PHYTOCHEMICALS – A GLOBAL PERSPECTIVE OF THEIR ROLE IN NUTRITION AND HEALTH

Edited by **Venketeshwer Rao**

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Preface

Since global recognition of the dietary guidelines that include increased consumption of plant-based foods for the prevention of chronic diseases and maintaining good health, there has been a considerable interest in the biologically active compounds that are present in plant foods. These compounds have been referred to, among other terms, as 'Phytochemicals', 'Phytonutrients', 'Nutraceuticals' and 'Functional ingredients". They include a multitude of compounds having different chemical identities, biological activities and mechanisms of action. The scope of 'Phytochemicals' has expanded beyond their initial applications to food to include therapeutics, pharmaceuticals and cosmeceuticals. Although advancements have been made in the field of phytochemicals in the past few decades, more information on the analytical methods of isolation and characterization, their occurrence, biological activity, mechanisms of action and applications to the food and other health industries need to be obtained through systematic scientific investigations. Recognizing this need for more information, a plan to publish a book that brings together up to date information on various aspects of phytochemicals was initiated. This book is a collection of several articles that range in scope from the diversity of their occurrence and chemical characteristics, analytical challenges in their isolation and characterization, and the undertaking of basic and clinical researches to evaluate their biological activities both in animal and human health. The book provides a global perspective related to the phytochemicals present not only in foods but also in medicinal plants. Internationally recognized authors that have expertise in their own respective areas within the phytochemical discipline have contributed to this book. Contents of this important and timely book are useful not only to the researchers but also to health professionals, government regulatory agencies and industrial personnel. It is with this vision that we present this book to our readers and are confident that it will serve as a standard reference book in this important field of human nutrition and health.

Dr. A. V. Rao
Professor Emeritus,
Department of Nutritional Sciences, Faculty of Medicine
University of Toronto,
Canada

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Phytochemicals: Extraction Methods, Basic Structures and Mode of Action as Potential Chemotherapeutic Agents

James Hamuel Doughari
Department of Microbiology, School of Pure and Applied Sciences,
Federal University of Technology, Yola
Nigeria

1. Introduction

Medicinal plants have been the mainstay of traditional herbal medicine amongst rural dwellers worldwide since antiquity to date. The therapeutic use of plants certainly goes back to the Sumerian and the Akkadian civilizations in about the third millenium BC. Hippocrates (ca. 460–377 BC), one of the ancient authors who described medicinal natural products of plant and animal origins, listed approximately 400 different plant species for medicinal purposes. Natural products have been an integral part of the ancient traditional medicine systems, e.g. Chinese, Ayurvedic and Egyptian (Sarker & Nahar, 2007). Over the years they have assumed a very central stage in modern civilization as natural source of chemotherapy as well as amongst scientist in search for alternative sources of drugs. About 3.4 billion people in the developing world depend on plant-based traditional medicines. This represents about 88 per cent of the world's inhabitants, who rely mainly on traditional medicine for their primary health care. According to the World Health Organization, a medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi synthesis. Such a plant will have its parts including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds, employed in the control or treatment of a disease condition and therefore contains chemical components that are medically active. These non-nutrient plant chemical compounds or bioactive components are often referred to as phytochemicals ('phyto-' from Greek - *phyto* meaning 'plant') or phytoconstituents and are responsible for protecting the plant against microbial infections or infestations by pests (Abo *et al*., 1991; Liu, 2004; Nweze *et al*., 2004; Doughari *et al*., 2009). The study of natural products on the other hand is called phytochemistry. Phytochemicals have been isolated and characterized from fruits such as grapes and apples, vegetables such as broccoli and onion, spices such as turmeric, beverages such as green tea and red wine, as well as many other sources (Doughari & Obidah, 2008; Doughari *et al*., 2009).

The science of application of these indigenous or local medicinal remedies including plants for treatment of diseases is currently called ethno pharmacology but the practice dates back since antiquity. Ethno pharmacology has been the mainstay of traditional medicines the

entire world and currently is being integrated into mainstream medicine. Different catalogues including *De Materia Medica, Historia Plantarum, Species Plantarum* have been variously published in attempt to provide scientific information on the medicinal uses of plants. The types of plants and methods of application vary from locality to locality with 80% of rural dwellers relying on them as means of treating various diseases. For example, the use of bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections is reported in different manuals of phytotherapy, while species such as lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tee tree (*Melaleuca alternifolia*) are described as broad-spectrum antimicrobial agents (Heinrich *et al*., 2004). A single plant may be used for the treatment of various disease conditions depending on the community. Several ailments including fever, asthma, constipation, esophageal cancer and hypertension have been treated with traditional medicinal plants (Cousins & Huffman, 2002; Saganuwan, 2010). The plants are applied in different forms such as poultices, concoctions of different plant mixtures, infusions as teas or tinctures or as component mixtures in porridges and soups administered in different ways including oral, nasal (smoking, snoffing or steaming), topical (lotions, oils or creams), bathing or rectal (enemas). Different plant parts and components (roots, leaves, stem barks, flowers or their combinations, essential oils) have been employed in the treatment of infectious pathologies in the respiratory system, urinary tract, gastrointestinal and biliary systems, as well as on the skin (Rojas *et al*., 2001; R´ıos & Recio, 2005; Adekunle & Adekunle, 2009).

Medicinal plants are increasingly gaining acceptance even among the literates in urban settlements, probably due to the increasing inefficacy of many modern drugs used for the control of many infections such as typhoid fever, gonorrhoea, and tuberculosis as well as increase in resistance by several bacteria to various antibiotics and the increasing cost of prescription drugs, for the maintenance of personal health (Levy, 1998; Van den Bogaard *et al*., 2000; Smolinski *et al*., 2003). Unfortunately, rapid explosion in human population has made it almost impossible for modern health facilities to meet health demands all over the world, thus putting more demands on the use of natural herbal health remedies. Current problems associated with the use of antibiotics, increased prevalence of multiple-drug resistant (MDR) strains of a number of pathogenic bacteria such as methicillin resistant *Staphylococcus aureus, Helicobacter pylori,* and MDR *Klebsiela pneumonia* has revived the interest in plants with antimicrobial properties (Voravuthikunchai & Kitpipit, 2003). In addition, the increase in cases of opportunistic infections and the advent of Acquired Immune Deficiency Syndrome (AIDS) patients and individuals on immunosuppressive chemotherapy, toxicity of many antifungal and antiviral drugs has imposed pressure on the scientific community and pharmaceutical companies to search alternative and novel drug sources.

2. Classes of phytochemicals

2.1 Alkaloids

These are the largest group of secondary chemical constituents made largely of ammonia compounds comprising basically of nitrogen bases synthesized from amino acid building blocks with various radicals replacing one or more of the hydrogen atoms in the peptide ring, most containing oxygen. The compounds have basic properties and are alkaline in reaction, turning red litmus paper blue. In fact, one or more nitrogen atoms that are present in an alkaloid, typically as 1°, 2° or 3° amines, contribute to the basicity of the alkaloid. The

degree of basicity varies considerably, depending on the structure of the molecule, and presence and location of the functional groups (Sarker & Nahar, 2007). They react with acids to form crystalline salts without the production of water (Firn, 2010). Majority of alkaloids exist in solid such as atropine, some as liquids containing carbon, hydrogen, and nitrogen. Most alkaloids are readily soluble in alcohol and though they are sparingly soluble in water, their salts of are usually soluble. The solutions of alkaloids are intensely bitter. These nitrogenous compounds function in the defence of plants against herbivores and pathogens, and are widely exploited as pharmaceuticals, stimulants, narcotics, and poisons due to their potent biological activities. In nature, the alkaloids exist in large proportions in the seeds

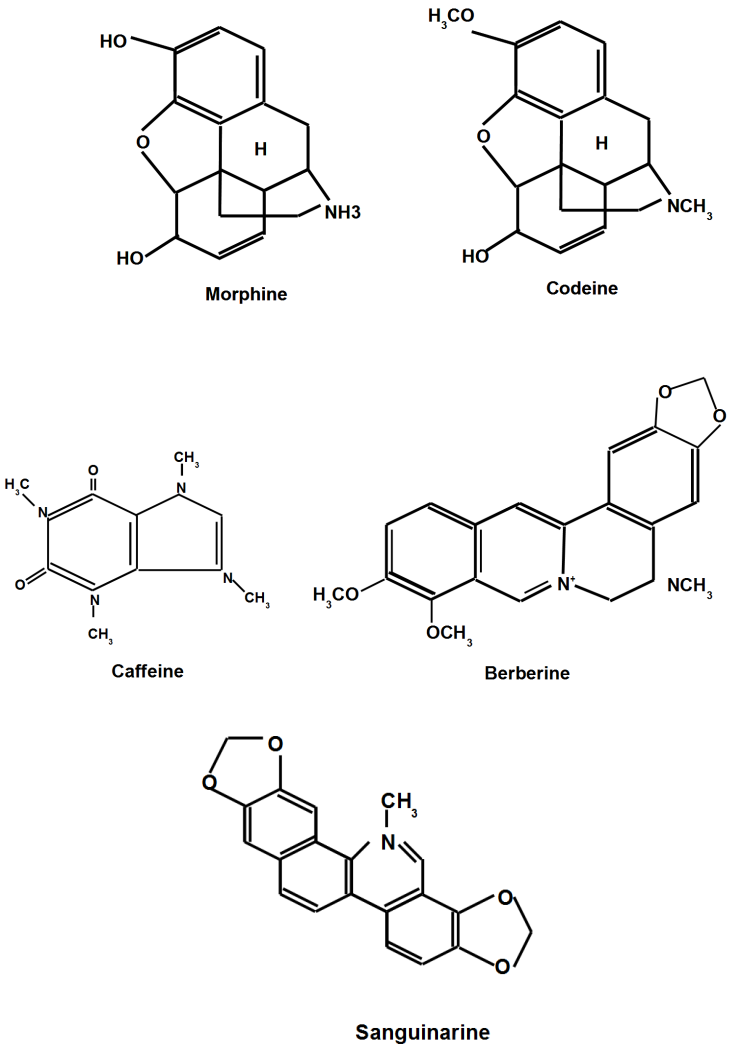


Fig. 1. Basic structures of some pharmacologically important plant derived alkaloids

and roots of plants and often in combination with vegetable acids. Alkaloids have pharmacological applications as anesthetics and CNS stimulants (Madziga *et al.,* 2010). More than 12,000-alkaloids are known to exist in about 20% of plant species and only few have been exploited for medicinal purposes. The name alkaloid ends with the suffix –*ine* and plant-derived alkaloids in clinical use include the analgesics morphine and codeine, the muscle relaxant (+)-tubocurarine, the antibiotics sanguinafine and berberine, the anticancer agent vinblastine, the antiarrythmic ajmaline, the pupil dilator atropine, and the sedative scopolamine. Other important alkaloids of plant origin include the addictive stimulants caffeine, nicotine, codeine, atropine, morphine, ergotamine, cocaine, nicotine and ephedrine (Fig. 1). Amino acids act as precursors for biosynthesis of alkaloids with ornithine and lysine commonly used as starting materials. Some screening methods for the detection of alkaloids are summarized in Table 1.

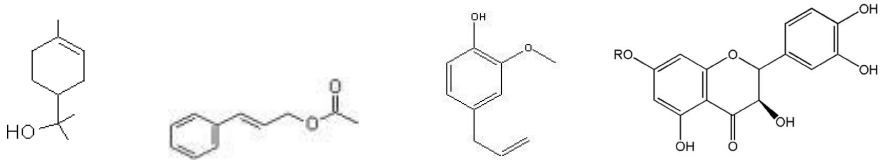
Reagent/test	Composition of the reagent	Result
Meyer's reagent	Potassiomercuric iodide solution	Cream precipitate
Wagner's reagent	Iodine in potassium iodide	Reddish-brown precipitate
Tannic acid	Tannic acid	Precipitation
Hager's reagent	A saturated solution of picric acid	Yellow precipitate
Dragendorff's reagent	Solution of potassium bismuth iodide potassium chlorate, a drop of hydrochloric acid, evaporated to dryness, and the resulting	Orange or reddish-brown precipitate (except with caffeine and a few other alkaloids)
Murexide test for caffeine	residue is exposed to ammonia vapour	Purine alkaloids produce pink colour

Table 1. Methods for detection of alkaloids

2.2 Glycosides

Glycosides in general, are defined as the condensation products of sugars (including polysaccharides) with a host of different varieties of organic hydroxy (occasionally thiol) compounds (invariably monohydrate in character), in such a manner that the hemiacetal entity of the carbohydrate must essentially take part in the condensation. Glycosides are colorless, crystalline carbon, hydrogen and oxygen-containing (some contain nitrogen and sulfur) water-soluble phytoconstituents, found in the cell sap. Chemically, glycosides contain a carbohydrate (glucose) and a non-carbohydrate part (aglycone or genin) (Kar, 2007; Firn, 2010). Alcohol, glycerol or phenol represents aglycones. Glycosides are neutral in reaction and can be readily hydrolyzed into its components with ferments or mineral acids. Glycosides are classified on the basis of type of sugar component, chemical nature of aglycone or pharmacological action. The rather older or trivial names of glycosides usually has a suffix 'in' and the names essentially included the source of the glycoside, for instance:

strophanthidin from *Strophanthus*, digitoxin from *Digitalis*, barbaloin from *Aloes*, salicin from *Salix*, cantharidin from *Cantharides*, and prunasin from *Prunus.* However, the systematic names are invariably coined by replacing the "ose" suffix of the parent sugar with "oside". This group of drugs are usually administered in order to promote appetite and aid digestion. Glycosides are purely bitter principles that are commonly found in plants of the Genitiaceae family and though they are chemically unrelated but possess the common property of an intensely bitter taste. The bitters act on gustatory nerves, which results in increased flow of saliva and gastric juices. Chemically, the bitter principles contain the lactone group that may be diterpene lactones (e.g. *andrographolide*) or triterpenoids (e.g. *amarogentin*). Some of the bitter principles are either used as astringents due to the presence of tannic acid, as antiprotozoan, or to reduce thyroxine and metabolism. Examples include cardiac glycosides (acts on the heart), anthracene glycosides (purgative, and for treatment of skin diseases), chalcone glycoside (anticancer), amarogentin, gentiopicrin, andrographolide, ailanthone and polygalin (Fig. 2). Sarker & Nahar (2007) reported that extracts of plants that contain cyanogenic glycosides are used as flavouring agents in many pharmaceutical preparations. Amygdalin has been used in the treatment of cancer (HCN liberated in stomach kills malignant cells), and also as a cough suppressant in various preparations. Excessive ingestion of cyanogenic glycosides can be fatal. Some foodstuffs containing cyanogenic glycosides can cause poisoning (severe gastric irritations and damage) if not properly handled (Sarker & Nahar, 2007). To test for O-glycosides, the plant samples are boiled with HCl/H_2O to hydrolyse the anthraquinone glycosides to respective aglycones, and an aqueous base, e.g. NaOH or NH_4OH solution, is added to it. For C-glycosides, the plant samples are hydrolysed using $FeCl_3/HCl$, and and an aqueous base, e.g. NaOH or NH_4OH solution, is added to it. In both cases a pink or violet colour in the base layer after addition of the aqueous base indicates the presence of glycosides in the plant sample.



α-Terpineol Cinnamyl acetate Eugenol Taxifolin-7-O- β-glucosid

Fig. 2. Basic structures of some pharmacologically important plant derived glycossides

2.3 Flavonoids

Flavonoids re important group of polyphenols widely distributed among the plant flora. Stucturally, they are made of more than one benzene ring in its structure (a range of C15 aromatic compounds) and numerous reports support their use as antioxidants or free radical scavengers (Kar, 2007). The compounds are derived from parent compounds known as flavans. Over four thousand flavonoids are known to exist and some of them are pigments in higher plants. Quercetin, kaempferol and quercitrin are common flavonoids present in nearly 70% of plants. Other group of flavonoids include flavones, dihydroflavons, flavans, flavonols, anthocyanidins (Fig. 3), proanthocyanidins, calchones and catechin and leucoanthocyanidins.

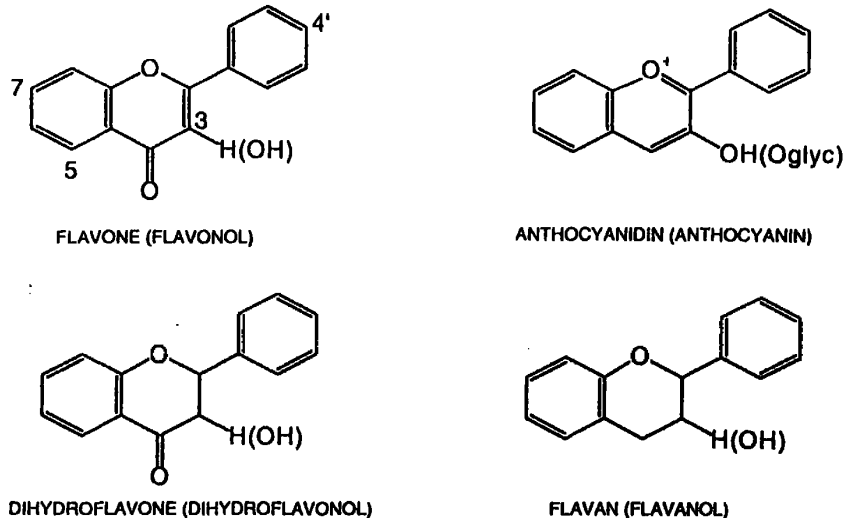


Fig. 3. Basic structures of some pharmacologically important plant derived flavonoids

2.4 Phenolics

Phenolics, phenols or polyphenolics (or polyphenol extracts) are chemical components that occur ubiquitously as natural colour pigments responsible for the colour of fruits of plants. Phenolics in plants are mostly synthesized from phenylalanine via the action of phenylalanine ammonia lyase (PAL). They are very important to plants and have multiple functions. The most important role may be in plant defence against pathogens and herbivore predators, and thus are applied in the control of human pathogenic infections (Puupponen-Pimiä *et al*., 2008). They are classified into (i) phenolic acids and (ii) flavonoid polyphenolics (flavonones, flavones, xanthones and catechins) and (iii) non-flavonoid polyphenolies. Caffeic acid is regarded as the most common of phenolic compounds distributed in the plant flora followed by chlorogenic acid known to cause allergic dermatitis among humans (Kar, 2007). Phenolics essentially represent a host of natural antioxidants, used as nutraceuticals, and found in apples, green-tea, and red-wine for their enormous ability to combat cancer and are also thought to prevent heart ailments to an appreciable degree and sometimes are anti-inflammatory agents. Other examples include flavones, rutin, naringin , hesperidin and chlorogenic (Fig. 4).

2.5 Saponins

The term saponin is derived from *Saponaria vaccaria* (*Quillaja saponaria*), a plant, which abounds in saponins and was once used as soap. Saponins therefore possess 'soaplike' behaviour in water, i.e. they produce foam. On hydrolysis, an aglycone is produced, which is called sapogenin. There are two types of sapogenin: steroidal and triterpenoidal. Usually, the sugar is attached at C-3 in saponins, because in most sapogenins there is a hydroxyl group at C-3. *Quillaja saponaria* is known to contain toxic glycosides quillajic acid and the sapogenin senegin. Quillajic acid is strenutatory and senegin is toxic. Senegin is also present in *Polygala senega*. Saponins are regarded as high molecular weight compounds in which, a

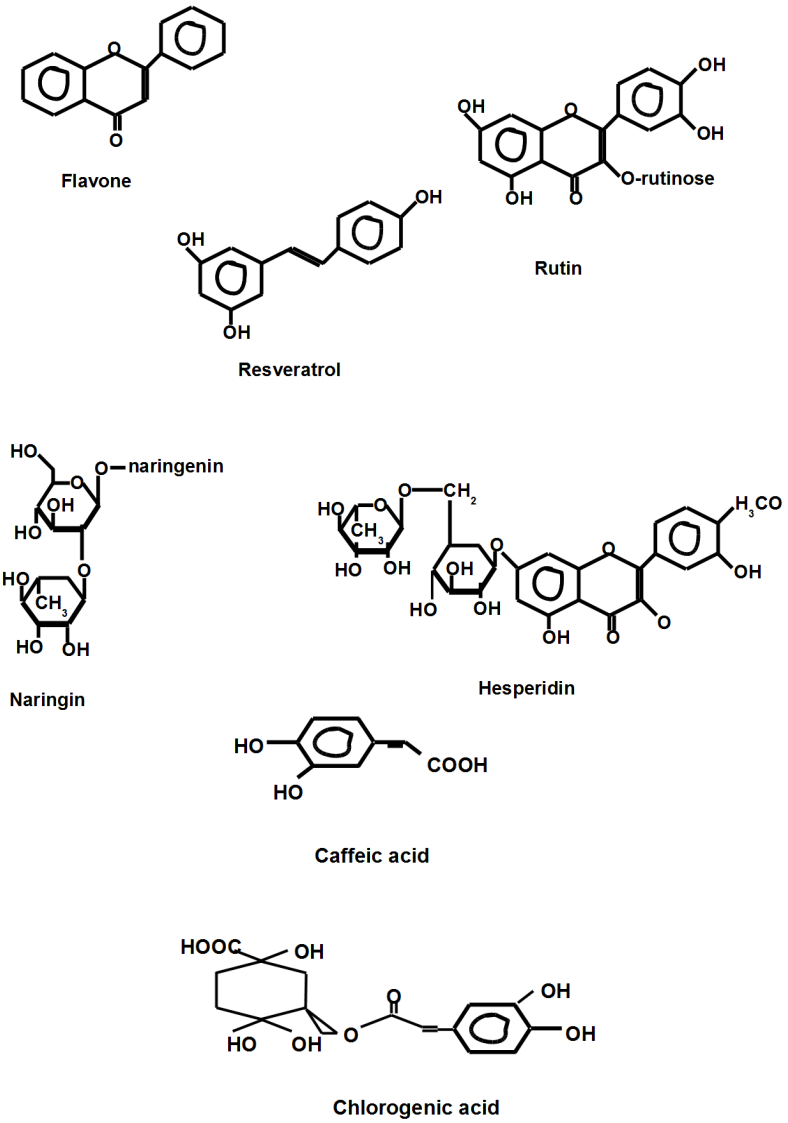


Fig. 4. Basic structures of some pharmacologically important plant derived phenolics

sugar molecule is combined with triterpene or steroid aglycone. There are two major groups of saponins and these include: steroid saponins and triterpene saponins. Saponins are soluble in water and insoluble in ether, and like glycosides on hydrolysis, they give aglycones. Saponins are extremely poisonous, as they cause heamolysis of blood and are known to cause cattle poisoning (Kar, 2007). They possess a bitter and acrid taste, besides causing irritation to mucous membranes. They are mostly amorphous in nature, soluble in alcohol and water, but insoluble in non-polar organic solvents like benzene and n-hexane.

