several drugs banned in the advanced west after appropriate analytical research are routed into India and on account of lack of control and sluggish enforcement of the law conveniently find their way into the market. What is poison to the human body in the west is equally poison to the people in India but not knowing the repercussion thereof on the human system, such drugs freely circulate and are even prescribed for patients.

The drug industry is totally profit-oriented and no care or attention is bestowed upon good health of the citizens of India. M/s. Nicholas Laboratories of India Ltd. of Bombay and M/s. Unichem Laboratories Ltd., filed writ petitions before the High Court at Bombay and obtained interim orders of stay; similarly M/s. Organon (India) Ltd., moved the Calcutta High Court and obtained an interim order of stay in regard to their preparations. Challenge in these writ petitions is to the vires of Section 10-A and 26-A of the Act (Writ Petition, 1983).

In view of such complications, import of herbal drugs like ganoderma, ginseng, ginkgo, Echinacea which are not native to India should be banned or should be allowed only after stringent quality control after import. Also, some herbs banned in west like aristolochia should be banned in India also.

PROPOSALS

The above mentioned lacunas should be taken care of and the necessary modification to the laws should be made to accommodate the herbal drug products which is about to grow in the future. Also, the following are suggested for a better protection.

1. A registry of all manufacturers of herbal drugs should be maintained which will be available even to the common man
2. GMP evidence on the product package
3. Labels to contain list of constituents by botanical and common names, herb drug interactions
4. Review of safety of all the herbal products by a blind testing at two places, one in India and other outside India
5. A rating system for companies manufacturing herbal drugs
6. Ethical criteria for medical drug promotion laid by the WHO (1988) should be followed. And the check list for assessing advertisements by WHO are as follows:

    6.1. Name of the product

    6.2. Name and the address of company

6.3. Name and address of the advertising agency

6.4. Are the following included in the advertisement

6.4.1. Generic name/constituents/active ingredients

6.4.2. Contents of active ingredients/ dosage form or regimen

6.4.3. Are the claims appropriate

6.4.4. Precautions

6.4.5. Contraindications

6.4.6. Adverse effects

6.4.7. Reference

6.4.8. Name and address of manufacturer

Pharmacists and Clinicians have an important role in communicating public health and safety information. So, there must be a board certified pharmacy clinician and medicinal chemist designed an elective course to address the issues and the pharmacist must learn to (1) distinguish between herbal and homeopathic (2) recognise toxicities by secondary use of herbals (3) identify which herbs are advocated for major medical and psycatric conditions (4) recognise established interactions between herbal allopathic interactions.

Also, acquiring information about patient use of natural products to be included in the patients database for pharmacy care and primary care. Monitoring patients for toxicities associated with natural product use, monitoring for drug-drug, drug-food and drug-disease interaction with natural product should be made. Counselling on efficacious use of natural product, provision of quality natural product should also be made. These can be achieved through consumer education.

We also suggest a logo for consumer identification like figure 1, 2, etc. for herbal products and a mark certification like ISI or AGMARK for herbal drugs and indigenous drugs should be formulated.

Another suggestion is an amendment to, or a law akin to prevention of food adulteration act 1954 may be enacted for herbal drugs to prevent any adulteration or unfair trade practices in the name of herbal drugs/products.

CONCLUSIONS

'I don't want to know what the law is, I want to know who the judge is'

-Roy. M. Cohn

This quotation has a hint of disrespect for the law. Such should not be the case with the regulations on herbs. There is too much at

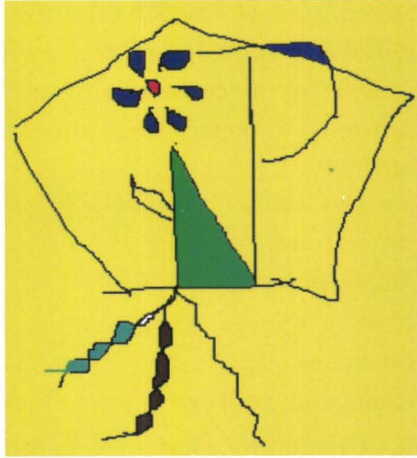

Fig 1.

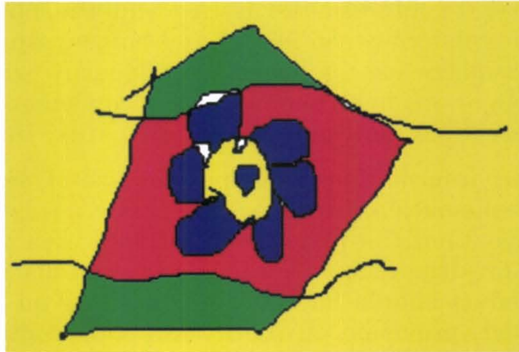

Fig 2.

stake for consumers and for the manufactures and distributors of herbal products.

Consumers are concerned about the cleanliness purity and potency of herbal products. GMP assures appropriate quality at all stages up to the final point of sale. Many organisations are developing guidelines for GMP and quality control and of standard and information monographs for herbals. However, emphasis varies among the parameters, particularly between analytical and therapeutic aspects. The greatest impediment to general improvement of herbal products is the lack of effective enforcement of QC and manufacturing standards.

There is much work to be done to refine and redefine the laws and regulations governing herbal drugs/products. This must be done at the national and international level because of the rapid growth in international trade in herbal products.

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