Saponins are also important therapeutically as they are shown to have hypolipidemic and anticancer activity. Saponins are also necessary for activity of cardiac glycosides. The two major types of steroidal sapogenin are diosgenin and hecogenin. Steroidal saponins are used in the commercial production of sex hormones for clinical use. For example, progesterone is derived from diosgenin. The most abundant starting material for the synthesis of progesterone is diosgenin isolated from *Dioscorea* species, formerly supplied from Mexico, and now from China (Sarker & Nahar, 2007). Other steroidal hormones, e.g. cortisone and hydrocortisone, can be prepared from the starting material hecogenin, which can be isolated from Sisal leaves found extensively in East Africa (Sarker & Nahar, 2007).

2.6 Tannins

These are widely distributed in plant flora. They are phenolic compounds of high molecular weight. Tannins are soluble in water and alcohol and are found in the root, bark, stem and outer layers of plant tissue. Tannins have a characteristic feature to tan, i.e. to convert things into leather. They are acidic in reaction and the acidic reaction is attributed to the presence of phenolics or carboxylic group (Kar, 2007). They form complexes with proteins, carbohydrates, gelatin and alkaloids. Tannins are divided into hydrolysable tannins and condensed tannins. Hydrolysable tannins, upon hydrolysis, produce gallic acid and ellagic acid and depending on the type of acid produced, the hydrolysable tannins are called gallotannins or egallitannins. On heating, they form pyrogallic acid. Tannins are used as antiseptic and this activity is due to presence of the phenolic group. Common examples of hydrolysable tannins include theaflavins (from tea), daidezein, genistein and glycitein (Fig. 5). Tannin-rich medicinal plants are used as healing agents in a number of diseases. In Ayurveda, formulations based on tannin-rich plants have been used for the treatment of diseases like leucorrhoea, rhinnorhoea and diarrhea.

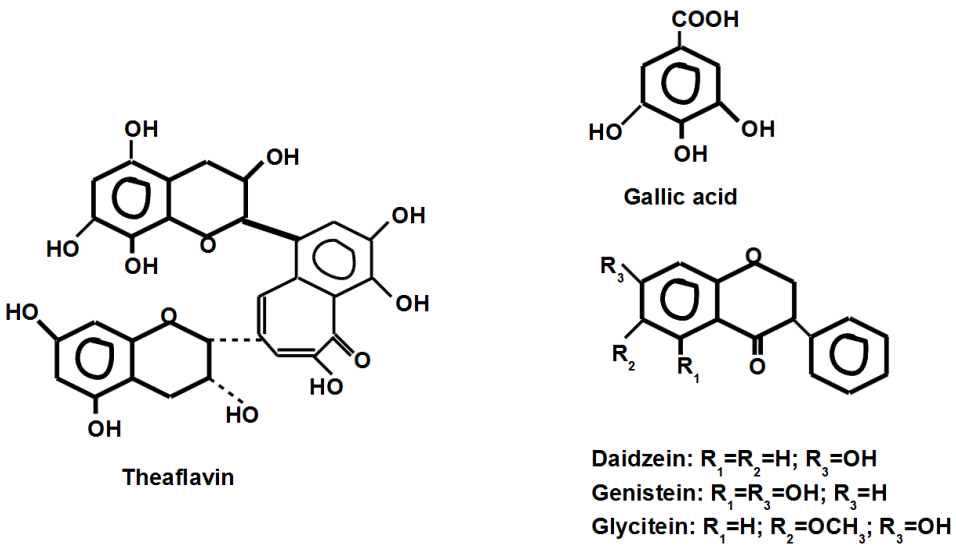
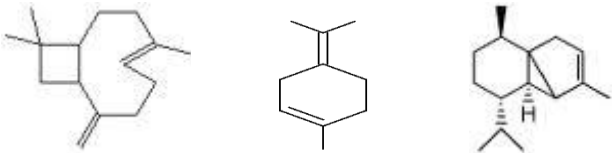


Fig. 5. Basic structures of some pharmacologically important plant derived tannins

2.7 Terpenes

Terpenes are among the most widespread and chemically diverse groups of natural products. They are flammable unsaturated hydrocarbons, existing in liquid form commonly found in essential oils, resins or oleoresins (Firn, 2010). Terpenoids includes hydrocarbons of plant origin of general formula (C5H8)n and are classified as mono-, di-, tri- and sesquiterpenoids depending on the number of carbon atoms. Examples of commonly important monterpenes include terpinen-4-ol, thujone, camphor, eugenol and menthol. *Diterpenes (C20)* are classically considered to be resins and taxol, the anticancer agent, is the common example. The t*riterpenes (C30)* include steroids, sterols, and cardiac glycosides with anti-inflammatory, sedative, insecticidal or cytotoxic activity. Common triterpenes: amyrins, ursolic acid and oleanic acid s*esquiterpene (C15*) like monoterpenes, are major components of many essential oils (Martinez *et al.,* 2008). The sesquiterpene acts as irritants when applied externally and when consumed internally their action resembles that of gastrointestinal tract irritant. A number of sesquiterpene lactones have been isolated and broadly they have antimicrobial (particularly antiprotozoal) and neurotoxic action. The sesquiterpene lactone, palasonin, isolated from *Butea monosperma* has anthelmintic activity, inhibits glucose uptake and depletes the glycogen content in *Ascaridia galli* (Fig. 6). Terpenoids are classified according to the number of isoprene units involved in the formation of these compounds. The major groups are shown in Table 2.



β-caryophyllene Terpenolen α-Cubebene

Fig. 6. Basic structures of some pharmacologically important plant derived tarpenes

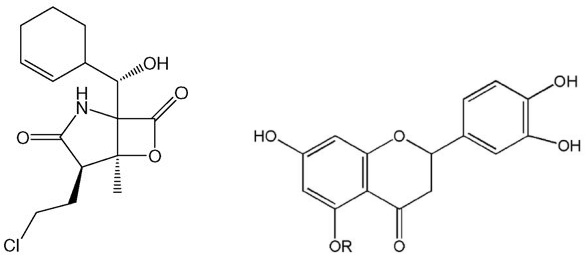
Type of terpenoids	Number of carbon atoms	Number of isoprene units	Example
Monoterpene	10	2	Limonene
Sesquiterpene	15	3	Artemisinin
Diterpene	20	4	Forskolin
Triterpene	30	6	a-amyrin
Tetraterpene	40	8	b-carotene
Polymeric terpenoid	several	several	Rubber

Table 2. Types of terpenoids according to the number of isopropene units

2.8 Anthraquinones

These are derivatives of phenolic and glycosidic compounds. They are solely derived from anthracene giving variable oxidized derivatives such as anthrones and anthranols (Maurya *et al.,* 2008; Firn, 2010). Other derivatives such as chrysophanol, aloe-emodin, rhein, salinos poramide, luteolin (Fig. 7) and emodin have in common a double hydroxylation at positions C-1 and C-8. To test for free anthraquinones, powdered plant material is mixed with organic solvent and filtered, and an aqueous base, e.g. NaOH or NH_4OH solution, is added to it. A

pink or violet colour in the base layer indicates the presence of anthraquinones in the plant sample (Sarker & Nahar, 2007).



Salinos poramide Luteolin

Fig. 7. Basic structures of some pharmacologically important plant derived anthraquinones

2.9 Essential oils

Essential oils are the odorous and volatile products of various plant and animal species. Essential oils have a tendency evaporate on exposure to air even at ambient conditions and are therefore also referred to as volatile oils or ethereal oils**.** They mostly contribute to the odoriferous constituents or *'essences'* of the aromatic plants that are used abundantly in enhancing the aroma of some spices (Martinez *et al.,* 2008). Essential oils are either secreted either directly by the plant protoplasm or by the hydrolysis of some glycosides and structures such as directly Plant structures associated with the secretion of essential oils include: Glandular hairs (Lamiaceae e.g. *Lavandula angustifolia*), Oil tubes (or vittae) (Apiaceae eg. *Foeniculum vulgare*, and Pimpinella anisum (Aniseed), modified parenchymal cells (Piperaceae e.g. *Piper nigrum* - Black pepper), Schizogenous or lysigenum passages (Rutaceae e.g. *Pinus palustris* - Pine oil. Essential oils have been associated with different plant parts including leaves, stems, flowers, roots or rhizomes. Chemically, a single volatile oil comprises of more than 200 different chemical components, and mostly the trace constituents are solely responsible for attributing its characteristic flavour and odour (Firn, 2010).

Essential oils can be prepared from various plant sources either by direct steam distillation, expression, extraction or by enzymatic hydrolysis. Direct steam distillation involves the boiling of plant part in a distillation flask and passing the generated steam and volatile oil through a water condenser and subsequently collecting the oil in florentine flasks. Depending on the nature of the plant source the distillation process can be either water distillation, water and steam distillation or direct distillation. Expression or extrusion of volatile oils is accomplished by either by sponge method, scarification, rasping or by a mechanical process. In the sponge method, the washed plant part e.g. citrous fruit (*e.g.*, orange, lemon, grape fruit, bergamot) is cut into halves to remove the juice completely, rind turned inside out by hand and squeezed when the secretary glands rupture. The oozed volatile oil is collected by means of the sponge and subsequently squeezed in a vessel. The oil floating on the surface is separated. For the the scarification process the apparatus Ecuelle a Piquer (a large bowl meant for pricking the outer surface of citrus fruits) is used. It is a large funnel made of copper having its inner layer tinned properly. The inner layer has numerous pointed metal needles just long enough to penetrate the epidermis. The lower stem of the apparatus serve two purposes; *first*, as a receiver for the oil; and *secondly*, as a

handle. Now, the freshly washed lemons are placed in the bowl and rotated repeatedly when the oil glands are punctured (scarified) thereby discharging the oil right into the handle. The liquid, thus collected, is transferred to another vessel, where on keeping the clear oil may be decanted and filtered. For the rasping process, the outer surface of the peel of citrus fruits containing the oil gland is skilfully removed by a grater. The 'raspings' are now placed in horsehair bags and pressed strongly so as to ooze out the oil stored in the oil glands. Initially, the liquid has a turbid appearance but on allowing it to stand the oil separates out which may be decanted and filtered subsequently. The mechanical process involves the use of heavy duty centrifugal devices so as to ease the separation of oil/water emulsions invariably formed and with the advent of modern mechanical devices the oil output has increased impressively. The extraction processes can be carried out with either volatile solvents (e.g hexane, petroleum ether or benzene) resulting into the production of 'floral concretes'- oils with solid consistency and partly soluble in 95% alcohol, or non volatile solvents (tallow, lard or olive oil) which results in the production of perfumes. Examples of volatile oils include amygdaline (volatile oil of bitter almond), sinigrin (volatile oil of black mustard), and eugenol occurring as gein (volatile oil of *Geum urbanum*) (Fig. 8).

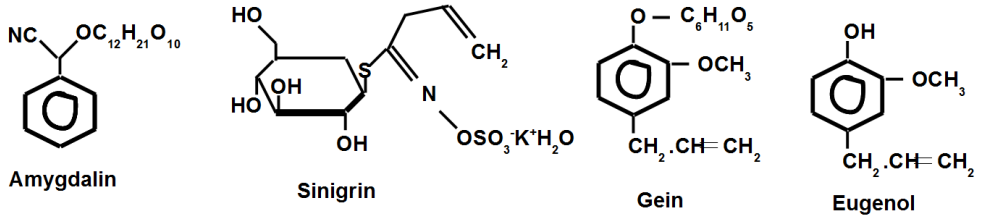


Fig. 8. Basic structures of some pharmacologically important plant derived essential oils

2.10 Steroids

Plant steroids (or steroid glycosides) also referred to as 'cardiac glycosides' are one of the most naturally occurring plant phytoconstituents that have found therapeutic applications as arrow poisons or cardiac drugs (Firn, 2010). The cardiac glycosides are basically steroids with an inherent ability to afford a very specific and powerful action mainly on the cardiac muscle when administered through injection into man or animal. Steroids (anabolic steroids) have been observed to promote nitrogen retention in osteoporosis and in animals with wasting illness (Maurya *et al.,* 2008; Madziga *et al.,* 2010). Caution should be taken when using steroidal glycosides as small amounts would exhibit the much needed stimulation on a diseased heart, whereas excessive dose may cause even death. Diosgenin and cevadine (from *Veratrum veride*) are examples of plant steroids (Fig. 9).

3. Mechanism of action of phytochemicals

Different mechanisms of action of phytochemicals have been suggested. They may inhibit microorganisms, interfere with some metabolic processes or may modulate gene expression and signal transduction pathways (Kris-Etherton *et al*., 2002; Manson 2003; Surh 2003). Phytochemicals may either be used as chemotherapeutic or chemo preventive agents with chemoprevention referring to the use of agents to inhibit, reverse, or retard tumorigenesis. In this sense chemo preventive phytochemicals are applicable to cancer therapy, since

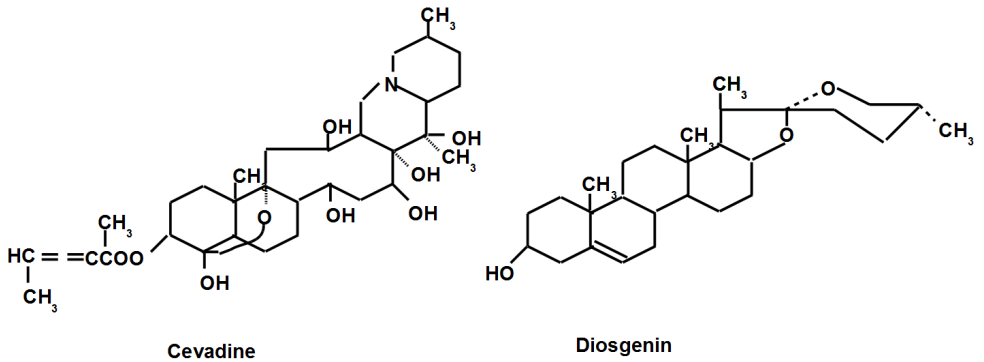


Fig. 9. Basic structures of some pharmacologically important plant derived steroids

molecular mechanisms may be common to both chemoprevention and cancer therapy (D'Incalci *et al*., 2005; Sarkar & Li, 2006). Plant extracts and essential oils may exhibit different modes of action against bacterial strains, such as interference with the phospholipids bilayer of the cell membrane which has as a consequence a permeability increase and loss of cellular constituents, damage of the enzymes involved in the production of cellular energy and synthesis of structural components, and destruction or inactivation of genetic material. In general, the mechanism of action is considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents (Kotzekidou *et al.,* 2008). Some specific modes of actions are discussed below.

3.1 Antioxidants

Antioxidants protect cells against the damaging effects of reactive oxygen species otherwise called, free radicals such as singlet oxygen, super oxide, peroxyl radicals, hydroxyl radicals and peroxynite which results in oxidative stress leading to cellular damage (Mattson & Cheng, 2006). Natural antioxidants play a key role in health maintenance and prevention of the chronic and degenerative diseases, such as atherosclerosis, cardiac and cerebral ischema, carcinogenesis, neurodegenerative disorders, diabetic pregnancy, rheumatic disorder, DNA damage and ageing (Uddin *et al*., 2008; Jayasri *et al*., 2009). Antioxidants exert their activity by scavenging the 'free-oxygen radicals' thereby giving rise to a fairly 'stable radical'. The free radicals are metastable chemical species, which tend to trap electrons from the molecules in the immediate surroundings. These radicals if not scavenged effectively in time, they may damage crucial bio molecules like lipids, proteins including those present in all membranes, mitochondria and, the DNA resulting in abnormalities leading to disease conditions (Uddin et al. 2008). Thus, free radicals are involved in a number of diseases including: tumour inflammation, hemorrhagic shock, atherosclerosis, diabetes, infertility, gastrointestinal ulcerogenesis, asthma, rheumatoid arthritis, cardiovascular disorders, cystic fibrosis, neurodegenerative diseases (e.g. parkinsonism, Alzheimer's diseases), AIDS and even early senescence (Chen *et al*., 2006; Uddin *et al*., 2008). The human body produces insufficient amount of antioxidants which are essential for preventing oxidative stress. Free radicals generated in the body can be removed by the body's own natural antioxidant defences such as glutathione or catalases (Sen, 1995). Therefore this deficiency had to be

compensated by making use of natural exogenous antioxidants, such as vitamin C, vitamin E, flavones, β-carotene and natural products in plants (Madsen & Bertelsen, 1995; Rice-Evans *et al*., 1997; Diplock *et al*., 1998).

Plants contain a wide variety of free radicals scavenging molecules including phenols, flavonoids, vitamins, terpenoids hat are rich in antioxidant activity (Madsen & Bertelsen, 1995; Cai & Sun, 2003). Many plants, citrus fruits and leafy vegetables are the source of ascorbic acid, vitamin E, caratenoids, flavanols and phenolics which possess the ability to scavenge the free radicals in human body. Significant antioxidant properties have been recorded in phytochemicals that are necessary for the reduction in the occurrence of many diseases (Hertog & Feskens, 1993; Anderson & Teuber, 2001). Many dietary polyphenolic constituents derived from plants are more effective antioxidants *in vitro* than vitamins E or C, and thus might contribute significantly to protective effects *in vivo* (Rice-Evans & Miller, 1997; Jayasri *et al.,* 2009). Methanol extract of *Cinnamon* contains a number of antioxidant compounds which can effectively scavenge reactive oxygen species including superoxide anions and hydroxyl radicals as well as other free radicals *in vitro.* The fruit of *Cinnamon*, an under-utilized and unconventional part of the plant, contains a good amount of phenolic antioxidants to counteract the damaging effects of free radicals and may protect against mutagenesis.

Antioxidants are often added to foods to prevent the radical chain reactions of oxidation, and they act by inhibiting the initiation and propagation step leading to the termination of the reaction and delay the oxidation process. Due to safety concerns of synthetic compounds, food industries have focused on finding natural antioxidants to replace synthetic compounds. In addition, there is growing trend in consumer preferences for natural antioxidants, all of which has given more impetus to explore natural sources of antioxidants.

3.2 Anticacinogenesis

Polyphenols particularly are among the diverse phytochemicals that have the potential in the inhibition of carcinogenesis (Liu, 2004). Phenolics acids usually significantly minimize the formation of the specific cancer-promoting nitrosamines from the dietary nitrites and nitrates. Glucosinolates from various vegetable sources as broccoli, cabbage, cauliflower, and Brussel sprouts exert a substantial protective support against the colon cancer. Regular consumption of Brussel sprouts by human subjects (up to 300 $g.day^{-1}$) miraculously causes a very fast (say within a span of 3 weeks) an appreciable enhancement in the glutathione-S-transferase, and a subsequent noticeable reduction in the urinary concentration of a specific purine meltabolite that serves as a marker of DNA-degradation in cancer. Isothiocyanates and the indole-3-carbinols do interfere categorically in the metabolism of carcinogens thus causing inhibition of procarcinogen activation, and thereby inducing the 'phase-II' enzymes, namely: NAD(P)H quinone reductase or glutathione S-transferase, that specifically detoxify the selected electrophilic metabolites which are capable of changing the structure of nucleic acids. Sulforaphane (rich in broccoli) has been proved to be an extremely potent phase-2 enzyme inducer. It predominantly causes specific cell-cycle arrest and also the apoptosis of the neoplasm (cancer) cells. Sulforaphane categorically produces d-D-gluconolactone which has been established to be a significant inhibitor of breast cancer. Indole-3-carbinol (most vital and important indole present in broccoli) specifically inhibits the Human Papilloma

Virus (HPV) that may cause uterine cancer. It blocks the estrogen receptors specifically present in the breast cancer cells as well as down regulates CDK6, and up regulates p21 and p27 in prostate cancer cells. It affords G1 cell-cycle arrest and apoptosis of breast and prostate cancer cells significantly and enhances the p 53 expression in cells treated with benzopyrene. It also depresses Akt, NF-kappaB, MAPK, and Bel-2 signaling pathways to a reasonably good extent. Phytosterols block the development of tumors (neoplasms) in colon, breast, and prostate glands. Although the precise and exact mechanisms whereby the said blockade actually takes place are not yet well understood, yet they seem to change drastically the ensuing cell-membrane transfer in the phenomenon of neoplasm growth and thereby reduce the inflammation significantly.

3.3 Antimicrobial activity

Phytoconstituents employed by plants to protect them against pathogenic insects, bacteria, fungi or protozoa have found applications in human medicine (Nascimento *et al*., 2000). Some phytochemicals such as phenolic acids act essentially by helping in the reduction of particular adherence of organisms to the cells lining the bladder, and the teeth, which ultimately lowers the incidence of urinary-tract infections (UTI) and the usual dental caries. Plants can also exert either bacteriostatic or bactericidal activity ob microbes. The volatile gas phase of combinations of *Cinnamon* oil and clove oil showed good potential to inhibit growth of spoilage fungi, yeast and bacteria normally found on IMF (Intermediate Moisture Foods) when combined with a modified atmosphere comprising a high concentration of CO_2 (40%) and low concentration of O_2 (<0.05%) (Jakhetia *et al.,* 2010). *A. flavus,* which is known to produce toxins, was found to be the most resistant microorganism. It is worthy of note that antimicrobial activity results of the same plant part tested most of the time varied from researcher to researcher. This is possible because concentration of plant constituents of the same plant organ can vary from one geographical location to another depending on the age of the plant, differences in topographical factors, the nutrient concentrations of the soil, extraction method as well as method used for antimicrobial study. It is therefore important that scientific protocols be clearly identified and adequately followed and reported.

3.4 Anti-ulcer

Plants extracts have been reported to inhibit both growth of *H. pylori in-vitro* as well as its urease activity (Jakhetia *et al.,* 2010). The efficiency of some extracts in liquid medium and at low pH levels enhances their potency even in the human stomach. Their inhibitory effect on the intestinal and kidney Na+/K+ ATPase activity and on alanine transport in rat jejunum has also been reported (Jakhetia *et al.,* 2010).

3.5 Anti-diabetic

Cinnamaldehyde, a phytoconstituent extracts have been reported to exhibit significant antihyperglycemic effect resulting in the lowering of both total cholesterol and triglyceride levels and, at the same time, increasing HDL-cholesterol in STZ-induced diabetic rats. This investigation reveals the potential of cinnamaldehyde for use as a natural oral agent, with both hypoglycaemic and hypolipidemic effects. Recent reports indicate that *Cinnamon* extract and polyphenols with procyanidin type-A polymers exhibit the potential to increase

the amount of TTP (Thrombotic Thrombocytopenic Purpura), IR (Insulin Resistance), and GLUT4 (Glucose Transporter-4) in 3T3-L1 Adipocytes. It was suggested that the mechanism of *Cinnamon*'s insulin-like activity may be in part due to increase in the amounts of TTP, IRβ, and GLUT4 and that *Cinnamon* polyphenols may have additional roles as anti-inflammatory and/or anti-angiogenesis agents (Jakhetia *et al.,* 2010).

3.6 Anti-inflammatory

Essential oil of *C. osmophloeum* twigs has excellent anti- inflammatory activities and cytotoxicity against HepG2 (Human Hepatocellular Liver Carcinoma Cell Line) cells. Previous reports also indicated that the constituents of *C. osmophloeum* twig exhibited excellent anti-inflammatory activities in suppressing nitric oxide production by LPS (Lipopolysaccharide)-stimulated macrophages (Jakhetia *et al.,* 2010).

3.7 Multifunctional targets

Multiple molecular targets of dietary phytochemicals have been identified, from pro- and anti-apoptotic proteins, cell cycle proteins, cell adhesion molecules, protein kinases, transcription factors to metastasis and cell growth pathways (Awad & Bradford, 2005; Aggarwal & Shishodia, 2006; Choi & Friso, 2006). Phytochemicals such as epigallocatechin-3-gallate (EGCG) from green tea, curcumin from turmeric, and resveratrol from red wine tend to aim at a multitude of molecular targets. It is because of these characteristics that definitive mechanisms of action are not available despite decades of research (Francis *et al*., 2002). The multi-target nature of phytochemicals may be beneficial in overcoming cancer drug resistance. This multi-faceted mode of action probably hinders the cancer cell's ability to develop resistance to the phytochemicals. It has also been demonstrated that EGCG has inhibitory effects on the extracellular release of verotoxin (VT) from *E. coli* 0157: H7 (Voravuthikunchai & Kitpipit, 2003). Ethanol pericarp extracts from *Punica granatum* was also reported to inhibited VT production in periplasmic space and cell supernatant. Mechanisms responsible for this are yet to be understood, however the active compounds from the plant are thought to interfere with the transcriptional and translational processes of the bacterial cell (Voravuthikunchai & Kitpipit, 2003). More work is needed to be done in order to establish this assumption. Phytochemicals may also modulate transcription factors (Andreadi *et al*., 2006), redox-sensitive transcription factors (Surh *et al*., 2005), redox signalling, and inflammation. As an example, nitric oxide (NO), a signalling molecule of importance in inflammation, is modulated by plant polyphenols and other botanical extracts (Chan & Fong, 1999; Shanmugam *et al*., 2008). Many phytochemicals have been classified as phytoestrogens, with health-promoting effects resulting in the phytochemicals to be marketed as nutraceuticals (Moutsatsou, 2007).

4. Methods of studying phytochemicals

No single method is sufficient to study the bioactivity of phytochemicals from a given plant. An appropriate assay is required to first screen for the presence of the source material, to purify and subsequently identify the compounds therein. Assay methods vary depending on what bioactivity is targeted and these may include antimicrobial, anti-malarial, anticancer, seed germination, and mammalian toxicity activities. The assay method however

should be as simple, specific, and rapid as possible. An *in vitro* test is more desirable than a bioassay using small laboratory animals, which, in turn, is more desirable than feeding large amounts of valuable and hard to obtain extract to larger domestic or laboratory animals. In addition, *in vivo* tests in mammals are often variable and are highly constrained by ethical considerations of animal welfare. Extraction from the plant is an empirical exercise in which different solvents are utilized under a variety of conditions such as time and temperature of extraction. The success or failure of the extraction process depends on the most appropriate assay. Once extracted from the plant, the bioactive component then has to be separated from the co extractives. Further purification steps may involve simple crystallization of the compound from the crude extract, further solvent partition of the co extractives or chromatographic methods in order to fractionate the compounds based on their acidity, polarity or molecular size. Final purification, to provide compounds of suitable purity for such structural analysis, may be accomplished by appropriate techniques such as recrystallization, sublimation, or distillation.

4.1 Extraction of phytochemicals

4.1.1 Solvent extraction

Various solvents have been used to extract different phytoconstituents. The plant parts are dried immediately either in an artificial environment at low temperature (50-60°C) or dried preferably in shade so as to bring down the initial large moisture content to enable its prolonged storage life and . The dried berries are pulverised by mechanical grinders and the oil is removed by solvent extraction. The defatted material is then extracted in a soxhlet apparatus or by soaking in water or alcohol (95% v/v). The resulting alcoholic extract is filtered, concentrated in vacuo or by evaporation, treated with HCl (12N) and refluxed for at least six hours. This can then be concentrated and used to determine the presence of phytoconstituents.

Generally, the saponins do have high molecular weight and hence their isolation in the purest form poses some practical difficulties. The plant parts (tubers, roots, stems, leave etc) are washed sliced and extracted with hot water or ethanol (95% v/v) for several hours. The resulting extract is filtered, concentrated *in vacuo* and the desired constituent is precipitated with ether.

Exhaustive extraction (EE) is usually carried out with different solvents of increasing polarity in order to extract as much as possible the most active components with highest biological activity.

4.1.2 Supercritical fluid extraction (SFE)

This is the most technologically advanced extraction system (Patil & Shettigar, 2010). Super Critical Fluid Extraction (SFE) involves use of gases, usually CO_2, and compressing them into a dense liquid. This liquid is then pumped through a cylinder containing the material to be extracted. From there, the extract-laden liquid is pumped into a separation chamber where the extract is separated from the gas and the gas is recovered for re-use. Solvent properties of CO_2 can be manipulated and adjusted by varying the pressure and temperature that one works at. The advantages of SFE are, the versatility it offers in pinpointing the constituents you want to extract from a given material and the fact that your

end product has virtually no solvent residues left in it (CO_2 evaporates completely). The downside is that this technology is quite expensive. There are many other gases and liquids that are highly efficient as extraction solvents when put under pressure (Patil & Shettigar, 2010).

a. **Coupled SFE-SFC** System in which a sample is extracted with a supercritical fluid which then places the extracted material in the inlet part of a supercritical fluid chromatographic system. The extract is than chromatographed directly using supercritical fluid.
b. **Coupled SFE-GC and SFE-LC** System in which a sample is extracted using a supercritical fluid which is then depressurized to deposit the extracted material in the inlet part or a column of gas or liquid chromatographic system respectively. SFE is characterized by robustness of sample preparation, reliability, less time consuming, high yield and also has potential for coupling with number of chromatographic methods.

4.1.3 Microwave-Assisted extraction

Patil & Shettigar (2010) reported an innovative, microwave-assisted solvent-extraction technology known as Microwave-Assisted Processing (MAP). MAP applications include the extraction of high-value compounds from natural sources including phytonutrients, nutraceutical and functional food ingredients and pharmaceutical actives from biomass. Compared to conventional solvent extraction methods, MAP technology offers some combination of the following advantages: 1. Improved products, increased purity of crude extracts, improved stability of marker compounds, possibility to use less toxic solvents; 2. Reduced processing costs, increased recovery and purity of marker compounds, very fast extraction rates, reduced energy and solvent usage. With microwave-derived extraction as opposed to diffusion, very fast extraction rates and greater solvent flexibility can be achieved. Many variables, including the microwave power and energy density, can be tuned to deliver desired product attributes and optimize process economics. The process can be customized to optimize for commercial/cost reasons and excellent extracts are produced from widely varying substrates. Examples include, but are not limited to, antioxidants from dried herbs, carotenoids from single cells and plant sources, taxanes from taxus biomass, essential fatty acids from microalgae and oilseeds, phytosterols from medicinal plants, polyphenols from green tea, flavor constituents from vanilla and black pepper, essential oils from various sources, and many more (Patil & Shettigar, 2010).

4.1.4 Solid phase extraction

This involves sorption of solutes from a liquid medium onto a solid adsorbent by the same mechanisms by which molecules are retained on chromatographic stationary phases. These adsorbents, like chromatographic media, come in the form of beads or resins that can be used in column or in batch form. They are often used in the commercially available form of syringes packed with medium (typically a few hundred milligrams to a few grams) through which the sample can be gently forced with the plunger or by vacuum. Solid phase extraction media include reverse phase, normal phase, and ion-exchange media. This is method for sample purification that separates and concentrates the analyte from solution of crude extracts by adsorption onto a disposable solid-phase cartridge. The analyte is

normally retained on the stationary phase, washed and then evaluated with different mobile phase. If an aqueous extract is passed down a column containing reverse phase packing material, everything that is fairly nonpolar will bind, whereas everything polar will pass through (Patil & Shettigar, 2010).

4.1.5 Chromatographic fingerprinting and marker compound analysis

Chromatographic fingerprint of an Herbal Medicine (HM) is a chromatographic pattern of the extract of some common chemical components of pharmacologically active and or chemical characteristics (Patil & Shettigar, 2010). This chromatographic profile should be featured by the fundamental attributions of "integrity" and "fuzziness" or "sameness" and "differences" so as to chemically represent the HM investigated. It is suggested that with the help of chromatographic fingerprints obtained, the authentication and identification of herbal medicines can be accurately conducted (integrity) even if the amount and/or concentration of the chemically characteristic constituents are not exactly the same for different samples of this HM (hence, "fuzziness") or, the chromatographic fingerprints could demonstrate both the "sameness" and "differences" between various samples successfully. Thus, we should globally consider multiple constituents in the HM extracts, and not individually consider only one and/or two marker components for evaluating the quality of the HM products. However, in any HM and its extract, there are hundreds of unknown components and many of them are in low amount. Moreover, there usually exists variability within the same herbal materials. Hence it is very important to obtain reliable chromatographic fingerprints that represent pharmacologically active and chemically characteristic components of the HM. In the phytochemical evaluation of herbal drugs, TLC is being employed extensively for the following reasons: (1) it enables rapid analysis of herbal extracts with minimum sample clean-up requirement, (2) it provides qualitative and semi quantitative information of the resolved compounds and (3) it enables the quantification of chemical constituents. Fingerprinting using HPLC and GLC is also carried out in specific cases. In TLC fingerprinting, the data that can be recorded using a high-performance TLC (HPTLC) scanner includes the chromatogram, retardation factor (Rf) values, the colour of the separated bands, their absorption spectra, λmax and shoulder inflection/s of all the resolved bands. All of these, together with the profiles on derivatization with different reagents, represent the TLC fingerprint profile of the sample. The information so generated has a potential application in the identification of an authentic drug, in excluding the adulterants and in maintaining the quality and consistency of the drug. HPLC fingerprinting includes recording of the chromatograms, retention time of individual peaks and the absorption spectra (recorded with a photodiode array detector) with different mobile phases. Similarly, GLC is used for generating the fingerprint profiles of volatile oils and fixed oils of herbal drugs. Furthermore, the recent approaches of applying hyphenated chromatography and spectrometry such as High-Performance Liquid Chromatography–Diode Array Detection (HPLC–DAD), Gas Chromatography–Mass Spectroscopy (GC–MS), Capillary Electrophoresis- Diode Array Detection (CE-DAD), High-Performance Liquid Chromatography–Mass Spectroscopy (HPLC–MS) and High-Performance Liquid Chromatography–Nuclear Magnetic Resonance Spectroscopy (HPLC–NMR) could provide the additional spectral information, which will be very helpful for the qualitative analysis and even for the on-line structural elucidation.

4.1.6 Advances in chromatographic techniques

4.1.6.1 Liquid chromatography

a. Preparative high performance liquid chromatography

There are basically two types of preparative HPLC. One is low pressure (typically under 5 bar) traditional PLC, based on the use of glass or plastic columns filled with low efficiency packing materials of large particles and large size distribution. A more recent form PLC, Preparative High Performance Liquid Chromatography (Prep.HPLC) has been gaining popularity in pharmaceutical industry. In preparative HPLC (pressure >20 bar), larger stainless steel columns and packing materials (particle size 10-30 µm are needed. The examples of normal phase silica columns are Kromasil 10 µm, Kromasil 16 µm, Chiralcel AS 20 µm whereas for reverse phase are Chromasil C18, Chromasil C8,YMC C18. The aim is to isolate or purify compounds, whereas in analytical work the goal is to get information about the sample. Preparative HPLC is closer to analytical HPLC than traditional PLC, because its higher column efficiencies and faster solvent velocities permit more difficult separation to be conducted more quickly (Oleszek & Marston, 2000; Philipson, 2007). In analytical HPLC, the important parameters are resolution, sensitivity and fast analysis time whereas in preparative HPLC, both the degree of solute purity as well as the amount of compound that can be produced per unit time i.e. throughput or recovery are important. This is very important in pharmaceutical industry of today because new products (Natural, Synthetic) have to be introduced to the market as quickly as possible. Having available such a powerful purification technique makes it possible to spend less time on the synthesis conditions.

b. Liquid Chromatography- Mass Spectroscopy (LC-MS)

In Pharmaceutical industry LC-MS has become method of choice in many stages of drug development. Recent advances includes electro spray, thermo spray, and ion spray ionization techniques which offer unique advantages of high detection sensitivity and specificity, liquid secondary ion mass spectroscopy, later laser mass spectroscopy with 600 MHz offers accurate determination of molecular weight proteins, peptides. Isotopes pattern can be detected by this technique (Oleszek & Marston, 2000; Philipson, 2007).

c. Liquid Chromatography- Nuclear Magnetic Resonance (LC-NMR)

The combination of chromatographic separation technique with NMR spectroscopy is one of the most powerful and time saving method for the separation and structural elucidation of unknown compound and mixtures, especially for the structure elucidation of light and oxygen sensitive substances. The online LC-NMR technique allows the continuous registration of time changes as they appear in the chromatographic run automated data acquisition and processing in LC-NMR improves speed and sensitivity of detection (Daffre *et al.,* 2008). The recent introduction of pulsed field gradient technique in high resolution NMR as well as three-dimensional technique improves application in structure elucidation and molecular weight information. These new hyphenated techniques are useful in the areas of pharmacokinetics, toxicity studies, drug metabolism and drug discovery process.

4.1.6.2 Gas chromatography

a. Gas Chromatography Fourier Transform Infrared spectrometry

Coupling capillary column gas chromatographs with Fourier Transform Infrared Spectrometer provides a potent means for separating and identifying the components of different mixtures.

B. Gas Chromatography-Mass Spectroscopy

Gas chromatography equipment can be directly interfaced with rapid scan mass spectrometer of various types. The flow rate from capillary column is generally low enough that the column output can be fed directly into ionization chamber of MS. The simplest mass detector in GC is the Ion Trap Detector (ITD). In this instrument, ions are created from the eluted sample by electron impact or chemical ionization and stored in a radio frequency field; the trapped ions are then ejected from the storage area to an electron multiplier detector. The ejection is controlled so that scanning on the basis of mass-to-charge ratio is possible. The ions trap detector is remarkably compact and less expensive than quadrapole instruments.GC-MS instruments have been used for identification of hundreds of components that are present in natural and biological system (Oleszek & Marston, 2000; Philipson, 2007; Daffre *et al.,* 2008).

4.1.6.3 Supercritical Fluid Chromatography (SFC)

Supercritical fluid chromatography is a hybrid of gas and liquid chromatography that combines some of the best features of each. This technique is an important third kind of column chromatography that is beginning to find use in many industrial, regulatory and academic laboratories. SFC is important because it permits the separation and determination of a group of compounds that are not conveniently handled by either gas or liquid chromatography. These compounds are either non-volatile or thermally labile so that GC procedures are inapplicable or contain no functional group that makes possible detection by the spectroscopic or electrochemical technique employed in LC. SFC has been applied to a wide variety of materials including natural products, drugs, foods and pesticides.

4.2 Other Chromato-Spectrometric studies

The NMR techniques are employed for establishing connectivities between neighbouring protons and establishing C-H bonds. INEPT is also being used for long range heteronuclear correlations over multiple bondings. The application of Thin Layer chromatography (TLC), High Performance Chromatography (HPLC) and HPLC coupled with Ultra violate (UV) photodiode array detection, Liquid Chromatography-Ultraviolet (LC-UV), Liquid Chromatography-Mass Spectrophotometry (LCMS), electrospray (ES) and Liquid Chromatography-Nuclear Magnetic Resonance (LC-NMR) techniques for the separation and structure determination of antifungal and antibacterial plant compounds is on the increase frequently (Oleszek & Marston, 2000; Bohlin and Bruhn, 1999). Currently available chromatographic and spectroscopic techniques in new drug discovery from natural products Currently, computer modelling has also been introduced in spectrum interpretation and the generation of chemical structures meeting the spectral properties of bioactive compounds obtained from plants (Vlietinck, 2000). The computer systems utilise 1H, 13C, 2D-NMR, IR and MS spectral properties (Philipson, 2007). Libraries of spectra can be searched for comparison with complete or partial chemical structures. Hyphenated chromatographic and spectroscopic techniques are powerful analytical tools that are combined with high throughput biological screening in order to avoid re-isolation of known compounds as well as for structure determination of novel compounds. Hyphenated chromatographic and spectroscopic techniques include LC–UV–MS, LC–UV–NMR, LC–UV–ES–MS and GC–MS (Oleszek & Marston, 2000; Philipson, 2007).

4.3 Simple assay methods

4.3.1 Antimicrobial assay

Common methods used in the evaluation of the antibacterial and antifungal activities of plant extracts and essential oils, include the agar diffusion method (paper disc and well), the dilution method (agar and liquid broth) (Yagoub, 2008; Okigbo *et al*., 2009; El-Mahmood, 2009; Aiyegoro *et al*., 2009), and the turbidimetric and impedimetric monitoring of microbial growth (R´ıos & Recio, 2005). These methods are simple to carry out under laboratory conditions.

4.3.2 Antioxidant assays

Most common spectrophotometric assay method applied is the DPPH radical scavenging system in which the hydrogen or electrons donation ability of plant extracts are measured from bleaching of purple methanol solution of 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) free radical (Changwei *et al*., 2006). This spectrophotometric assay uses the stable radical DPPH as a reagent. DPPH absorbs at 517 nm, and as its concentration is reduced by existence of an antioxidant, the absorption gradually disappears with time. A 2-ml aliquot of a suspension of the ethanol extracts is mixed with 1 ml of 0.5 mM 2,2- diphyenyl-1-picrylhydrazyl (DPPH) solution and 2 ml of 0.1 M sodium acetate buffer (pH 5.5). After shaking, the mixture is incubated at ambient temperature in the dark for 30 minutes, following which the absorbance is measured at 517 nm using a UV-160A spectrometer. A solvent such as ethanol can be used as negative control. Radical scavenging activity is often expressed as percentage inhibition and is often calculated using the formula:

$$\%\ \text{radical scavenging activity} = [(A_{control} - A_{test})/A_{control}] \times 100$$

Where $A_{control}$ is the absorbance of the control (DPPH solution without test sample) and A_{test} is the absorbance of the test sample (DPPH solution plus antioxidant).

Phenolics content and reducing power of extracts is often determined using the Folin-Ciocalteu method. Equal volumes of Folin-Ciocalteu reagent and given quantity (mg) of plant extracts of different concentrations (e.g. 0.4, 0.3, 0.2, 0.1 and 0.05 mg/ml) are often mixed in different sets of test tubes shaken thoroughly, and left to stand for 1 min. Ten percent of $NaHCO_3$ is then added and the mixture once again allowed to stand for 30 minutes, after which the absorbance (725 nm) is measured spectrophotometrically. Gallic acid (0.05-0.5 mg/ml) is often used to produce standard calibration curve and the total phenolic content expressed as mg equivalent of gallic acid (mg GAE) per gram dry weight of the extract by computing with standard calibration curve (Djeridane *et al.,* 2006).

For determination of reducing power of plant extracts, the ferric reducing/antioxidant power (FRAP) assay method can be applied. The assay is based on the reducing power of a compound (antioxidant). A potential antioxidant reduces the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}); the later forms a blue complex (Fe^{2+}/2, 4, 6, tripyridyl-s-triazine (TPTZ)), which increases the absorption at 593 nm. Stronger absorption at this wavelength indicates higher reducing power of the phytochemical, thus higher antioxidant activity. Reaction mixture containing test extract sample at different concentrations (10-100µl) in phosphate buffer (0.2 M, pH 6.6) and equal amounts of 1% (w/v) potassium ferricyanide are incubated at 50^{o}C for

20 minutes and then the reaction terminated by the addition of equal volumes of 10% (w/v) tricarboxyllic acid (TCA) solution and the mixture centrifuged at 3000rpm for 20 minutes. The supernatant is mixed with equal volume of distilled water and 0.1 % (w/v) ferric chloride solution and the absorbance measured at 700 nm. Increased absorbance of the mixture with concentration indicates the reducing power of extract (Jayasri, 2009).

4.3.3 Toxicological studies

These are often carried out to determine the toxicity of a plant part. Usually animal models such as mice, guinea pigs or rabbits are often employed. In these procedures, the LD_{50} of the extracts in the experimental animal is often determined via either oral or intradermal administration. The toxic response of experimental animals to the administration of plant alkaloids is usually detected by assay of the serum ALT and AST of the animal as sensitive indicators of hepatocellular damage (Chapatwala *et al*., 1982). Any toxicity usually results in distortion of hepatocytes membrane integrity due to hepatocellular injury and plasma levels rise, as a consequence of high toxin levels present within hepatocytes.

5. Safety concerns for phytochemicals

Plants are natural reservoir of medicinal agents almost free from the side effects normally caused by synthetic chemicals (Fennel *et al*., 2004). The World Health Organization estimates that herbal medicine is still the main stay of about 75-80% of the world population, mainly in the developing countries for primary health care because of better cultural acceptability, better compatibility with the human body, and lesser side-effects (Kamboj, 2000; Yadav & Dixit, 2008). The over use of synthetic drugs with impurities resulting in higher incidence of adverse drug reactions, has motivated mankind to go back to nature for safer remedies. Due to varied locations where these plants grow, coupled with the problem of different vanacular names, the World Health Organization published standards for herbal safety to minimize adultartion and abuse (WHO, 1999).

A number of modern drugs have been isolated from natural sources and many of these isolations were based on the uses of the agents in traditional medicine (Rizvi *et al*., 2009). Antimicrobial properties of crude extracts prepared from plants have been described and such reports had attracted the attention of scientists worldwide (Falodun *et al*., 2006; El-Mahmood & Amey, 2007; El-Mahmood, 2009). Herbs have been used for food and medicinal purposes for centuries and this knowledge have been passed on from generation to generation (Adedapo *et al*., 2005). This is particularly evident in the rural areas where infectious diseases are endemic and modern health care facilities are few and far thus, compelling the people to nurse their ailments using local herbs. Herbal treatments have been adjudged to be relatively safe (WHO, 1999). For instance, daily oral doses of epigallocatechin-3-gallate (EGCG) for 4 weeks at 800 mg/day in 40 volunteers only caused minor adverse effects (Phillipson, 2007). In a 90-day study of polyphenon E (a formulation of green tea extract with 53% EGCG), the oral no effect level (NOEL) values are 90 mg/kg/day for rats and 600 mg/kg/day for dogs (Boocock *et al*., 2007). For curcumin given to cancer patients at 3600 mg/day for 4 months or 800 mg/day for 3 months, only minor adverse effects are seen. For resveratrol, a single oral dose at 5 g in 10 volunteers only causes minor adverse effects (Boocock *et al*., 2007). Though herbs are relatively safe to use, their combined

use with orthodox drugs should be done with extreme caution. Concomitant use of conventional and herbal medicines is reported to lead to clinically relevant herb–drug interactions (Liu et al., 2009). The two may interact either pharmacokinetically or pharmacodynamically resulting into adverse herbal-drug interactions (Izzo, 2005). St John's wort (*Hypericum perforatum*), used for the treatment of mild to moderate depression, interacts with digoxin, HIV inhibitors, theophylline and warfarin. Some medicinal herbs, when ingested, either affect cytochrome P450 isoenzymes by which drugs are metabolised, or, phosphoglycoprotein transporter systems that affect drug distribution and excretion. Concurrent use of some herbal medicines with other medicines may either lower blood plasma concentrations of medicinal drugs, possibly resulting in suboptimal therapeutic amounts, or lead to toxic concentrations in the blood, sometimes with fatal consequences (Phillipson, 2007).

Despite this observation however, it has been reported that phytochemicals act in synergy with chemotherapeutic drugs in overcoming cancer cell drug resistance and that the application of specific phytochemicals may allow the use of lower concentrations of drugs in cancer treatment with an increased efficacy (Liu, 2004).

Another advantage with phytochemicals is that, among an estimated 10,000 secondary products (natural pesticides), it has been proposed that human ancestors evolved a generalized defense mechanism against low levels of phytochemicals to enable their consumption of many different plant species containing variable levels of natural pesticides (carcinogens) without subsequent ill health (Liu, 2004). Traces of phytochemicals found in fruits and vegetables may potentiate the immune system and help to protect against cancer (Trewavas and Stewart, 2003). Phytochemicals show biphasic dose responses on mammalian cells. Though at high concentrations they can be toxic, sub-toxic doses may induce adaptive stress response (Ames & Gold, 1991). This includes the activation of signaling pathways that result in increased expression of genes encoding cytoprotective proteins. It is therefore suggested that hormetic mechanisms of action may underlie many of the health benefits of phytochemicals including their action against cancer drug resistance (Mattson, 2008).

Molecular mechanisms of herb–drug interaction occur, the most notable is the ATP-binding cassette drug transporters such as P-glycoprotein (You & Moris, 2007) and the drug metabolizing enzymes (known as phase I and phase II enzymes), especially cytochrome P450 3A4 (CYP3A4) (Pal & Mitra, 2006; Meijerman *et al*., 2006).

6. Future prospects of phytochemicals as sources of antimicrobial chemotherapeutic agents

Though there are few disadvantages associated with natural products research. These include difficulties in access and supply, complexities of natural product chemistry and inherent slowness of working with natural products. In addition, there are concerns about intellectual property rights, and the hopes associated with the use of collections of compounds prepared by combinatorial chemistry methods. Despite these limitations, over a 100 natural-product-derived compounds are currently undergoing clinical trials and at least 100 similar projects are in preclinical development (Phillipson, 2007). Among these products the highest number are from plant origin (Table 3). Most are derived from plants and microbial sources. The projects based on natural products are predominantly being studied

for use in cancer or as anti-infectives. There is also, a growing interest in the possibility of developing products that contain mixtures of natural compounds from traditionally used medicines (Charlish, 2008), while, a defined mixture of components extracted from green tea (Veregen TM) has been approved by the US Food and Drug Administration (FDA) and has recently come on the market.

Development stage	Plant	Bacterial	Fungal	Animal	Semi-synthetic	Total
Preclinical	46	12	7	7	27	99
Phase I	14	5	0	3	8	30
Phase II	41	4	0	10	11	66
Phase III	5	4	0	4	13	26
Pre-registration	2	0	0	0	2	4
Total	108	25	7	24	61	225

Table 3. Drugs based on natural products at different stages of development

Most of the leads from natural products that are currently in development have come from either plant or microbial sources. Earlier publications have pointed out that relatively little of the world's plant biodiversity has been extensively screened for bioactivity and that very little of the estimated microbial biodiversity has been available for screening (Doughari *et al.,* 2009). Hence, more extensive collections of plants (and microbes) could provide many novel chemicals for use in drug discovery assays. With the growing realization that the chemical diversity of natural products is a better match to that of successful drugs than the diversity of collections of synthetic compounds and with the global emergence of multidrug resistant pathogens (Feher and Schmidt, 2003) the interest in applying natural chemical diversity to drug discovery appears to be increasing once again (Galm & Shen, 2007).

With advances in fractionation techniques to isolate and purify natural products (e.g. counter-current chromatography (Doughari *et al.,* 2009) and in analytical techniques to determine structures (Singh & Barrett, 2006), screening of natural product mixtures is now more compatible with the expected timescale of high-throughput screening campaigns. Singh and Barrett (2006) point out that pure bioactive compound can be isolated from fermentation broths in less than 2 weeks and that the structures of more than 90% of new compounds can be elucidated within 2 weeks. With advances in NMR techniques, complex structures can be solved with much less than 1 mg of compound. It has recently been demonstrated that it is possible to prepare a screening library of highly diverse compounds from plants with the compounds being pre-selected from an analysis of the Dictionary of Natural Products to be drug-like in their physicochemical properties (Oleszek & Marston, 2000; Doughari *et al.,* 2009). It will be interesting to see if such a collection proves to be enriched in bioactive molecules. Several alternative approaches are also being explored in efforts to increase the speed and efficiency with which natural products can be applied to drug discovery. For instance, there is an attraction to screen the mixtures of compounds obtained from extracts of plant material or from microbial broths to select extracts from primary screens that are likely to contain novel compounds with the desired biological activity using the concept of 'differential smart screens'. This approach involves screening extracts of unknown activity against pairs of related receptor sites. By the comparison of the ratios of the binding potencies at the two receptor sites for a known selective ligand and for an extract, it is possible to predict which extract was likely to contain components with the

appropriate pharmacological activity (McGaw *et al*., 2005; Doughari *et al.,* 2009; Okigbo *et al*., 2009). Another approach is the use of 'chemical-genetics profiling' (Doughari *et al.,* 2009). In this method, by building up a database of the effects of a wide range of known compounds, it is possible to interrogate drugs with unknown mechanisms or mixtures of compounds such as natural product mixtures. The technique highlighted unexpected similarities in molecular effects of unrelated drugs (e.g. amiodarone and tamoxifen) and also revealed potential anti-fungal activity of crude extracts. This activity was confirmed by isolation and testing of defined compounds, stichloroside and theopalauamide (Fig. 10).

Because these compounds are not structurally similar, they would not have been expected to act via the same biological target, thus providing more chances for a very versatile drug component with high efficacy against antibiotic resistant bacteria. It's been reported that despite the popularity of chemical drugs, herbal medicine in Africa and the rest of the world, continued to be practiced due to richness of certain plants in varieties of secondary metabolites such as alkaloids, flavonoids, tannins and terpenoids (Cowan, 1999; Lewis & Ausubel, 2006; Adekunle & Adekunle, 2009). Stapleton *et al.* (2004) reported that aqueous extracts of tea (*Camellia sinensis*) reversed methicillin resistance in methicillin resistant *Staphylococcus aureus* (MRSA) and also to some extent reduced penicillin resistance in beta-lactamase-producing *Staphylococcus aureus*. Also, Betoni *et al*. (2006) reported synergistic interactions between extracts of guaco (*Mikania glomerata*), guava (*Psidium guajava*), clove (*Syzyguim aromaticum*), garlic (*Allium sativum*) lemon grass (*Cymbopogon citratus*) ginger (*Zingiber officinale*) cargueja (*Baccharis trimera*), and mint (*Mentha pieria*) and some antibiotics against *S. aureus*. However, these are preliminary investigations and more works are needed to actually determine the active ingredients in these plants extracts and this may help in improving management of the different infectious diseases that are developing resistance to commonly used antibiotics and possibly to verocytotoxuic bacteria. Furthermore, toxicological studies can also be carried out to determine the reliance on these herbs without many side effects.

Researchers have also devised cluster of chemically related scaffolds which are very useful in guiding the synthesis of new compounds. In an attempt to combine the advantages of virtual screening of chemically diverse natural products and their synthetic analogues (scaffolds) with the rapid availability of physical samples for testing, an academic collaboration has established the Drug Discovery Portal (http://www.ddp.strath.ac.uk/). This brings together a wide variety of compounds from academic laboratories in many different institutions in a database that can be used for virtual screening. Academic biology groups can also propose structures as targets for virtual screening with the Portal's database (and with conventional commercially available databases). Access to the Portal is free for academic groups and the continued expansion of the chemical database means that there is a valuable and growing coverage of chemical space through many novel chemical compounds (Feher & Schmidt, 2003; Galm & Shen, 2007).

Despite all of the advances made by the pharmaceutical industry in the development of novel and highly effective medicines for the treatment of a wide range of diseases, there has been a marked increase in the use of herbal medicines even including the more affluent countries of the world. Germany has the largest share of the market in Europe and it was reported that the sales of herbal medicinal products (HMPs) in 1997 were US$ 1.8 billion (Barnes *et al*., 2007).Numerous scientific medical/pharmaceutical books have been

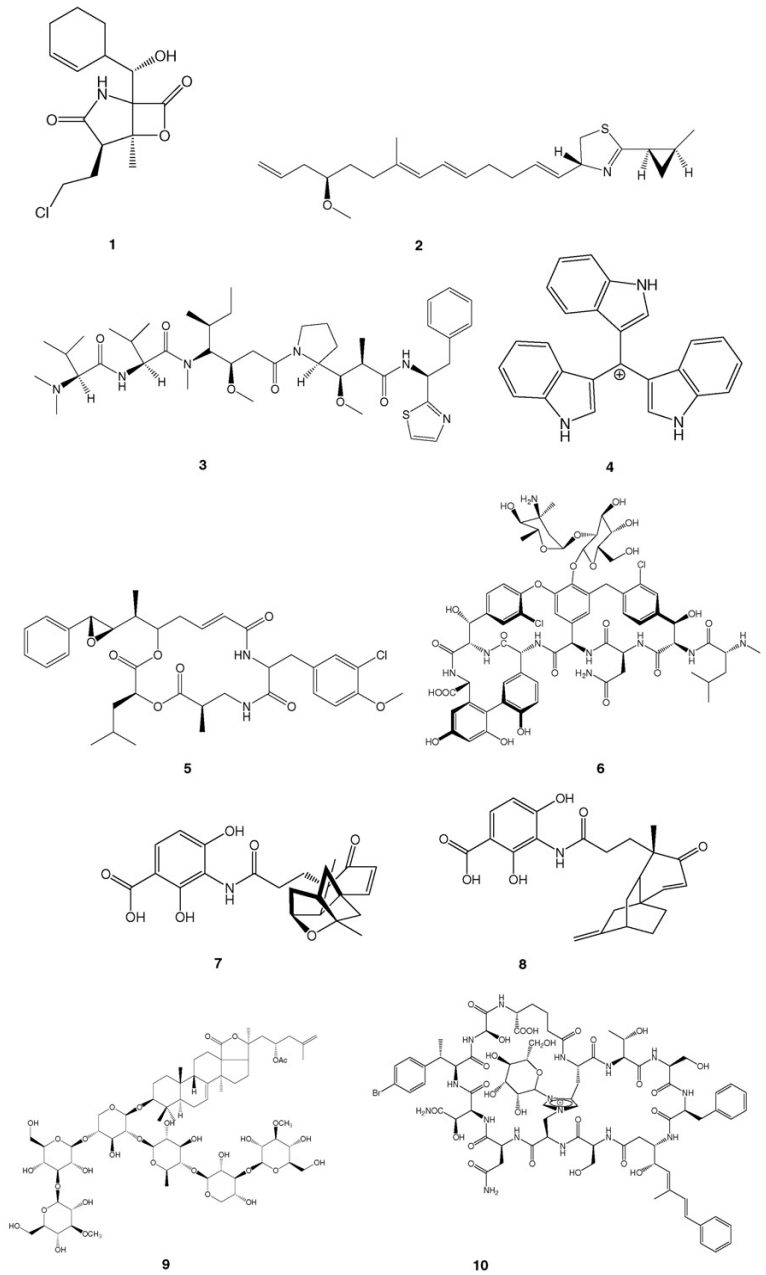


Fig. 10. Natural products – recently discovered and/or in development. (1) Salinosporamide A; (2) curacin A; (3) dolastatin 10; (4) turbomycin A; (5) cryptophicin; (6) vancomycin; (7) platensimycin; (8) platencin; (9) stichloroside; (10) theopalauamide (Source; Doughari *et al.,* 2009).

published in recent years aiming to provide the general public and healthcare professionals with evidence of the benefits and risks of herbal medicines (Barnes *et al*., 2007; Phillipson, 2007). The pharmaceutical industry has met the increased demand for herbal medicines by manufacturing a range of HMPs many of which contain standardized amounts of specific natural products. In the 1950s, it would not have been possible to predict that in 50 years time there would be a thriving industry producing HMPs based on the public demand for herbal medicines. To date European Pharmacopoeia has even published up to 125 monographs on specific medicinal herbs with another 84 currently in preparation (Mijajlovic *et al*., 2006; Phillipson, 2007. The monographs are meant to provide up-to-date knowledge of phytochemistry for defining the chemical profiles of medicinal herbs and an understanding of analytical tests for identification of the herbs and for the quantitative assessment of any known active ingredients (Phillipson, 2007). Several regulatory bodies incuding Traditional Medicines Boards (TMBs, in Nigeria and other African Countries), Medicines and Healthcare products Regulatory Agency (MHRA), Herbal Medicines Advisory Committee (HMAC) (Uk) and American Herbal Products Association (AHPA) and several other pharmacopoeia (British, Chinese, German, Japanese) provide guidelines and advice on the safety, quality and utilization of the plant herbal products in several countries (Yadav & Dixit, 2008). Scientific and Research communities are currently engaged in phytochemical research, and pharmacognosy, phytomedicine or traditional medicine are various disciplines in higher institutions of learning that deals specifically with research in herbal medicines. It is estimated that >5000 individual phytochemicals have been identified in fruits, vegetables, and grains, but a large percentage still remain unknown and need to be identified before we can fully understand the health benefits of phytochemicals (Liu, 2004).

7. Concluding remarks

With the increasing interest and so many promising drug candidates in the current development pipeline that are of natural origin, and with the lessening of technical drawbacks associated with natural product research, there are better opportunities to explore the biological activity of previously inaccessible sources of natural products. In addition, the increasing acceptance that the chemical diversity of natural products is well suited to provide the core scaffolds for future drugs, there will be further developments in the use of novel natural products and chemical libraries based on natural products in drug discovery campaigns.

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2

Structural Analysis of Flavonoids and Related Compounds – A Review of Spectroscopic Applications

Pedro F. Pinheiro and Gonçalo C. Justino
Centro de Química Estrutural, Instituto Superior Técnico, Technical University of Lisbon
Portugal

1. Introduction

In 1936 St. Rusznyák and A. Szent-Györgyi described, in a paper in Nature (Rusznyàk & Szent-György, 1936), the relief of certain pathological conditions, characterized by an increased permeability or fragility of the capillary wall, by extracts of Hungarian red pepper containing flavonols, a type of flavonoids, which were then named "vitamin P". The following five decades saw a slow but steady rise in the interest in the group of flavonoids, and their benefits in the treatment of a vast number of diseases and conditions, including pregnancy toxaemia, rheumatic fever, diabetes and cancer. In the late 1980s and throughout the 1990s flavonoids were intensely studied concerning their actions as mutagenic agents and as antioxidants and pro-oxidants as their likely roles in biological systems (for example, Aviram & Fuhrman, 1998; Lambert & Elias, 2010). In the early 90s the antioxidant activity of flavonoids was extensively studied *in vitro*, and it was assumed that such activity would be at the basis of the health promoting benefits of these compounds. However, in the late 90s and early 00s the metabolism of flavonoids was deeply scrutinized, and the results indicated that their antioxidant activity *in vivo* could not account for the overall actions attributed to them (Fraga et al., 2010). The paradigm for flavonoid action changed towards the establishment of flavonoids as inflammation modulators, and more recently their role in neuroprotection, memory and cognition has been under scrutiny (Gomes et al., 2008; Spencer et al., 2009; Spencer, 2010). However, exact mechanisms for many of the actions attributed to flavonoids have not yet been established, but the relationship between their activity and the presence of specific functional groups in the molecules is undeniable. Moreover, each role attributed to flavonoids has been linked to different structural features – for example, while antioxidant activity depends essentially on the number and location of OH groups in the molecules, their antagonist effect towards adenosine receptors depends more on the overall planarity than on the hydroxyl groups; in fact, the latter even appear to be counter-productive (González et al., 2007).

The work developed in this area strongly depends on powerful analytical techniques for quantification and structural identification, as circulating forms are usually found in the low micromolar range, and intracellular levels are even lower. This chapter will briefly review the analytical techniques employed to determine the flavonoids structure from *in vitro* and *in vivo* studies. Albeit it will be focused on the more common classes of flavonoids (flavones,

flavonols, flavanones, catechins, isoflavones and anthocyanidins), it will also address recent developments in minor flavonoid classes. Two fundamental works must be distinguished here. The first is the 1982 book by Markham entitled *Techniques of Flavonoid Identification*, which addresses the state-of-the-art of flavonoid structural identification at the time, and focused largely on UV spectroscopy (Markham, 1982). More recently, in 2005, Markham and Andersen have edited *Flavonoids: Chemistry, Biochemistry and Applications*, which is a reference book for those studying either structure or activity (or both) of flavonoids, containing large tables of collected NMR data and addressing many of the topics under research at that time (Fossen & Andersen, 2005). Hence, this chapter will be mainly focused on developments in the field from 2005 onwards.

2. Overview of flavonoid structural classification

Flavonoids are polyphenols of plant origin that are among the most important compounds in human diet due to their widespread distribution in foods and beverages. They can occur both in the free form (aglycones) and as glycosides, and differ in their substituents (type, number and position) and in their insaturation. As mentioned, the most common classes are the flavones, flavonols, flavanones, catechins, isoflavones and anthocyanidins, which account for around 80 % of flavonoids. Figure 1 shows the basic structure of the different flavonoid classes addressed in this chapter.

All flavonoids share a basic C6-C3-C6 phenyl-benzopyran backbone. The position of the phenyl ring relative to the benzopyran moiety allows a broad separation of these compounds into flavonoids (2-phenyl-benzopyrans), isoflavonoids (3-phenyl-benzopyrans) and neoflavonoids (4-phenyl-benzopyrans) (Figure 1). Division into further groups is made

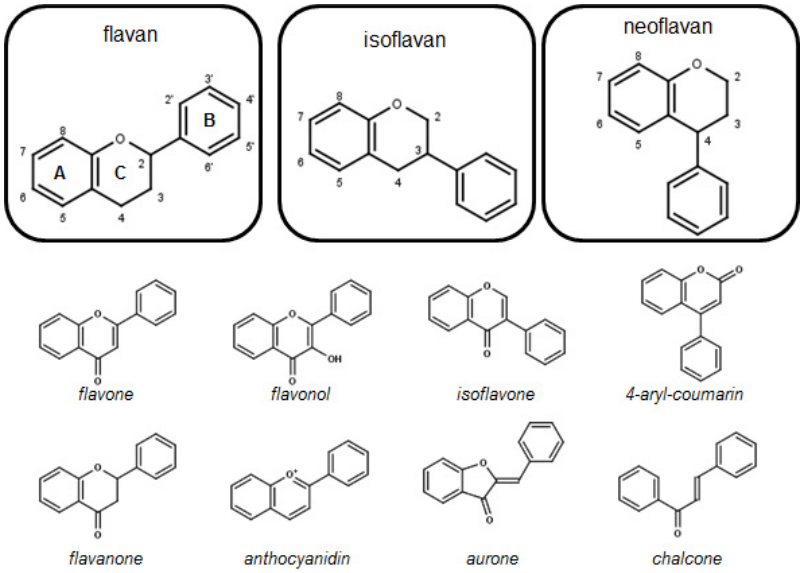

Fig. 1. Structure of the structural backbones of the main flavonoid groups (flavan, isoflavan and neoflavan) and of relevant flavonoid classes. Atom numbering and ring nomenclature are also included.

on the basis of the central ring oxidation and on the presence of specific hydroxyl groups. Most common flavonoids are flavones (with a C2-C3 double bond and a C4-oxo function), flavonols (flavones with a 3-OH group) and flavanones (flavone analogues but with a C2-C3 single bond), and abundant isoflavonoids include isoflavones (the analogue of flavones). 4-arylcoumarin (a neoflavonoid with a C3-C4 double bond) and its reduced form, 3,4-dihydro-4-arylcoumarin, are the major neoflavonoids. Other natural compounds, such as chalcones and aurones also possess the C6-C3-C6 backbone, and are henceforth included in the general group of flavonoids.

3. Mass Spectrometry

Mass Spectrometry (MS) has proved to be one of the most effective techniques in biomedical research, in special when complex matrixes of biological samples must be analysed. The main advantages of MS are its high sensitivity, which allows analysis of compounds present in the μg scale, and high specificity, as it is able to separate molecules of the same molecular weight but different atom composition, and sometimes even to differentiate stereoisomeric compounds. Its easy coupling to separation techniques such as liquid and gas chromatography is also an excellent advantage. A review of separation methods, applied to flavonols, isoflavones and anthocyanidins, has been recently published (Valls et al., 2009). Sample preparation may also be critical, but that varies from sample to sample; nevertheless, some general guidelines have been reviewed (Prasain et al., 2004). For more detail on mass spectrometry equipment and experiments, an excellent book has been published in 2007 (de Hoffmann & Stroobant, 2007). Specifically for electrospray and MALDI mass spectrometry applications in the biology area, the work edited by Cole (Cole, 2010) is highly adequate.

Gas Chromatography (GC) is one of the key techniques for the separation of organics and, coupled to MS, one of the most common techniques of structural identification. However, flavonoids are largely nonvolatile, and need be derivatized; also, they are usually thermally unstable. Both these characteristics have led to the establishment of Liquid Chromatography, in particular High Performance (HPLC), as the fundamental separation technique for flavonoids. Consequently, LC-MS coupling is routinely used for the overall structure elucidation of flavonoids (Fossen & Andersen, 2005).

3.1 Ionization techniques

Various ionization techniques are available, and each has their own specificities which make them more or less useful depending on the molecules under study and on the aim of such study. The ones most applied to flavonoid research are hereafter presented.

3.1.1 FAB-MS and LSIMS

Fast-Atom Bombardment (FAB) and Liquid Secondary Ion Mass Spectrometry (LSIMS) are ionization techniques used in Secondary Mass Ion Spectrometry (SIMS) in which secondary ions, emitted by sample irradiation with a beam of energetic (primary) ions, are analyzed. Typically, these techniques are able to produce ions from polar compounds with molecular weights up to 10 kDa, but they require the analyte to be dissolved in a matrix, which may lead to the formation of more complex spectra. Also, being soft techniques, ion abundance is

low, and it has been used essentially to identify flavonoid glycosides and for molecular weight determination (Stobiecki, 2000; Prasain et al., 2004).

3.1.2 ESI and APCI

Electrospray Ionization (ESI) is a technique in which ions are generated by solvent evaporation under a high voltage potential, and can be applied directly, by infusion of the sample with a flow-controlled syringe, or coupled to separation techniques such as LC or capillary electrophoresis. In both cases, a steady liquid stream enters the system, allowing multiple analyses to be performed over a relatively large period of time. ESI interfaces are mostly coupled to quadrupole mass spectrometers; both are simple and robust equipments, able to produce either positive or negative ions, and their main limitation is the relatively limited *m/z* range, usually below 2 kDa. In Atmospheric Pressure Chemical Ionization (APCI) sources ionization occurs via a corona discharge on a heated solvent spray, which produces solvent-derived primary ions that will, in turn, ionize the solute (Prasain et al., 2004; de Hoffmann & Stroobant, 2007). Both ESI and APCI use atmospheric pressure and high collision frequency, and thus generate large amounts of ions; as they involve solvent evaporation, the decomposition of the analytes is reduced, and full scans show limited fragmentation. The main disadvantage of both these techniques is that some HPLC solvents interfere with the ionization process, and thus chromatographic separations need to be specifically designed (Prasain et al., 2004).

3.1.3 MALDI

Matrix-Assisted Laser Desorption/Ionization (MALDI) is a soft ionization technique in which the analytes are co-crystallized with a matrix; this mixture is deposited on a plate upon which a laser beam is aimed. The laser discharge ultimately leads to analyte ionization and projection from the matrix and onto the analyser. Typical matrixes are derivatives of 4-hydroxycinnamic acid, and also 2,5-dihydroxybenzoic acid (2,5DHB) (de Hoffmann & Stroobant, 2007). Due to the structural similarity between these matrixes and the flavonoids, only recently has MALDI been applied to flavonoid structural elucidation, using a FT-ICR spectrometer, and a significant, although still informative, number of flavonoid-matrix clusters are observable (Madeira & Florêncio, 2009).

3.2 Mass analysis

Mass analysis is the second step in a MS experiment. Following ion generation, mass analysers measure the mass-to-charge ratio, *m/z*, of the ions, by using a combination of electromagnetic fields. There are many types of mass analysers, as there are of ion sources and detectors. The most common are of the ion type and of the quadrupole type, which analyse *m/z* ratios by the resonance frequency and by the trajectory stability, respectively, and time-of-flight (TOF) analysers, which measure ion velocity (or flight time). More recent resonance frequency analysers, namely Fourier Transform (FT) ion cyclotron resonance (FT-ICR) and FT orbitraps, are now starting to be applied to flavonoids (de Hoffmann & Stroobant, 2007). A recent work has compared the performance of different mass analysers coupled to the same ion source and it was concluded that fragmentation patterns are transferable among different mass analysers, only the relative abundances are changed; although applied to cyano dyes (Volná et al., 2007),

the conclusions also apply to other classes of compounds, such as flavonoids, where different mass analyzers lead to similar fragmentation patterns.

3.3 Tandem mass spectrometry

Tandem mass spectrometry, usually abbreviated MS/MS, or MS^n for n^{th} order fragmentation, is any method that involves at least two stages of mass analysis, in conjunction with a fragmentation process, either dissociation of reaction, which causes a change in the *m/z* ratio on an ion. Most commonly, a mass analyser is used to isolate a precursor ion, which is then fragment to yield product ions (and, eventually, neutral fragments) that will be detected in the second mass analysis – a typical MS^2 experiment. This can, at least conceptually, be expanded with further successive modification and detection steps, giving rise to MS^3,… , MS^n. However, as only a very small fraction of ions detected in one analyser makes it to the following analyzers, MS^3 is usually the highest order achieved. This spatial arrangement of equipment, analyser-modified-analyser, corresponds to tandem MS in space, where ions are treated in different regions of space. Alternatively, tandem MS can be performed in time, with analysers such as ion traps, orbitraps or FT-ICR, where the same analyser performs different tasks successively (de Hoffmann & Stroobant, 2007).

m/z modification can be achieved by various techniques, but the most common is Collision Induced (or Activated) Dissociation (CID or CAD), where precursor ions undergo collisional activation with neutral atoms or molecules (such as inert gases) in the gas phase. CID is an example of a post-source fragmentation, in which energy is added to the already vibrationally excited ions. An alternative to CID is ECD (Electron Capture Dissociation), in which multiply charged positive ions are submitted to a beam of low energy electrons, producing radical cations. In opposition to post-source fragmentation, in in-source fragmentation, ions already possess sufficient internal energy and fragment spontaneously within the mass spectrometer. Although usually this is an undesired effect, because it leads to lower abundance of precursor ions, it may in some cases become useful (de Hoffmann & Stroobant, 2007; Abrankó et al., 2011).

3.3.1 Scan modes

Four general types of tandem MS scans are possible, and all may generate valuable information. A *product ion scan* analyses all the fragment ions resulting from a single selected precursor ion (these are usually called *MS^2 spectra*). Conversely, a *precursor ion scan* will identify all the precursors of a selected product ion; a *neutral loss scan* is performed from a selected neutral fragment and will identify the fragmentations leading to the loss of that neutral fragment; these two techniques cannot be performed in time-based analysers (de Hoffmann & Stroobant, 2007). A particular application of neutral loss scans is in the identification of phase II conjugation metabolites, that can be identified by specific neutral losses (Table 1) (Prasain & Barnes, 2007). Neutral loss scans are widely used to detect phase II conjugation metabolites, such as glucuronides (loss of 176 Da) and sulfates (loss of 80 Da), as well as for the detection of glutathione adducts (loss of 129 Da). Scanning for neutral losses of 162 and 132 Da has also been used to separate flavonoids with hexose residues from those with pentose residues, respectively. More selective than these three techniques, *selected reaction monitoring* (SRM) will analyse if a specific product ion comes from the fragmentation of a specific precursor ion; although more sensitive, it is much more specific.

Metabolic Reaction	Mass change (Neutral loss)	Metabolic Reaction	Mass change (Neutral loss)
Glucuronidation	+176 (176)	Methylation	+14 (–)
Glycosylation, hexose	+162 (162)	Hydroxylation	+16 (–)
Glycosylation, deoxyhexose	+146 (146)	Acetylation	+42 (60)
Glycosylation, pentose	+132 (132)	Carboxylation	+44 (44)
Sulfation	+80 (80)	Decarboxylation	-44 (44)
Glutathionylation	+129 (129)	Demethylation	-14 (–)

Table 1. Mass shift associated with possible metabolic reactions of flavonoids and correspondent detection by neutral loss scanning. Adapted from Prasain & Barnes, 2007.

The following subsections will address the various applications of mass spectrometry to flavonoid structural elucidation, and are organized as a potential guide to explore the structure of novel compounds.

3.4 Flavonoid glycosides – differentiation and characterization

In plants, flavonoids are often found to be glycosylated; the glycoside residues can be attached to O and C atoms of the flavonoids, giving rise to *O*-glycosides, *C*-glycosides and *O*-*C*-glycosides. These can be differentiated by soft ionization techniques, with low fragmentation energy, usually by FAB-MS, in which glycoside loss from *O*-glycosides undergo heterolysis of their hemi-acetal O-C bonds, gives rise to Y_i^+ ions; at low energy, *C*-glycosides only produce $[M+H]^+$ ions, and, at higher energies, intraglycosidic cleaveages rive rise to $^{i,j}X$ fragments and water loss gives rise to characteristic ions (Cuyckens et al., 2000; Li & Claeys, 1994; Vukics & Guttman, 2010). Higher fragmentation energies lead to intraglycosidic cleavage in *O*-glycosides, to the generation of Y_i fragments in *C*-glycosides, and to both $^{i,j}X$ and Y_i fragments in *O*-*C*-glycosides, all of which correspond to complex, often misleading, mass spectra. (Li & Claeys, 1994). This nomenclature, proposed by Domon & Costello (1988) for the MS study of glycoconjugates, is presented in Figure 2.

The sugar type can be easily determined by the characteristic *m/z* values of the A_i, B_i and C_i fragments arising from hexoses, deoxyhexoses and pentoses, which are not directly observable in the mass spectra but can be computed from the *m/z* differences of the parent ions and corresponding X_i, Y_i and Z_i fragments (Vukics & Guttman, 2010; Ferreres et al., 2007; Li & Claeys, 1994). The *m/z* values for these fragments are presented in Table 2.

Flavonoid glycosides usually contain one or two glycoside residues, but molecules with more residues have been identified in nature. By definition, diglycosides can have the residues attached at different positions (di-*O*-glycosides and di-*C*,*O*-glycosides) or at the same position (*O*-diglycosides and *C*,*O*-diglycosides). The differentiation of the different types can be made from the product ions identified in the spectra, particularly from the Z_i

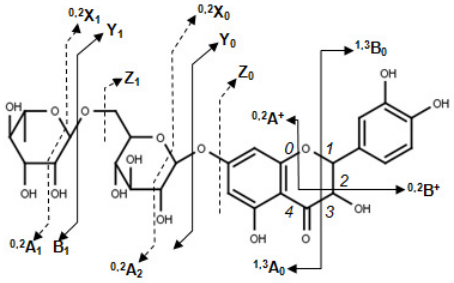

Fig. 2. Ion nomenclature used for flavonoid glycosides. Fragmentation of quercetin-7-*O*-rutinoside is depicted. Glycoside ions are named according to Domon & Costello, 1988; aglycone ions follow the nomenclature of Ma et al., 1997.

Fragments	**- ^{0,1}X**	**- ^{0,2}X (- H_2O) (- $2H_2O$)**	**- ^{0,3}X**	**- ^{1,5}X**	**- ^{2,3}X – $2H_2O$ (- $3H_2O$)**	**- ^{0,4}X - 2 H_2O**	**- Y_i**	**- X_i**
Hexose	150	120 (138) (156)	90	134	66 (84)	96	162	180
Deoxyhexose	134	104 (122) (140)	74	120	66 (84)	80	146	164
Pentose	120	90 (108) (126)	60	104	– (–)	66	132	150

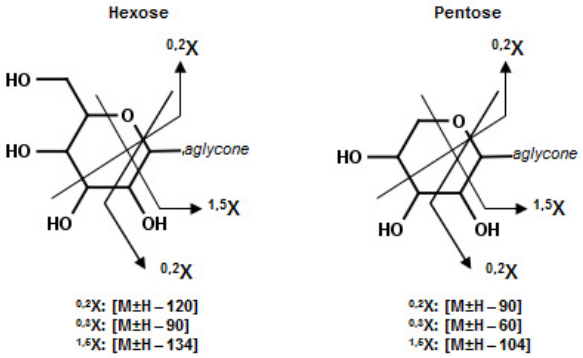

Table 2. Characteristic mass losses of flavonoid glycosides and characteristic cross-ring cleavages in the sugar rings of hexoses and pentoses (Vukic & Guttman, 2010; Cuyckens & Claeys, 2004).

ions – for example, Z_1 fragments do not occur in monoglycosides. Also, Yi fragments undergo well-known losses to form radical ions, which can be used to characterize the distribution of sugar residues. It must be noted that in some *O*-glycosides an internal sugar loss may take place, in which the aglycone-bound sugar is released first and, simultaneously, the other residue attaches itself to the aglycone; although this may lead to more complex spectra, this is well described in the literature, and can be overcome by the study of flavonoid-sodium adducts (Vukics & Guttman, 2010; Ma et al., 2000; Brüll et al., 1998; Cuyckens et al., 2001).

Concerning the glycoside sequence of di-, tri- and tetra-glycosides, there is no general procedure to apply for the ordering of the different residues, and it must be done rationally case by base. However, it has been shown that the interglycosidic linkage is easily accessible by MS; although these bonds can be of different types, 1→2 and 1→6 bonds are the preferred ones. For these two bond types, the Y_0^+/Y_1^+ is always larger for a 1→2 bond than for a 1→6 bond – the Y_0^+ ion is more abundant than the Y_1^+ ion for glycosides with a 1→2 bond, and the relative abundance is reversed in the case of a 1→6 bond. The negative ionisation mode may be particularly important in this case as the Z_1^- appears to be exclusive of 1→2 bonded glycosides. In the case of *C*,*O*-diglycosides, it is usually necessary to obtain MS^3 spectra to confirm the various interglycosidic linkages, because while 1→2 glycosides yield, by internal cleavage of the C-glycosyl moiety, $^{0,2}X_0^-$ ions, all other possibilities (1→3, 1→4 and 1→6) do not have characteristic fragmentation products (Ma et al., 2000; Cuyckens et al., 2000; Vukics & Guttman, 2010).

In theory, flavonoids could be glycosylated in any position, but *O*-glycosylation occurs mainly at position 7, as in flavones, isoflavones, flavanones and flavonols; *O*-glycosylation in positions 3 and 5 is also frequent, albeit less than in position 7. *C*-glycosyl flavonoids are usually flavones, and the glycosyl moieties are attached at positions 6 or 8; the literature has two reports of 3-*C*-glycosyl-flavones. Positions can be identified with basis of the product ions of the compounds because each glycosylation site appears to yield specific fragmentations. The $[Y_0\text{-CO}]^-$ is specific of 7-*O*-monoglycosides, while 3-*O*-monoglycosides are characterized by the $[Y_0\text{-2H-CO}]^-$ ion. Also, higher energy fragmentations yield product ions that contain both the glycoside moiety and fragments of the aglycone, particularly B-ring derived fragments. In what concerns *C*-glycosides, 6-*C*-glycosides usually undergo more extent fragmentations than 8-*C*-glycosides, and the former are typically associated with a $^{2,3}X^+\text{-}2H_2O$ fragment and an abundant $^{0,3}X^-$ ion, while for the latter no typical product ions have been out forward and the $^{0,3}X^-$ ion usually has a low abundance (Li & Claeys, 1994; Vukics & Guttman, 2010; Ferreres et al., 2007; Cuyckens & Claeys, 2005).

Many flavonoid glycosides possess acylated glycosyl moieties. Various acyl groups have been reported in the literature, and they are usually identifiable by characteristic mass losses, which are shown in Table 3. However, the position of glycoside acylation is not easily accessible by mass spectrometry, except for the cases in which a $^{0,4}X$ fragment is present in the spectra of a hexose-containg flavonoid as it inequivocally establishes that the hexosyl moiety is acylated in position 6 (Cuyckens & Claeys, 2004).

3.5 Aglycones – identification of flavonoid classes

The identification of the diverse flavonoid classes is often achieved by the MS^2 spectra of the various compounds, because, as a general rule, each class of flavonoids is characterized by

specific fragmentation patterns. This is especially true in the case of flavan-derived flavonoids, and, to a lesser extent, in the case of some isoflavan-derived flavonoids. For the remaining classes, qualitative MS spectra are still scarce, and, in particular, systematic studies of compounds of the same class are still to be performed.

3.5.1 Flavones, flavonols, flavanones and flavanonols

The most useful fragmentations of flavonoids, in terms of structural elucidation, are those that involve breaking the C-ring bonds, which are termed retro Diels-Alder (RDA) by analogy with the Diels-Alder cycloaddition. These fragmentations (included in Figure 2) give rise to product ions containing the A or B ring and part of the C ring; for example, the $^{1,3}A^+$ ion derived from a flavone, formed by a 1/3 fragmentation (cleavage of bonds 1 and 3), will contain the whole A ring plus the O1, C4 and O4 atoms.

These RDA fragmentations allow the establishment of diagnostic product ions for the various types of flavonoids. These are valid for a large of experimental MS conditions, but it must be kept in mind that the higher fragmentation energies will lead to increased

Acyl group	Characteristic fragments (mass change, amu)	
Acetyl	[M+H-acetylhexose]$^+$	(204)
Malonyl	[M±H–CO2]	(44)
	[M+H-malonyl]$^+$	(86)
	[M+H-malonylhexose]$^+$	(248)
Benzoyl	[M+H-benzoylhexose]$^+$	(266)
	[benzoyl]$^+$	*m/z*=105
	[benzoylhexose]$^+$	*m/z*=267
Galloyl	[M±H-galloyl]	(152)
	[M-H-gallic acid]$^-$	(170)
	[M+H-galloylhexose]$^+$	(314)
	[Gallic acid – H]$^+$	*m/z*=169
Coumaroyl	[M+H-coumaroyl]$^+$	(146)
	[M+H-coumaroylhexose]$^+$	(308)
Feruloyl	[M±H-feruloyl]	(176)
	[M±H-feruloylhexose]	(338)
	[feruloyl]$^+$	m/z=177
	[ferulic acid]$^{\bullet+}$	m/z=194
	[feruloylhexose]$^+$	m/z=339
Sinapoyl	[M±H-sinapoyl]	(206)
	[M±H-sinapoylhexose]	(368)
	[sinapoyl]$^+$	m/z=207
	[sinapic acid]$^+$	m/z=224
	[sinapoylhexose]$^+$	m/z=369
	[sinapoylhexose]$^-$	m/z=367

Table 3. Characteristic acyl groups found in acylated glycosyl flavonoids and corresponding characteristic fragmentations. ± stands for either + or –. Adapted from Cuyckens & Claeys, 2004.

fragmentation and a higher abundance of ions with lower masses, deviating from these diagnostic ions and fragmentation pathways.

The most important RDA fragmentations in flavones and flavonols (3-hydroxyl-flavones) are the 0/2, the 0/4, the 1/3. While the 0/4 pathway appears to be exclusive of flavones, according to a low-energy CID study, where it leads to the $^{0,4}B^+$ ion, which looses water to form the $^{0,4}B^+ - H_2O$, the $^{0,2}A^+$ is an exclusive of flavonols; also, the $^{1,3}B^+$ ion appears to be exclusive of flavones. In common, flavonoids from both classes give rise to the product ions $^{1,3}A^+$, and $^{0,2}B^+$ (Cuyckens et al., 2005). This information is essential when analysing, for example, flavonoid metabolites by MS. Quercetin metabolism leads to the formation of, among others, conjugates with sulphate and glucuronic acid, as well as to methoxy-quercetin. Considering a full scan MS of quercetin (molecular weight of 302 g/mol) metabolites collected from rat plasma, an ion at $m/z = 479$ corresponds to a protonate quercetin glucoronide; the 1/4 RDA fragmentation produces a $^{1,4}A^+$ at $m/z = 303$, that bears the mass increase, meaning the glucuronosyl moiety is present in this ion; therefore, it must be located at either position 5 or 7 (Justino et al., 2004). Similar rationales for other ions lead to the identification of the positions of metabolisation in these compounds.

More recently, a mixed ESI-MS and quantum chemical approach has analysed the fragmentation pathways of flavones and flavanols, and has proposed structures for other ions, other than RDA-derived, that are also informative in terms of structural elucidation. In particular, losses of one and two C_2H_2O moieties from the precursor ion, involving all the rings, and the formation of $^{1,3}A^+ - C_2H_2O$ ion, are the most useful (Justino et al., 2009). Similar methodology has been applied in other cases (Madeira et al; 2010).

Flavones and flavonols constitute the vast majority of the flavan-based flavonoids studies. Flavanones, which lack the C ring 2-3 double bond, are less abundant than those two and have received less attention. In terms of MS fragmentations, in particular RDA pathways, flavanone itself undergoes cleavages to yield the $^{1,3}A^+$ and $^{1,4}B^+$ ions; these same ions are also observed for other flavanones, such as 5,7,4′-tri-hydroxy-flavanone (Nikolic & van Breemen, 2004). Studies on flavanone derivatives have evidenced that the positive mode CID spectra of isoprenylated flavanones is dominated by isoprenyl loss and $^{1,3}A^+$ ion formation, while the negative mode is dominated by $^{1,4}A^-$, [$^{1,3}A^-$ - isoprenyl] and [$^{1,4}A^-$ - isoprenyl] ions. (Zhang et al., 2008) Derived ions, such as $[^{1,3}A + H]^+$ and $[^{1,4}B - H_2 + H]^+$, have also been identified. (Zhou et al., 2007). Negative mode ESI has also allowed dividing flavanones into two groups, one with a 2′-OH group, with a spectra dominated by the $^{1,4}A^-$ ion (for 5-OH-containing molecules) or the 1,4B- (otherwise), and the other with no 2′ substituents, for which the spectra were dominated by an intense $^{1,3}A^-$ ion and a low abundant $^{1,3}B^-$ ion. (Zhang et al., 2008) a 0/3 fragmentation, origination a $^{0,3}B^-$ ion, has also been observed (Xu et al., 2009); an equivalent fragmentation has also been observed in the positive mode , to produce a $^{0,3}B^+$ derived ion (Kéki et al., 2007). Flavanols (3-hydroxy-flavanones) show fragmentation patterns similar to those of flavanones; the main difference is that positive ESI spectra of flavanols are dominated by loss of water and homolytic H loss from the 3-hydroxy group to generate the $[M-H]^{\bullet+}$ ion (Zhang et al., 2008).

3.5.2 Isoflavones and Isoflavonols

Unlike flavan derived flavonoids, the fragmentation pathways of isoflavan derived flavonoids have only been explored more recently, but a good set of systematic studies are

published. Besides common neutral losses, isoflavones undergo RDA fragmentation to yield the $^{0,4}B^+$-H_2O and the $^{1,3}A^+$ ions; a full B ring loss, yielding the [M-*B ring*-CO]+ ion, is also frequently reported. Also characteristic is the loss of a CO group from Ring C oxo group, leading to ring contraction (Madeira et al., 2010; Heinonen et al., 2003; Borges et al., 2001; Simons et al., 2011).

In the negative mode, the 1/3 and 0/3 RDA fragmentations are predominant, and the 0/4 fragmentation is also observed sometimes, accompanied by extensive losses of CO, CO_2 and C_3O_2 moieties. C ring expansion is also commonly observed (Vessecchi et al., 2011; Kang et al., 2007; March et al., 2004; Ablajan, 2011). For isoflavones, rare RDA fragmentations have been described in the literature, such as a 2/3 fragmentation that has also been described for various acylated 7,2′-hydroxy-3′,4′-dimethoxyisoflavan glycosides (Qi et al., 2008) and a 2/4 fragmentation of daidzein (Wei et al., 2000).

The main difference in the fragmentation pathways of flavan- and isoflavan-derived flavonoids appears to be that in the latter the 0/3 RDA fragmentation occurs frequently while it appears not to occur for flavan-compounds; also, the 1/3 pathway appears to be much more important in isoflavan-compounds, in particular in those without a 2-OH group. Differentiation of isomeric aglycones of flavones and isoflavones is also possible based on a double neutral loss of CO (Kuhn et al., 2003).

3.5.3 Chalcones

Chalcones exist in nature with a variety of substituents. The MS spectra of these compounds are characterized by substituent loss, fragmentations of the substituents and chalcone fragmentations. Chalcone fragmentations are dominated by cleavage of a single bond, yielding a B ring derived ion with the attached C=O group in the charged form ($C{\equiv}O^+$), from which a CO loss yield the free B ring (Nowakowska & Pankiewicz, 2008). Besides that, 2′-OH-chalcones, the most common ones, with an OH group in ring B adjacent to the propenone chain, are known to be converted to the corresponding flavanones by various processes, and that has also been observed to occur in ESI MS; the patter of chalcone fragmentation will then be the same of flavanone fragmentation (Zhang et al., 2008).

3.5.4 Other classes

Many other flavonoid classes exist. Many of those have been isolated and characterized, but no useful fragmentation pathways have been established.

For aurones, a positive mode of two aurone glycosides was identified as main product ions the ones formed by the loss of the glycosyl moiety, followed by a CO loss involving the heterocyclic O atom in the 5-member ring, and, from the $[M\text{-glycosyl+H}]^{\bullet+}$ ion, the $^{1,3}A^+$ ion, which may lose a further CO group, and the $^{1,3}B^+$ ion (Kesari et al., 2004).

Coumarins show a wide diversity of substituents, and many even have fused rings attached. This variability does not allow overall fragmentation patterns, but many studies observe that in many cases the heterocyclic ring undergoes a contraction by CO loss (from the C=O group) to yield an ion containing a five membered ring (for example, Nowakowska & Pankiewicz, 2008; Zhang et al., 2007; Zhang et al., 2008).

Pterocarpans, isoflavonoid derivatives, show much more complex fragmentation pathways than the above analysed classes of flavonoids; MS studies of different deuterated peterocarpan derivatives, as well as of pterocarpan glycosides, points out that these are dominated by various and successive ring openings and/or contractions (Tóth et al., 2000; Zhang et al., 2007). A rare 2/4 RDA fragmentation, like that of isoflavan-derived flavonoids, has also been observed for pterocarpans in the negative mode (Simons et al., 2011).

Neoflavonoids, in which the B ring is attached in position 4, and include 4-aryl-coumarins, have been poorly studied by MS. The few reports available indicate, however, that neoflavonoid fragmentation is dominated by loss of the CH_3 radical in methoxylated compounds and by B ring loss, to yield the [M+H-B ring] fragment (Charles et al., 2005; Hulme et al., 2005; Liu et al., 2005). Figure 3 summarizes the characteristic RDA fragmentation pathways for flavonoid classes for which there is reliable information.

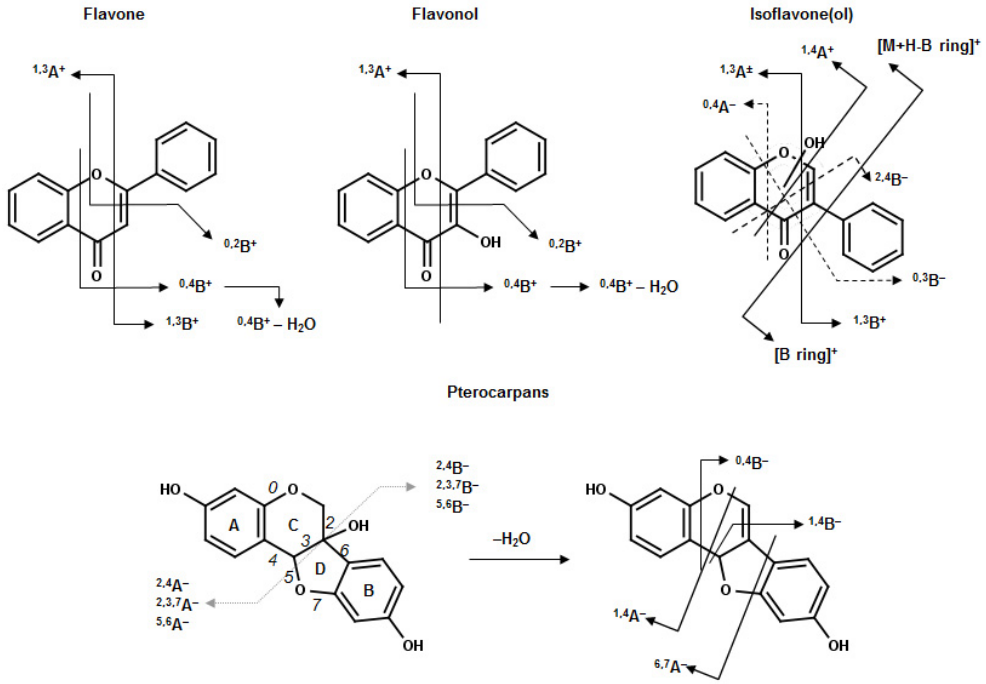

Fig. 3. Diagnostic products of flavones, flavonols, isoflavones, isoflavonols and pterocarpans. ± stands for either + or –. Adapted from Cuyckens et al, 2000; Kuhn et al., 2003; Madeira et al, 2010; Wei et al., 2000; Kang et al., 2007 and Simons et al., 2011.

3.6 Flavonoid-metal complexes

Flavonoids are good chelating agents towards metal ions and, in the case of iron and copper, the favoured places of chelation are catechol groups, hydroxyl groups adjacent to oxo groups, and 1-oxo-3-hydroxyl-containing moieties (Ren et al., 2008; Fernandez et al., 2002). This ability to chelate metals has been used to enhance the capabilities of MS; it has been

used to assist the elucidation of flavonoid glucuronides (Davis et al., 2006; Davis & Brodbelt, 2007) and various diglycosides (Pikulski et al., 2007). This is a result of the spectral changes observed when flavonoids are complexed with metals, giving rise to simpler yet more intense spectra (Satterfield & Brodbelt, 2000).

4. Nuclear magnetic resonance spectroscopy

Nuclear Magnetic Resonance spectroscopy, hereafter simply designated by NMR, is one the most powerful research techniques used to investigate the structure and some properties of molecules. One of the main applications of NMR in flavonoid research is the structural elucidation of novel compounds, for which nothing is known; although NMR traditionally requires large amounts of sample, which is not easy to obtain when analysing novel compounds, the technical developments in the last decade, both in NMR instrumentation, pulse programs and in computing power, have allowed the complete assignment of all proton and carbon signals using amounts in the order of 1 mg (Fossen & Andersen, 2005).

The major goal of this section is to summarize the applications of the various NMR experiments to flavonoid research, together with the information (other than atom connectivity) that can be taken from these experiments. Flavonoid NMR data are not presented here, as these are easily accessible, for a vast number of compounds, from the literature (reviewed in Fossen & Andersen, 2005).

4.1 One Dimensional NMR: ^{1}H and ^{13}C

The two most basic NMR experiments are the ^{1}H and the ^{13}C NMR experiments, which are aimed at the determination of the resonance frequency of each ^{1}H or ^{13}C nucleus in the molecule.

^{1}H NMR experiments register the chemical shifts (δ) and spin-spin couplings, the latter described by the coupling constants (*J*). This provides valuable information about the relative number of hydrogens and also their type, by comparison of the recorded chemical shifts with compiled data. This is particularly useful in establishing the aglycone type and the acyl groups attached to it, as well as in identifying the number and the anomeric configuration of the glycoside moieties attached to the aglycone. ^{13}C NMR data is used to complement ^{1}H NMR data, and is particularly useful at establishing the type of groups present in the samples' molecules by comparison with compiled data; however, it must be noted that ^{13}C NMR is much less sensitive due to the abundance of ^{13}C (1.1 %) when compared to ^{1}H (99.9 %) (Claridge, 1999).

Together, these two 1D experiments are used primarily to identify aglycone types and substituent groups, but a definite structural elucidation, which the accurate location of the various groups, requires various 2D experiments.

4.2 Homonuclear 2D NMR

2D NMR experiments generate contour maps that show the correlations between different nuclei in the molecules, and can be either homonuclear or heteronuclear, depending on whether the interacting nuclei are of the same or different elements (Claridge, 1999). COSY (COrrelation SpectroscopY) was one of the first multidimensional systems. COSY

crosspeaks are between protons that are coupled to each other, usually two bonds apart ($^2J_{HH}$), but sometimes also three and four bonds apart ($^3J_{HH}$ and $^4J_{HH}$); the intensity of coupling affects the intensity of the peak. DQF-COSY (Double Quantum Filter COSY) is an improvement of the COSY experiment in which non-coupled proton signals, such as those from solvent, are eliminated as they may overlap signals from the analyte (Claridge, 1999). A further improvement is the TOCSY (TOtal Correlation Spectroscopy) experiment, which creates correlations between *all* protons in a given spin system, as long as there are couplings between every intervening protons; this is extremely useful to identify protons on sugar rings – every proton from one sugar ring will have a correlation with all other protons from the same ring but not with those of other rings. Magnetization is transferred over up to 5 or 6 bonds, and is interrupted by small or null ^{1}H-^{1}H couplings and hetero-atoms; also, the number of transfer steps can be adjusted by changing the spin-lock time (Fossen & Andersen, 2005). A good reference for the TOCSY transfer in various sugars is Gheysen's work (Gheysen et al., 2008). Selective 1D TOCSY (also known as HOHAHA, homonuclear Hartman-Hahn) is particularly useful in compounds with more than one sugar moiety, in which overlap occurs; in this experiment, one peak is selected and that magnetization is transferred stepwisely to the protons in the same spin system; instead of crosspeaks, transfer is shown by increased multiplet intensity (Fossen & Andersen, 2005).

4.3 Heteronuclear 2D NMR

Heteronuclear 2D NMR experiments correlate nuclei of different elements. The most powerful techniques of all are undoubtedly the 2D proton–carbon experiments HMQC/HSQC (Heteronuclear Multiple Quantum Coherence/Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) as they provide an opportunity to dovetail proton and carbon NMR data directly.

HMQC and HSQC establish one bond correlations between the protons of a molecule and the carbons to which they are attached ($^1J_{CH}$). Both these are much more sensitive than the correspondent 1D ^{13}C experiments; while in 1D experiment the low abundance of the isotope leads to a low signal-to-noise ratio, in the heteronuclear 2D experiment the initial magnetization occurs on the highly sensitive ^{1}H nuclei and is then transferred to the ^{13}C atoms that are connected to each proton. A similar NMR experiment is ^{1}H-^{13}C HMBC (Heteronuclear Multiple Bond Correlation), in which long-range interactions (typically $^2J_{CH}$ and $^3J_{CH}$) are analyzed; HMBC is usually more sensitive to 3-bond correlations than to 2-bond correlations, but this depends on the overall signal-to-noise ratios and on the adjustable parameters of each experiment. A newer experiment, $^2J_{CH}$,$^3J_{CH}$-HBMC, has been designed to differentiate these two types of correlations (Claridge, 1999; Krishnamurthy, 2000; Fossen & Andersen, 2005).

HBMC application to flavonoids usually addresses assignment on nonprotonated C atoms, from both the aglycones and acyl groups. Unlike TOCSY, HMBC transfer is not stopped by heteroatoms, and so it can also be used to determine the linkage points of heteroatom-containing groups such as sugar residues. HMBC is also useful to distinguish some classes of flavonoids, as flavones from aurones, which have similar ^{1}H and ^{13}C NMR spectra but very different HMBC spectra. Currently, only enhanced variants of the HSQC and HMBC experiments, namely gradient enhanced (*ge*) ones, are used, due to their higher sensitivity and capacity. These have been used to establish strong intramolecular H bonding between the 4-oxo and 5-hydroxy groups in flavonoids (Exarchou et al., 2002; Kozerski et al., 2003).

Further developments in NMR experiments, using new 2D and 3D techniques, have been developed in recent years, and are starting to be used for flavonoid analysis. In particular, 2D and 3D HSQC-TOCSY experiments are capable of assigning all ^{13}C signals of individual glycosides in polyglycosilated flavonoids (Fossen & Andersen, 2005).

4.4 Connectivity through space – the nuclear overhauser effect

While the above mentioned 2D techniques are useful to establish the connectivities between atoms through bonds, the Nuclear Overhauser Effect (NOE), which can be summarized as "A change in the intensity of an NMR signal from a nucleus, observed when a neighboring nucleus is saturated", is useful at establishing non-bonded connectivities, or connectivities through space. The crosspeaks in a ^{1}H-^{1}H NOESY (NOE SpectroscopY) spectrum correspond to correlations between protons that are close to each other in space (up to 4 Å) but not necessarily connected through bonds; these correlations may arise from both intramolecular and intermolecular proton interactions, and has been successfully used to establish rotational conformers and restrictions, establish intermolecular associations and even solve protein-ligand and DNA-ligand structures. A 2D NOE experiment, ROESY (Rotating Overhauser Effect SpectroscopY), has been used in flavonoid research mainly to establish the stereochemistry of various flavonoids (Claridge, 1999; Fossen & Andersen, 2005). Figure 4 presents sample NMR spectra of anthocyanidine glucosides (Jordheim et al., 2006).

4.5 Solid state NMR

X-ray crystallography depends on the ability to obtain flavonoid crystals, which has only been achieved for a small number of flavonoids; in alternative, the ^{1}H CP-MAS (Cross Polarization Magic Angle Spinning) NMR techniques have been used to elucidate the solid state conformation of flavonoids, either pure or, for example, in tissues, providing enough sample is available (typically in the 10 mg scale). In particular, such an experiment had provided information on the planarity of the flavonoid rings, on the intramolecular H bonding between the 4-oxo group and the 3- and 5-hydroxy groups, and on intermolecular association. (Fossen & Andersen, 2005; Olejniczak & Potrzebowski, 2004)

5. UV-vis spectrophotometry

Ultraviolet and visible spectroscopy was one of the earliest techniques routinely used for flavonoid analysis due to the existence of two characteristic UV/Vis bands in flavonoids, band I in the 300 to 550 nm range, arising from the B ring, and band II in the 240 to 285 nm range, arising from the A ring. For examples, while the band I of flavones and flavonols lies in the 240 – 285 nm range, that of flavanone (no C ring instauration) lies in the 270 – 295 nm range; conversely, the band II of flavones and flavanones (no 3-OH group) lies around 303 – 304 nm, and that of 3-hydroxylated flavonoles is centred around 352 nm.

Shift reagents, such as sodium methoxide and aluminium chrolide, lead to shifts in the maximum wavelength of these bands due to methoxide-induced deprotonation of OH groups or Al^{3+} complexation by OH groups, were also routinely used to study flavonoid structure. Nowadays these techniques are not routinely used but still continue to be applied in some cases, in particular to HPLC eluates - a hyphenated LC-UV-MS has been developed using post-column UV shift reagents for the flavonoid analysis of crude extracts.

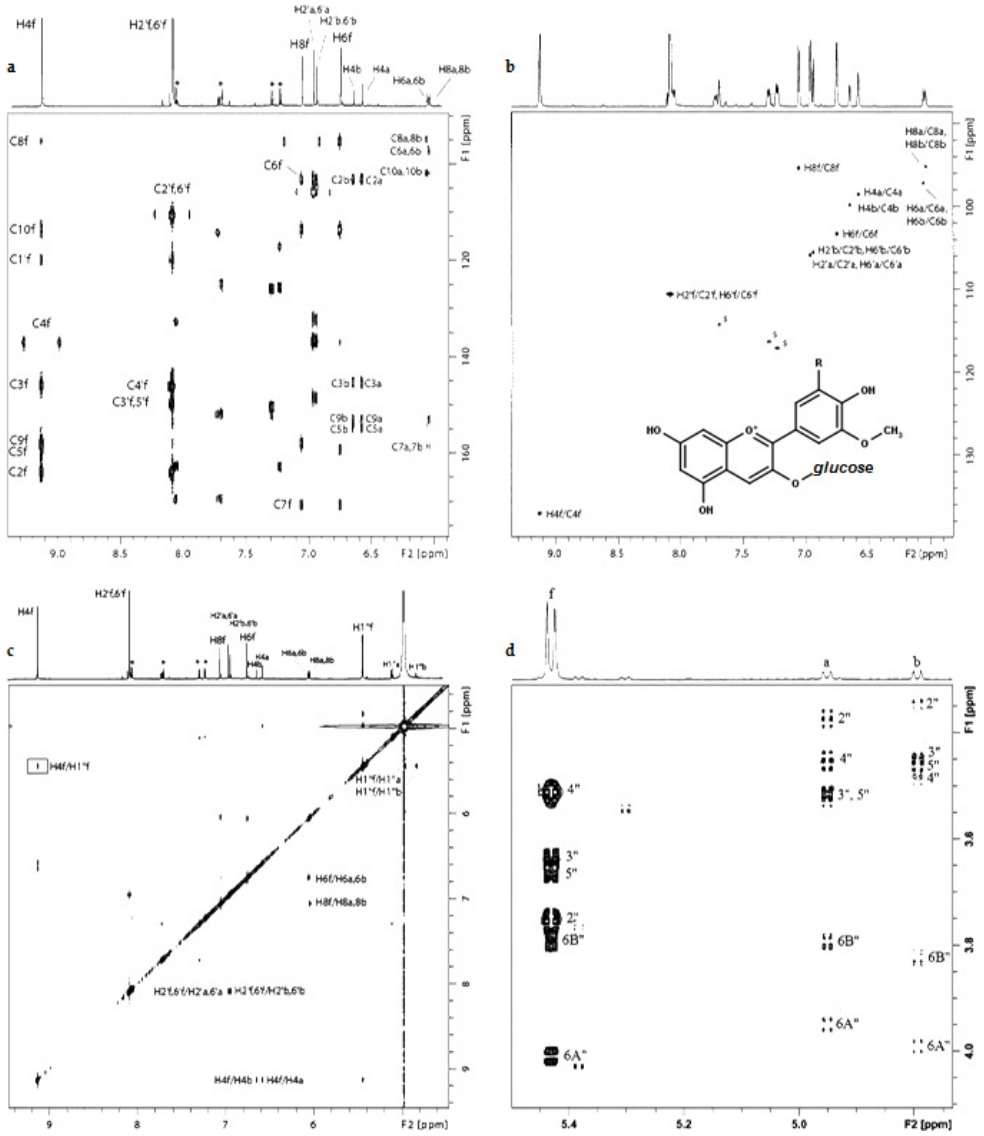

Fig. 4. Sample NMR 2D spectra of malvidin 3-*O*-β-glucopyranoside (R=OCH_3) (**a**, **b** and **c**) and of petunidin 3-*O*-β-glucopyranoside (R=OH) (**d**) obtained at 600.13 MHz, at about 11 mM at 25 °C in CD_3OD. a) ^1H-^{13}C HMBC spectrum; b) ^1H-^{13}C HSQC spectrum; c) NOESY spectrum; A negative cross-peak due to NOE correlation between H-4 and H-1'' of the flavylium cation, locating the position of the monosaccharide to the aglycone, is enclosed in a box. Other labelled cross-peaks are positive and are caused by chemical exchange; d) ^1H−^1H TOCSY NMR spectrum of petunidin 3-O-β-glucopyranoside. f = assignment for the flavylium; a = assignment for the hemiacetal **a** (major); b = assignment for the hemiacetal **b** (minor); */s = impurities. Adapted from Jordheim et al., 2006.

UV/Vis spectrophotometry is still widely used to study anthocyanidins, which change their form and colour depending on pH, concentration, metal ions and copigmentation (Giusti & Wrolstad, 2001). This multistate behaviour has been used to derive molecular machines based on flavonoids, particulary flavylium containing ones like anthocyanins (Melo et al., 2000; Moncada et al., 2004).

6. Other techniques

MS, NMR and UV/Vis are the most commonly used techniques to elucidate the structure of flavonoids. Three other techniques are also used: X-ray crystallography, although with the potential to solve complete structures, is hampered by the difficulty to obtain good crystals; circular dichroism and vibrational spectroscopies are used to solve specific structural details.

6.1 X-ray crystallography

X-ray crystallography is able to detect the arrangement of atoms within a crystal by the atom-induced diffraction of X-rays. Many materials form crystals, such as salts, metals and organic and biological molecules, in particular proteins. Flavonoids, however, only form crystals in sporadic conditions, and the number of reported flavonoid crystals is very low (Fossen & Andersen, 2005).

Nevertheless, traditional X-ray crystallography has been used to identify the intermolecular π-π interactions that guide the stacking of parallel aglycones to form supramolecular layers, and to identify aminoacyl residues involved in the formation of protein-flavonoid complexes, which are critical to the circulation of flavonoids in mammals (Rolo-Naranjo et al., 2010); this is one of the strongest applications of X-ray crystallography to the flavonoid area.

More recently, X-ray powder diffraction has been used, either alone or in association with solid-state NMR, to obtain structures of flavonoids, in particular of catechins (Harper et al., 2010).

6.2 Circular dichroism spectroscopy

Circular dichroism (CD) is a spectroscopic technique that allows the analysis of the differential absorption of left and right circularly polarized light. The major advantage of CD over optical rotation measurements is that CD absorption is confined to the narrow absorption range of each individual chromophore, and so it can be used to determine the contribution of individual chromophores and to access their possible substitution patterns (Wallace & Janes, 2009).

One of the most immediate applications of electronic CD and vibrational CD is the determination of the absolute configuration of quiral flavonoid molecules, such as isoflavan-4-ols (Kim et al., 2010). Slade et al., 2005, have reviewed the CD configuration characterization of most classes of flavonoids, and in particular of flavan-3,4-diols, which bear three quiral centers (Ferreira et al., 2004), and more recently proanthocyanidins analysis has also been reviewed, with a focus on CD results (Hümmer & Schreier, 2008).

CD is also routinely used to study the interaction of many flavonoids with biomolecules, providing valuable information on biomolecule-drug interaction, such as DNA binding of

quercetin (Ahmadi et al., 2011), binding to serum albumin (di Bari et al., 2009) and hemoglobin (Chauduri et al., 2011), inhibition of β-amyloid toxicity and fibrillogenesis (Thapa et al., 2011). It must be noted that many of the CD studies of protein-flavonoid association studies are usually accompanied by UV-VIS and/or fluorescence studies, such as the probing of kaempferol interaction with human serum albumin (Matei & Hillebrand, 2010).

6.3 Vibrational spectroscopy

Vibrational spectroscopy, in its infra-red and Raman variants, is a spectroscopic technique that analyses the vibrational modes of molecules and molecular groups, allowing bond characterization, and, by comparison with known tabulated data, identification of functional groups; in the case of flavonoids, vibrational spectroscopy has been systematically used to study hydroxyl and carbonyl groups, but more recent technical developments have allowed its application to a broader set of research goals. Raman spectra are much less complex than the IR spectra of the same molecules, and for that reason Raman spectroscopy has been gradually taking over IR spectroscopy, although it is common to use both techniques as complement of each other. Vibrational spectroscopy is seldom used alone, and most studies are accompanied by other spectroscopic approaches and/or quantum chemical computations (Siebert & Hildebrandt, 2007).

Both these spectroscopies are routinely applied to study the effects of substituents on the geometry of the molecule, in particular of dihedral angles, and also on the analysis of intramolecular (Li et al., 2011; Erdogdu et al., 2010) and intermolecular H bonding, either to other flavonoid moleculer or to solvent molecules. Similarly, metal complexation by flavonoids is also routinely assessed by vibrational spectroscopy (O'Coinceanainn et al., 2004).

7. Conclusion

The role of flavonoids in biological systems appears yet to be far from definitively determined, involving a large number of research groups all over the world. Interestingly, although many new actions of flavonoids *in vivo* have been put forward, the previously proposed actions are never dismissed, only relegated to secondary ways of flavonoid action, usually considered to be important in pathological conditions (Gomes et al., 2008).

As described above, various physical-chemistry techniques have been used as means of characterization of flavonoids. The great amount of work developed since the 1940s yielded a vast library of structural and spectroscopic information about these compounds, making the identification of new isolated species an easy and quick task. However, some limitations have yet to be overcome. For instance, the maximum molecular size allowed in mass spectroscopy (*ca.* 10 kDa) and in NMR spectroscopy (*ca.* 30 kDa) limits the role of these techniques in the characterization of some more complex molecules and molecular complexes, nevertheless, these two techniques have lead to great breakthrough in terms of structure elucidation that could not be achieved with the classical spectroscopic techniques like UV/Vis and Vibrational (Raman and Infra-Red) spectroscopy or by X-ray diffraction.

It must be noted that, in many cases, information obtained by NMR or MS needs to be correlated with data from other structural analysis techniques, such as CD, in order to confirm some feeble data. Nevertheless, it has been demonstrated that MS and NMR are the most suitable techniques to determine the chemical structure of flavonoids and its

derivatives. While NMR spectroscopy returns information about atoms and bonding between them, MS gives data about molecular and ion/fragment masses, leading to a more complex and laborious data analysing. This problem can be overcome with the construction of structural databases, which allow an easier and quicker data annotation, as well for NMR spectroscopy. NMR has yet a relevant advantage in the biological studies – the ability of studying them in their natural media. Solid-state NMR can be used to observe flavonoid behaviour in tissues, while solution NMR is useful to determine ligand-acceptor interactions though 2D-NMR experiments such as NOESY, being this one of the greatest tools to undergo protein activity inhibition that can be in the base of the flavonoid biological activity.

It is fair to conclude that although MS methods rarely provide a full molecular determination they are, due to their intrinsic characteristics, the best approach to study flavonoid structures, in complement, when possible, with NMR experiments. For faster cruder screenings, UV absorption data can be used to develop appropriate methods to achieve initial flavonoid class identification.

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3

Analytical Methods for Isolation, Separation and Identification of Selected Furanocoumarins in Plant Material

Katarzyna Szewczyk and Anna Bogucka - Kocka
Chair and Department of Pharmaceutical Botany, Medical University of Lublin, Poland

1. Introduction

Coumarins are α–pyrone derivatives synthesized as secondary metabolites in plants. They occur as free compounds or glycosides in plants. They have been isolated from A. Vogel, since 1820, from the tonka beans (*Coumarouna odorata* Aubl. = *Dipteryx odorata* Will.) and they have been synthesized in 1868 from W. H. Perkin, through the famous Perkin reaction (Dewick, 2009).

Furanocoumarins are one of the coumarin derivatives. They can be grouped into the linear type, where the furan ring (dihydro) is attached at C(6) and C(7), and the angular type, carrying the substitution at C(7) and C(8).

The most abundant linear furanocoumarins are psolaren, xanthotoxin, bergapten and isopimpinellin, whereas the angular type is mostly represented by angelicin, sphondin, and pimpinellin. Some structures of furanocoumarins are presented in table 1. As was mentioned for the simple coumarins, numerous minor furanocoumarins have been described in the literature, for example bergamottin (5-geranoxy-psolaren) (Stanley & Vannier, 1967), which has received attention recently as a major grapefruit component interfering with drug metabolism by intestinal CYP3A4 (Bourgaud et al., 2006; Wen et al., 2008).

2. Distribution of furanocoumarins in plants

Linear furanocoumarins (syn. psolarens) are principally distributed in four angiosperm families: Apiaceae (Umbelliferae), Moraceae *(Brosimum, Dorstenia, Fatoua* and *Ficus),* Rutaceae and Leguminose (restricted to Psoralea and Coronilla generae). The angular (dihydro) furanocoumarins are less widely distributed and primarily confined to the Apiaceae and Leguminosae (Berenbaum et al., 1991; Bourgaud et al., 1995).

Moreover, furanocoumarins have been reported from Asteraceae (Compositae), Pittosporaceae, Rosaceae, Solanaceae and Thymelaeaceae (Milesi et al., 2001; Murray et al., 1982). Certain precursors to this group of compounds are found in the Cneoraceae (Murray, 1982).

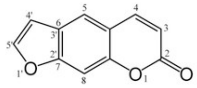
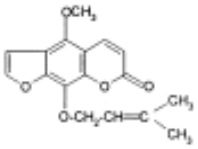
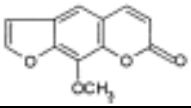
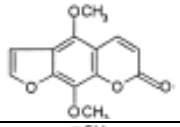
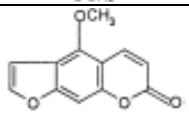
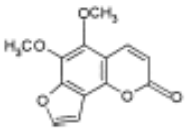
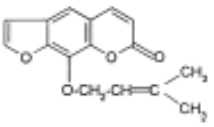
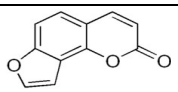
Name of compound	Structure
Psoralen	
Phellopterin	
Xanthotoxin	
Isopimpinellin	
Bergapten	
Pimpinellin	
Imperatorin	
Angelicin	

Table 1. The structure of some furanocoumarins

Coumarins are distributed across different parts of the plants, and they have specific histological locations in the tissues. Within the plant they are most abundant in fruits and roots. However, in flowers and leaves they are evident in fewer quantities. In some plant species coumarins were also found in the bark or stems (Głowniak, 1988).

The amount of particular furanocoumarins depends on the enzymes active in plants secondary metabolism. Plants with similar enzyme profiles contain comparable amount of secondary metabolites that are products of chemical reactions induced by these enzymes. Thus, furanocoumarins' content, in different species, varieties and forms may contribute to their better distinction, and better understanding of the taxonomy of genuses within which they are present.

Diawara et al. (1995) examined the relative distribution of furocoumarins in celery (*Apium graveolens* L. var. *dulce* Miller) plant parts and found that leaves of the outer petioles contained significantly higher levels of the three phototoxic constituents than did other plant parts, followed by leaves of the inner petioles.

On the other hand, levels of furanocoumarins observed in plants grown in the field are higher than those observed in plants grown in laboratory or greenhouse conditions and may fluctuate over the season (Trumbe et al., 1992; Diawara et al., 1995). In most studies, bergapten has been found to occur in highest concentrations, followed by xanthotoxin, but psoralen is often observed only in trace quantities (Trumbe et al., 1990; Trumbe et al., 1992; Diawara et al., 1993). However, other studies have found that xanthotoxin (Beier et al., 1983) or psoralen (Diawara et al., 1993; Trumble et al., 1990) is most abundant (Stanley-Horn, 1999).

Considering the histological location of furanocoumarins in plant tissues, they are arranged differently. For example, celery contains schizogenous canals scattered throughout the pericycle, which are secretory and are thought to extend through the stem and foliage (Maksymowych & Ledbetter, 1986). Furanocoumarins are thought to be restricted to schizogenous canals in seeds of celery (Berenbaum, 1991) and accumulate primarily in petiolar and foliar canals in cow-parsnip, *Heracleum lanatum* Michx. (Apiaceae). However, there is also evidence suggesting that this group of compounds occur in and on the surfaces of tissues as well. A study of several apiaceous and rutaoeous species by Zobel and Brown (1990) revealed that a large proportion of each furocoumarin was located on the leaf surface in most of the plants studied. Furanocoumarins of *Ruta graveolens* L. are present in the epidermal layer of both stems and leaves and in the mesophyll directly below the epidermis, while glands of leaves contain only traces of furanocoumarins. In fact, the cuticular layer contains 15.60% of the psoralens found in leaves (Zobel et al., 1989). The occurrence of bergapten and xanthotoxin in the surface wax of leaves of wild carrot, *Daucus carota* L., a plant containing only trace levels of furanocoumarins has also been reported (Ceska et al., 1986; Stanley-Horn, 1999).

The content of coumarins in plants is conditioned by the degree of the development of the plant and its vegetation stage, too. Concentrations of linear furanocoumarins increase dramatically with plant age between 8 and 18 weeks (Reitz et al., 1997) with a subsequent decline in bergapten concentrations in the last six to eight weeks before harvest (Trumble et al., 1992). Significant decreases in levels of furanocoumarins were also observed both in and on senescing leaves of *Ruta graveolens* (Zobel & Brown, 1991). The content of some furanocoumarins in *Apium graveolens* and *Petroselinum sativum* decreases in summer and in autumn increases (Kohlmünzer, 2010).

3. Biosynthesis of furanocoumarins

The biosynthesis of linear and angular furanocoumarins is still poorly understood at the molecular level. They are produced *via* the shikimic acid biosynthetic pathway beginning with the conversion of phenyloalanine to trans-cinnamic acid by phenylalanine ammonia lyase. Orto-hydroxylation of trans-cinnamic acid yields 2'-hydroxycinnamic acid, which is converted to its cis form, the precursor to coumarin, in the presence of UV light. Alternatively, trans-cinnamic acid may undergo parahydroxylation to yield p-coumaric

acid. P-coumaric acid may undergo 2'–hydroxylation followed by conversion by 4-coumarate: CoAligase to 4-coumaryl CoA. This compound is intermediate in the biosynthesis of both flavonoids and phenylpropanoids, including 7-hydroxycoumarin (umbelliferone). Umbelliferone is the precursor to both the angular and linear furanocoumarins. The production of the latter involves prenylation to form marmesin, followed by oxidative loss of the hydroxypropyl group in marmesin by 'psoralensynthase' to yield psoralen (Berenbaum, 1991; Stanley-Horn, 1999). A second cytochrome P-450-dependent monooxygenase enzyme then cleaves off the hydroxyisopropyl fragment (as acetone) from marmesin to give the furocoumarin psoralen. Psoralen can act as a precursor for the further substituted furanocoumarins bergapten, xanthotoxin and isopimpinellin. On the other hand, angular furanocoumarins, such an angelicin can arise by a similar sequence of reactions, but these involve initial dimethylallylation at the alternative position *ortho* to the phenol (Dewick, 2009).

4. Biological activities of furanocoumarins

Due to their biological activities, furanocoumarins are very interesting compounds and widely investigated. The various biological and pharmacological activities of coumarins, have been known for a long time.

They play the role of phytoalexin in plants (Szakiel, 1991), which can be synthesized as a result of elicitation by microorganisms, insects, fungi as well as abiotic elicitors such as UV radiation, environment pollutants and mechanical breakage. Defensive activity of furanocoumarins consists in their toxicity against phytopathogens (e.g. retardation of DNA synthesis) (Waksmundzka-Hajnos et al., 2004).

Linear furocoumarins can be troublesome to humans since they can cause photosensitization towards UV light, resulting in sunburn or serious blistering. Used medicinally, this effect may be valuable in promoting skin pigmentation and treating psoriasis. Plants containing psoralens have been used internally and externally to promote skin pigmentation and suntanning. Bergamot oil obtained from the peel of *Citrus aurantium* ssp. *bergamia* (Rutaceae) can contain up to 5% bergapten and is frequently used in external suntan preparations. The psoralen absorbs in near UV light and allows this radiation to stimulate formation of melanin pigments (Dewick, 2009).

Methoxsalen (xanthotoxin; 8-methoxypsoralen), a constituent of the fruit of *Ammi majus* (Umbelliferae/Apiaceae), is used medically to facilitate skin repigmentation where severe blemishes exist (vitiligo). An oral dose of methoxsalen is followed by long – wave UV irradiation, though such treatments must be very carefully regulated to minimize the risk of burning, cataract formation, and the possibility of causing skin cancer. The treatment is often referred to as PUVA (psoralen + UV-A). PUVA is also of value in the treatment of psoriasis, a widespread condition characterized by proliferation of skin cells. Similarly, methoxsalen is taken orally, prior to UV treatment. Reaction with psoralens inhibits DNA replication and reduces the rate of cell division. Because of their planar nature, psoralens intercalate into DNA, and this enables a UV – initiated cycloaddition reaction between pyrimidine bases (primarily thymine) in DNA and the furan ring of psoralens. A second cycloaddition can then occur, this time involving the pyrone ring, leading to interstrand cross – linking of the nucleic acid (Dewick, 2009; Żołek et al., 2003).

A troublesome extension of these effects can arise from the handling of plants which contain significant levels of furocoumarins. *Apium graveolens* (= celery; Umbelliferae/Apiaceae) is normally free of such compounds, but fungal infection with the natural parasite *Sclerotinia sclerotiorum* induces the synthesis of furanocoumarins as a response to the infection. Field workers handling these infected plants may become very sensitive to UV light and suffer from a form of sunburn termed photophytodermatitis. Infected parsley (*Petroselinum crispum*) can give similar effects. Handling of rue (*Ruta graveolens;* Rutaceae) or giant hogweed (*Heracleum mantegazzianum*; Umbelliferae/Apiaceae), which naturally contain significant amounts of psoralen, bergapten, and xanthotoxin, can cause similar unpleasant reactions, or more commonly rapid blistering by direct contact with the sap. The giant hogweed can be particularly dangerous. Individuals vary in their sensitivity towards furanocoumarins, some are unaffected, whilst others tend to become sensitized by an initial exposure and then develop the allergic response on subsequent exposures (Dewick, 2009).

Methoxsalen in combination with ultraviolet light is also used for antineoplastic effects and for treating certain skin disorders, including alopecia, cutaneos T-cell lymphoma, excema, lichen planus, mycosis fungoides and psoriaris. A recent report has found that this drug inhibits the enzyme, CYP2A6, which is responsible for the metabolism of nicotine. When 8-methoxypsoralen is taken with oral nicotine, this drug can reduce the number of cigarettes smoked by about one quarter and decrease overall levels of tobacco smoke exposure by almost half in tobacco dependent individuals (Lehr et al., 2003).

Xanthotoxin is used orally or topically in combination with controlled exposure to long wavelength ultraviolet radiation (UVA) or sunlight to repigment vitiliginous skin in patients with idiopathic vitiligo. Many studies have shown that naturally occurring furocoumarins, e.g. imperatorin and isopimpinellin, inhibit P450-mediated enzyme activities *in vitro*. Imperatorin and isopimpinellin have also the potential chemopreventive effects when administered in the diet. The stimulation of melanogenesis by bergapten is related to increased tyrosinase synthesis. In addition, bergapten stimulated TRP-1 synthesis and induced a dose-dependent decrease of DCT activity without modification of protein expression. Osthole could prevent postmenopausal osteoporosis. It can also delay aging, build up strength, enhance immune function, and adjust sex hormone levels (Chen et al., 2007).

Psoralen and bergapten exert their photosensibilising effects through a covalent interaction with DNA triggered by light of a specific wavelength (320-400 nm). The resulting complex blocks the DNA interaction with transcriptases and polymerases, avoiding cell replication. This mechanism consist of three steps, i.e., (1) drug intercalation between DNA nucleotide bases, (2) drug absorption of a UVA photon and covalent bond formation between the furan ring double bond and a thymine base (T2) of the DNA molecule, (3) absorption of a second photon (UVA) and covalent bonding between the lactone ring double bond and another thymine base (T1), which, in the end, results in a psoralen cross-linked DNA (da Silva et al., 2009; Panno et al., 2010; Cardoso et al., 2002). The same effects have been alternatively utilized for the treatment of human lymphoma and of autoimmune diseases through extracorporeal photochemotherapy (Panno et al., 2010).

Panno et al. (2010) investigated the pro-apoptotic effects induced by high doses of bergapten (methoxypsoralen; 5-MOP), in the absence of UV rays, in human breast cancer cells. The same authors examined the effects of bergapten, alone and in combination with UV light, on

the cellular growth of breast tumoral cells. Their study suggested that bergapten alone, or as a photoactivated product, could be used as an active molecule able to counteract effectively the survival and growth of breast hormone-responsive tumors.

Furanocoumarins isolated from fruits of *Heracleum sibiricum* L. inducing apoptosis by forming adducts with DNA. Bogucka – Kocka (2004) reported a visible influence of these compounds on the inhibition of the proliferation and on induction of apoptosis processes in the human HL-60 cell lines. Moreover, compounds isolated from *Angelica dahurica* (Apiaceae) were examined regarding their cytotoxic activity against L1210, HL-60, K562, and B16F10 tumor cells lines using the MMT cell assay. It was discovered that pangelin and oxypeucedanin hydrate acetonide exhibited the most cytotoxic activity against all selected tumor cell lines (Heinrich et al., 2004). Um and co-authors (2010) were isolated four furonocoumarins (bergapten, isopimpinellin, xanthotoxin and imperatorin) from *Glehnia littoralis* F. Schmidt ex Miquel (Apiaceae), which exhibited dose-dependent inhibitory effectson the cell proliferation. Their study demonstrated that *G. littoralis* has potent inhibitory effect on proliferation of HT-29 human colon cancer cells.

In addition, Oxypeucedanine (= prangolarin), which was isolated from *Prangos, Hippomarathrum, Angelica* and *Ferulago* (genera of Apiaceae) and *Ruta* genus of Rutaceae, has pharmacological and biological activities. It was reported to have antiarrhytmic, channel blocer and antiestrogenic activity. Razavi et al. (2010) studied phytotoxic, antibacterial, antifungal, antioxidant and cytotoxic effects of oxypeucedanin. Their results revealed that this compound exhibits considerable phytotoxic activity and might play an allelopathic role for plants. On the other hand, oxypeucedanin exhibits considerable cytotoxicity against Hela cell line (IC_{50} value of 314 μg/ml).

The ethanol extract of the *Cnidii* fructus and coumarins separated from it have growth-inhibitory effects on the tumor cells (Chen et al., 2007).

One of the major bioactive components of the fruits of *Cnidium monnieri* (L.) Cusson, bergapten, possesses antiinflammatory and analgesic activities. However, imperatorin exhibits strong cytotoxic activity on human leukemia, chemopreventive effects on hepatitis and skin tumor, and antiinflammatory activity (Li & Chen, 2004).

In addition of bergapten, this plant also contained numbers of others coumarins, such as xanthotoxin, isopimpinellin, bergapten, imperatorin and osthole. These constituents regarded for biological activity of this crude drug, which is used for treatment of pain in female genitalia, impotence and supportive (Chen et al., 2007).

Pharmacological studies have indicated that coumarins such as isoimperatorin, notopterol and bergapten possess anti-inflammatory, analgesic, anti-cancer and anti-coagulant activities (Qian et al., 2007).

Imperatorin (8-isopentenyloxypsoralen; 9-(3-methylbut-2-enyloxy)-7H-furo [3,2-g]chromen-7-one) is a bio-active furanocoumarin isolated e.g. from roots of *Angelica dahurica* and fruits of *Angelica archangelica* (Umbelliferae) (Baek et al., 2000). Experimental evidence indicates that imperatorin irreversibly inactivates γ-aminobutyric acid (GABA)-transaminase (the enzyme responsible for the degradation of GABA) and thus, increases the GABA content in the synaptic clefts of neurons and elevates the inhibitory neurotransmitter GABA level in the brain (Choi et al., 2005; Łuszczki et al., 2007).

Quite recently, it has been documented that imperatorin in a dose-dependent manner increased the threshold for maximal electro-convulsions in mice (Łuszczki et al., 2007a). The time–course and dose–response relationship analyses revealed that the time to peak of the maximum anticonvulsant effect for imperatorin was established at 30 min after its systemie (i.p.) administration in mice (Łuszczki et al., 2007a).

Recently results indicate that imperatorin administered at subthreshold doses enhanced the anticonvulsant effects of carbamazepine, phenytoin and phenobarbital, but not those of valproate against maximal electroshock-induced seizures in mice. It is important to note that the anti-seizure effects of carbamazepine combined with imperatorin were greater than those observed for the combinations of phenobarbital and phenytoin with imperatorin.

The difference in the anti-seizure effects of carbamazepine and phenytoin or phenobarbital in the maximal electroshock seizure test may be explained through pharmacokinetic interaction between imperatorin and carbamazepine. It was found that imperatorin significantly increased total brain carbamazepine concentrations, having no impact on the total brain phenytoin and phenobarbital concentrations in experimental animals. The selectivity in the increase in total brain carbamazepine concentration one can try to explain through the fact that imperatorin probably enhances the penetration of carbamazepine into the brain by modifying the blood–brain barrier permeability. On the other hand, it may be hypothesized that the selective increase in carbamazepine content in the brain tissue resulted from imperatorin-induced inhibition of multi-drug resistance proteins or P-glycoproteins that normal physiological activity is related to the removal of drugs from the brain tissue. Thus, inhibitors of these proteins may contribute to the accumulation of antiepileptic drugs in the brain (Brandt et al., 2006; Łuszczki et al., 2007).

Considering molecular mechanisms of the action of conventional antiepileptic drugs and imperatorin, one can ascertain that imperatorin-induced irreversible inactivation of GABA-transaminase and subsequent increases in GABA content in the brain, as well as, the enhanced GABA-mediated inhibitory neurotransmitter action through the interaction of imperatorin with benzodiazepine receptors. This may exhibit complementary potentials to the anticonvulsant activity of carbamazepine, phenytoin and phenobarbital shown in experimental animals testing. Noteworthy, the main anticonvulsant mechanism of the action of carbamazepine and phenytoin is related to the blockade of Na+ channels in certain neurons (Łuszczki et al., 2007).

It is interesting to note that imperatorin did not potentiate the protective action of valproate against maximal electroshock-induced seizures. This apparent lack of effects of imperatorin on the antiseizure action of valproate, one can try to explain by the fact that valproate possesses a number of variousmechanisms of action that contribute to its anti-seizure activity in both rodents and humans (Łuszczki et al., 2007).

The evaluation of acute adverse effect potentials is exhibited within combinations of imperatorin with conventional antiepileptic drugs revealing that the combinations did not disturb long-term memory, impair motor co-ordination, or change neuromuscular grip-strength in experimental animals. Therefore, the investigated combinations seem to be secure and well tolerated by experimental animals (Łuszczki et al., 2007).

It was shown that imperatorin enhances the protective action of carbamazepine, phenytoin and phenobarbital, but not that of valproate against maxima electroshock-induced seizures

in mice. The lack of any changes in total brain phenytoin and phenobarbital concentrations suggested that the observed interactions of imperatorin with phenytoin and phenobarbital were pharmacodynamic in nature and thus, they deserve more attention from a preclinical viewpoint. If the results from the study of Łuszczki and co-authors (2007) can be extrapolated to clinical settings, a novel therapeutic option in the management of epilepsy may be created for epileptic patients.

Piao et al. (2004) assayed eleven furanocoumarins, isolated from *Angelica dahuricae* to determine its antioxidant activities. 9-hydroxy-4-methoxypsoralen inhibited DPPH formation by 50% at a concentration of 6.1 μg/ml (IC_{50}), and alloisoimperatorin 9.4 μg/ml, thus the other nine furanocoumarins (oxypeucedanin hydrate, byakangelicol, pabulenol, neobyakangelicol, byakangelicin, oxypeucedanin, imperatorin, phellotorin, and isoimperatorin), with an IC_{50} values higher than 200 μg/ml, showed only a little DPPH radical-scavenging activities.

Tosun et al. (2008) evaluated the anticonvulsant activity of the furanocoumarins among others compounds, obtained from the fruits of *Heracleum crenatifolium*. This activity was estimated against maximal electroshock seizures induced in mice. Among analyzed compounds, bergapten showed significant anticonvulsant activity.

Osthole, a coumarin derivative extracted from many plants, such as *Cnidium monnieri* and *Angelica pubescens*, has been showed to exhibit estrogen-like effects and prevent postmenopausal osteoporosis in overiectomized rats. The latest research suggested that this compound can alleviate hyperglycemia and could be potentially developed into a novel drug for treatment of diabetes mellitus (Liang et al., 2009).

Tang et al. (2008) have reported that imperatorin and bergapten induce osteoblast differentiation and maturation in primary osteoblasts. These compounds increased also BMP-2 (bone morphogenetic protein type 2) expression via p38 and ERK-dependent (extracellular signal-regulated protein) pathways. Long–term administration of imperatorin and bergapten into the tibia of young rats also increased the protein level of BMP-2 and bone volume of secondary spongiosa.

However, the toxic effects of furanocoumarins are also well known. Da Silva et al. (2009) at computational analysis of psoralen, bergapten and their predicted metabolites revealed the presence of six toxicophoric groups related to carcinogenicity, mutagenicity, photoallergenicity, hepatotoxicity and skin sensitization.

Numerous studies have indicated that furanocoumarins are carcinogenic, and their ability to intercalate into DNA in the presence of long wave UV light accounts for their mutagenicity. Linear furocoumarins have been shown to exhibit varying levels of phototoxicity. It must be stated that with isopimpinellin, it results in having the least photosensitizing activity (Lehr et al., 2003).

Moreover, coumarin derivatives in high doses can produce significant side effects. They may induce headaches, nausea, vomiting, sleepiness, and in extreme cases, serious liver damage with potential hemorrhages as a result of hypoprothrominemia (Lozhkin & Sakanyan, 2006).

5. Analytical methods of furanocoumarins isolation

5.1 Extraction from plant material

As furanocoumarins have wide applications in biology and have many therapeutic activities, the study of isolation and identification of these compounds is very important. In this part of our work, review of possible methods of isolation of furanocoumarins will be described as follows.

Coumarins typically appear as colorless or yellow crystalline substances, well soluble in organic solvents (chloroform, diethyl ether, ethyl alcohol), as well as in fats and fatty oils. Coumarin and its derivatives exhibit sublimation on heating to 100˚C (Lozhkin & Sakanyan, 2006).

In this process of quantitative analysis of plant secondary metabolites, preliminary treatment of the plant materials is one of the most time-consuming steps. The first problem is the extraction of the compounds from the plant material – usually performed by liquid – solid extraction (LSE).

In research of the content of pharmacologically active compounds in medicinal plants, the routine procedure of extraction from plant tissues is usually applied. The extraction from plant material is frequently carried out by means of "classic" solvent-based procedures, in Soxhlet apparatus, or more simply, in laboratory flask at the temperature of the solvent's boiling under reflux (de Castro & da Silva, 1997; Saim et al., 1997). The imperfection of the time and solvent-consuming methods consists of poor penetration of the tissues by the solvent and also possible destruction of thermolabile compounds. Advantages of conventional extraction methods result from basic, inexpensive and simple equipment to operate. In the Soxhlet extraction, the sample is repeatedly contacted with fresh portions of the solvent in relatively high temperature and with no filtration required after the leaching step (de Castro & da Silva, 1997; de Castro & Garcia-Ayuso, 1998). Recently, modern alternative extraction methods, applied in the environmental analysis and in phyto-chemistry, are sometimes reported: (1) ultrasonification (USAE) (maceration in ultrasonic bath at various temperatures) (de Castro & Garcia-Ayuso, 1998; Court et al. 1996; Saim et al., 1997); (2) microwave-assisted solvent extraction in closed and open systems (MASE) (de Castro & Garcia-Ayuso, 1998; Saim et al., 1997); (3) accelerated solvent extraction (ASE) (called also PLE, pressurized solvent extraction) (Boselli et al., 2001; de Castro & Garcia-Ayuso, 1998; Ong et al., 2000; Papagiannopoulos et al., 2002; Saim et al., 1997); and (4) supercritical fluid extraction (SFE) (Saim et al., 1997). The above methods give better penetration of solvents into plant tissues or other solid matrices that are rapid and solvent saving. ASE apart from this advantage is dynamic, fast and also enables automatization of extraction and analysis procedures (Waksmundzka-Hajnos et al., 2004; Waksmundzka-Hajnos et al., 2007).

Coumarins are usually isolated from plants by extraction with solvents such as ethanol, methanol, benzene, chloroform, diethyl and petroleum ethers, or their combinations (Lozhkin & Sakanyan, 2006). The most exhaustive extraction of coumarins is achieved with ethanol and its aqueous solutions, either in cold or on heating. The total dense extract obtained after the evaporation of extractant is purified by treatment with chloroform and diethyl or petroleum ethers (Lozhkin & Sakanyan, 2006).

Petroleum ether is the extractant usually used in selective extraction of furanocoumarin fraction from plant tissues (Głowniak, 1988), whereas more polar coumarins—hydroxyderivatives are extracted with methanol. Methanol, used after petroleum ether on the same plant material, extracts more hydrophylic coumarins, but also the residual of furanocoumarins.

Historically, exhaustive extraction with different solvents, which can be performed in Soxhlet apparatus, proved to be the most accurate method of isolation of these groups of compound (Głowniak, 1988; Hadacek et al., 1994). The extraction of the same plant material is usually continued with methanol. For example, peucedanin was successfully isolated using this type of extraction with methanol (Lozhkin & Sakanyan, 2006).

Waksmundzka-Hajnos et al. (2004) compared methods of extraction of furanocoumarins. Some of furanocoumarins from *Pastinaca sativa* fruits were extracted using exhaustive extraction with petroleum ether in Soxhlet apparatus, ultrasonification (USAE), accelerated solvent extraction (ASE) and microwave-assisted solvent extraction (MASE).

USAE was performed with petroleum ether in ultrasonic bath at an ambient temperature of 20˚C or at a temperature of 60˚C for 30 min three times.

In the ASE method, the plant material was mixed with neutral glass and placed into a stainless steel extraction cell. The application of neutral glass, playing the role of dispersion agent, is recommended to reduce the volume of the solvent used for the extraction (ASE 200, 1995). This extraction was performed with pure methanol or petroleum ether at the same pressure (60 bar).

MASE was also used in the isolation of furocoumarin fractions performing with 80% methanol in a water bath using a two-step extraction with results of 40% generator power during 1 min and by 60% generator power during 30 mins in open and closed systems.

In most cases of the Waksmundzka-Hajnos et al. (2004) experiment, exhaustive extraction in Soxhlet apparatus indicates low yields of furanocoumarins. For example, the use of ultrasonification at 60˚C gives, in most cases a higher yield than the exhaustive Soxhlet method. In some cases, this method gives the highest yield of extraction (for xanthotoxin and for isopimpinellin) in comparison to all methods used in experimentation. Also, the use of ASE gives, in most cases, higher yields than the Soxhlet extraction (compare yield of extraction of isopimpinellin, bergapten, imperatorin and phellopterin). In case of bergapten, imperatorin, and phellopterin the yield of extraction by ASE was highest in comparison to all extraction methods used in experiments.

Microwave-assisted solvent extraction gives fair extraction yield for more polar furanocoumarins, probably because of the necessary use of more polar extractant (80% MeOH in water). From the gathered data, it is seen that the extraction yield of phellopterin and imperatorin in pressurized MASE is distinctly lower than in open systems. It shows that in a closed system, the extracted compounds were changed by microwaves. Hence, pressurised MASE cannot be recommended as a leaching method of furanocoumarin fraction (Waksmundzka-Hajnos et al., 2004).

These results are similar to those obtained from the same authors in previous investigations, in which they isolated furanocoumarins from *Archangelica officinalis* fruits. This study indicated the highest yield of psoralens by ASE, using methanol or petroleum ether as the extractant. It was also reported that microwave-assisted solvent extraction in the closed system probably causing the change of analytes (Waksmundzka-Hajnos et al., 2004a).

Soxhlet extraction, ultrasound-assisted extraction and microwaves-assisted extraction in the closed system have been investigated to determine the content of coumarins in flowering tops of *Melilotus officinalis*. Soxhlet extraction was performed in a Soxhlet apparatus equipped with cellulose extraction thimbles. Extraction was performed with ethanol (85˚C). Ultrasound-assisted extraction was conducted with 50% (v/v) aq. ethanol, in an ultrasonic bath, and MASE with 50% (v/v) aq. ethanol was performed using a closed-vessel system (Martino et al., 2006).

Soxhlet extraction was used in the isolation of oxypeucedanin from *Prangos uloptera*. Dried and powdered leaves were extracted with n-hexane, dichloromethane and methanol (Razavi et al., 2010).

Celeghini et al. (2001) studied the extraction conditions for coumarin analysis in hydroalcoholic extracts of *Mikania glomerata* Spreng leaves. Maceration, maceration under sonication, infusion and supercritical fluid extraction (SFE) were compared. In SFE method, the solvent extraction system was pressurized in the high pressure vessel with the aid of a nitrogen cylinder. Several solvent mixtures were used including CO_2:EtOH (95:5), (90:10), (85:15) and CO_2:EtOH:H_2O (95:2.5:2.5). The experiment was conducted at the same pressure and temperature. The evaluation of these methods showed that maceration under sonication had the best results.

Kozyra & Głowniak (2006) examined the influence of using solvent in the isolation of furanocoumarins. They carried out extraction techniques with different eluents such n-heptane, dichloromethane and methanol. These extractions were performed on a water bath with boiling eluent and on an ultrasonic bath, for 12 and 24 hrs. The more efficient for bergapten was extraction with dichloromethane.

In another study, six solvents (n-hexane, chloroform, ethyl acetate, ethanol, acetonitrile and water) were used to extract *Cnidii Fructus* in order to evaluate their efficiency in extracting osthole. A comparative evaluation showed that aqueous alcoholic solvent was the most efficient solvent (100%) (Yu et al., 2002).

The furanocoumarin determination from air-dried plant material was also performed using 75% methanol in an ultrasonic ice–water bath (Yang et al., 2010), with 100% methanol (Cardoso et al., 2000; Ojala, 2000), 70% methanol (Chen & Sheu, 1995), with hot (70˚C) pure methanol on a water bath (Bartnik & Głowniak, 2007), with pure ethanol in heated reflux (Yu et al., 2002), with 95% ethanol at 80˚C (Wang et al., 2007; Zheng et al., 2010), with acetone at room temperature (Taniguchi et al., 2011), with ether at 40˚C (Liu et al., 2004a), with dichloromethane at room temperature (Um et al., 2010), with petroleum ether at room temperature (Tosun et al., 2008), with chloroform in a sonic bath (Cardoso et al., 2000).

The extraction with all solvents was usually done 2–5 times, obtaining solutions that were filtered and evaporated under reduced pressure. Frequently, residuals after methanol/ethanol extractions were suspended in water and portioned a few times with chloroform or petroleum ether (Wang et al., 2007; Zheng et al., 2010).

5.2 Sample purification

The next step in sample preparations is the purification of the crude extract. Plant extracts contain much ballast material, both non-polar (chlorophylls, waxes) and polar such as

tannins or sugars. Most often liquid-liquid extraction (LLE) is used, which takes advantages of solubility differences of hydrophobic substances, which have affinity for non-polar solvents, and hydrophobic substances, which have an affinity for aqueous solutions. Although the analyses can be easily obtained by evaporation of the solvent, the method has many disadvantages – for example emulsions can be formed and the process is time-consuming. Purification can also be achieved by solid-phase-extraction (SPE). This method uses a variety of adsorbents and ion-exchangers and is widely used for a variety of purposes (Fritz & Macha, 2000; Hennion, 1999; Nilsson, 2000; Snyder et al., 1997; Waksmundzka-Hajnos et al., 2007).

The SPE method is very often used in sample pre-treatment for HPLC. This method has been developed for the purification of furanocoumarins from *Peucedanum tautaricum* Bieb. In the first step, aqueous methanol (50%; v/v) solutions of the samples were passed through conditioned microcolumns to adsorb furanocoumarins on the adsorbent bed. The microcolumns were washed with 50% methanol (Zgórka & Głowniak, 1999), and the compounds of interest group were separated from fatty components and chlorophyll by use of SPE microcolumns (LiChrolut RP-18 E; 500 mg, 3 mL). In the next step, the absorbed furanocoumarins were eluted at a flow-rate of 0.5 mL min^{-1} with 80% methanol into vials previously calibrated with a pipette (Bartnik & Głowniak, 2007).

Sidwa-Gorycka et al. (2003) used SPE for purification furanocoumaric fractions obtained from *Ammi majus* L. and *Ruta graveolens* L. methanolic (30%) extracts. They were loaded into octadecyl-SPE microcolumns activated previously with 100% methanol, followed by the selective elution of compounds. The cartridges were washed with 20 ml of 60% methanol to elute the coumarins. The eluting solvents were passed through the sorbent beds at a flow rate of 0.5 ml min^{-1}.

In addition, the SPE has been developed for purification of furanocoumarin fractions from creams and pomades. The obtained samples were cleaned-up using two methods. Each extracted sample was re-dissolved in chloroform and fractionated on cartridges, which were previously conditioned with chloroform and sequentially eluted with chloroform (first fraction), chloroform:methanol (90:10; v/v) (second fraction, furanocoumarins), chloroform:methanol (1:1; v/v) (third fraction) and methanol (fourth fraction). Next, each sample extracted above was re-dissolved in methanol in a sonic bath and fractionated on cartridges, which were previously conditioned with methanol and sequentially eluted with methanol (first fraction), methanol:chloroform (80:20; v/v) (second fraction, furanocoumarins), methanol:chloroform (1:1; v/v) (third fraction) and chloroform (fourth fraction). All fractions were evaporated to dryness in a stream of nitrogen (Cardoso et al., 2000).

5.3 Chromatographic methods in the analysis of furanocoumarins

5.3.1 Column Chromatography (CC)

The good results for purification, separation of the total furanocoumarins and the isolation of individual compounds give column chromatography (CC) a significant advantage of the use of various sorbents and solvent systems.

Furanocoumarins can be fractionated on an aluminum oxide column eluted with petroleum ether, petroleum ether-chloroform (2:1), chloroform, and chloroform-ethanol (9:1; 4:1; 2:1)

mixtures or on silica gel column eluted sequentially with hexane-chloroform and chloroform-ethanol systems with increased proportion of a more hydrophilic component (Lozhkin & Sakanyan, 2006).

Separation of the psoralens from *Heracleum sibiricum* L. (Apiaceae) fruits was performed by gravitation column chromatography. Glass columns were filled with silica gel (230-400 mesh) and run, under UV-lamp control, in the following eluents: 1) benzene-ethyl acetate; mixtures of increasing polarity (12.5 to 77.5%); 2) benzene-ethyl acetate (17:3); 3) benzene-chloroform-ethyl acetate (1:1, v/v, 5%). Chromatographically pure clean compounds, in this study, were crystallized from 96% ethanol (Bogucka-Kocka, 1999).

In another investigation, the coumarin mixture from fruits of *Heracleum crenatifolium* was subjected to CC on silica gel and eluted successively with an n-hexane-ethyl acetate solvent system, with increasing polarity (99:1 to 80:20). The collected fractions were applied to preparative-TLC on silica gel plates and pure furanocoumarins were obtained. After chromatography with the use of n-hexane-ethyl acetate (3:1), isobergapten and pimpinellin were obtained. Fractions, which were chromatographed with n-hexane-dichloromethane-ethyl acetate (4:4:2) resulted in a production of bergapten, and fractions, after chromatography using toluene-ethyl acetate (9:1), resulted in a production of yielded isopimpinellin, sphondin and byak-angelicol (Tosun et al., 2008).

On silica-gel column chromatography was also subjected to chloroform residue from roots of *Angelica dahurica*. The furanocoumarins were eluted stepwise with petroleum ether-acetone mixtures (Wang et al., 2007).

Similar techniques were performed for furanocoumarins from the roots *Angelicae dahuricae*. The methylene chloride soluble was chromatographed using column chromatography over silica gel. In this study, a stepwise gradient solution with hexane-ethyl acetate (5:1 to 0:1) was used. Repeated column chromatography of obtained fractions produced an isoimperatorin, imperatorin, oxypeucedanin, phellotorin, byakangelicol, neobyakangelicol, alloisoimperatorin, pabulenol, byakangelicin, and 9-hydroxy-4-methoxypsoralen (Piao et al., 2004).

A vacuum liquid chromatography on silica gel was developed for the isolation of oxypeucedanin from the leaves of *Prangos uloptera.* Hexane extract was subjected, starting with 100% hexane, followed by step gradient of ethyl acetate mixtures (1:99; 5:95; 10:90; 20:80; 40:60; 60:40; 80:20; 100) and finally methanol. The obtained fractions were purified by preparative-TLC on silica gel using $(CH_3)_2CO$-$CHCl_3$, 5:95 as the mobile phase to yield oxypeucedanine (Razavi et al., 2010).

The CC technique was used to separate furanocoumarins from roots of several *Dorstenia* species. The afforded hexane residues were chromatographed on silica gel (230-400 mesh) eluting with hexane-chloroform mixtures (1:1) (gives psoralen) or with hexane-ethyl acetate mixtures of an increasing polarity to give bergapten, 4-[3-(4,5-Dihydro-5.5-dimethyl-4-oxo-2-furanyl)-butoxy]=7H-furo[3,2-g][1]benzopyran-7-one, psoralen and 7-hydroxycoumarin. The chloroform extracts were eluted using hexane-chloroform mixtures to give psoralen, 7-hydroxycoumarin and 4-[3-(4.5-Dihydro-5.5-dimethyl-4-oxo-2-furanyl)-butoxy]=7H-furo[3.2-g][1]benzopyran-7-one or hexane-ethyl acetate of increasing polarity to give psoralen, psoralen dimer and 7-hydroxycoumarin. The polar fractions from the methanol

extract were acetylated using pyridine and Ac_2O to give (2'S, 1''S)-2.3-dihydro-2(2-acetoxy-1-hydroxymethylethyl)-7H-furo [3.2-g][1]benzopyran-7-one (Rojas-Lima et al., 1999).

Another useful adsorbent for column chromatography is Florisil (100-200 mesh), which was used to fractionate furanocoumarins obtained from fruits of *Peucedanum alsaticum* L. and *P. cervaria* (L.) Lap. Concentrated petroleum ether extracts were fractionated on this sorbent with a dichloromethane-ethyl acetate (0-50%) gradient, then ethyl acetate and methanol as mobile phases. After CC separation, the fractions richest in coumarins were analyzed by preparative-TLC on silica gel. Separated zones of selected furocoumarins were eluted from the plates (Skalicka-Woźniak et al., 2009).

The Florisil was also used in an investigation performed by Suzuki et al. (1979). Bergamot oil was eluted on this column with methylene chloride and ethyl acetate. The ethyl acetate fractions were re-chromatographed with methylene chloride. The obtained residue was analyzed by preparative-TLC on silica gel using cyclohexane-tetrahydrofuran (1:1) as eluent. The bergapten zone was scraped and eluted with acetone.

Isolation of the furanocoumarins from grapefruit juice was accomplished by preparative thin layer chromatography. The obtained fractions were applied to tapered silica gel GF TLC plates with a fluorescent indicator. Resolution of compounds was accomplished by using solvent systems consisting of hexane:ethyl acetate (3:1 to 2:3; v/v), chloroform, chloroform/methanol (95:5), and benzene: acetone (9:1). The zones containing furanocoumarins were scraped and extracted with acetone (Manthey et al., 2006).

5.3.2 Thin Layer Chromatography (TLC)

The physicochemical properties of coumarins depend upon their chemical structure, specifically, the presence and position of functional hydroxy or methoxy groups, and methyl or other alkyl chains. As a result of these differences, group separation of the all groups of coumarins does not cause any difficulties (Jerzmanowska, 1967; Waksmundzka-Hajnos et al., 2006). Separation of individual compounds in each group – structural analogs, i.e. closely related compounds – is, however, a difficult task.

Several analytical methods for the quality control of furanocoumarins in plant materials, such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), high performance liquid chromatography–mass spectrometry (HPLC–MS), high-speed counter-current chromatography (HSCCC), gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), capillary electrophoresis (CE)), and pressurized capillary electrochromatography (pCEC), has been reported (Chen et al., 2009; Wang et et., 2007).

The oldest publications recommended one- or two- thin layer chromatography for separation and identification of furanocoumarins. This method provides a quite rapid separation of components in a sample mixture. Fractions obtained from column chromatography were usually checked with the use of TLC technique.

Several adsorbents have been applied for the chromatographic analysis of furanocoumarins, e.g. silica gel, C18 layers, alumina, poliamide, Florisil, etc. (Cieśla & Waksmundzka-Hajnos, 2009). Analyzed fractions of studied compounds are eluted using several solvent systems. Borkowski (1973) proposed the following eluents: 1) benzene-acetone (90:10, v/v); 2) toluene-acetone (95:5, v/v); 3) benzene-ethyl acetate (9:1, v/v); 4) benzene-ethylic ether-

methanol-chloroform (20:1:1:1, v/v); 5) chloroform, and 6) ethyl acetate-hexane (25:75, v/v) for analysis of coumarins.

The spots of coumarins on thin-layer and paper chromatograms are usually revealed by UV fluorescence at certain characteristic wavelengths, before or after the treatment with an aqueous-ethanol solution of potassium hydroxide or with ammonia vapor, or using some other color reactions. The fluorescent color does not provide accurate identification of the structure of coumarins; nevertheless, sometimes it is possible to determine the type of functional groups (Celeghini et al., 2001; Lozhkin & Sakanyan, 2006).

Joint TLC – colorimetric methods based on the azo-addition reaction with TLC separation on an aluminum oxide layer eluted in the hexane – benzene – methanol (5:4:1) system were developed for the quantitative determination of peucedanin in *Peucedanum morrissonii* (Bess.) and for the analysis of beroxan, pastinacin, and psoralen preparations (Lozhkin & Sakanyan, 2006). Colorimetric determination of xanthotoxin, imperatorin, and bergapten in *Ammi majus* (L.) fruits can be performed after TLC separation on silica gel impregnated with formamide and eluted in dibutyl ether. In order to determine psoralen alone and together with bergapten in *Ficus carica* (L.) leaves, the extract was purified from ballast substances and chromatographed in a thin layer of aluminum oxide in diethyl ether (Lozhkin & Sakanyan, 2006).

Thin layer chromatographic analyses were made by Celeghini and co-authors (2001) on silica gel 60G. As eluent a mixture of toluene:ethyl ether (1:1) saturated with 10% acetic acid was used. The plates were sprayed with an ethanolic solution (5% v/v) of KOH and examined under UV light at 366 nm.

In the other investigation, purification of the eluted furanocoumarins from leaves of *Conium maculatum* was also carried out on TLC plates (Si gel 60, f-254). The chromatograms were developed at room temperature using one of the following solvent systems: chloroform:ethyl acetate (2:1), or chloroform, or toluene:ethyl acetate (1:1) (Al-Barwani & Eltayeb, 2004).

Bogucka-Kocka (1999) analyzed furanocoumarins in fruits of *Heracleum sibiricum* L., after silica gel column chromatography, using 2D-TLC. Two-dimensional thin-layer chromatography was conducted of silica gel plates and run in the following phases: 1) benzene-chloroform-acetonitrile (1:1, v/v, 5%) or 2) benzene-chloroform-ethyl acetate (1:1, v/v, 5%) (first direction); 3) benzene-ethyl acetate (1:1) (second direction). The chromatograms were analyzed using UV light and daylight, after spaying with one of the derivativers: 1) 0.5% I dissolvent in KI; 2) Dragendorf's reagent or 25% $SbCl_5$ in CCl_4.

Unfortunately, in furocoumarin' group, these substances have comparable polarity and similar chemical structures. As a result, multi-dimensional separations are required in such cases.

Thin-layer chromatography gives the possibility of performing multi-dimensional separation – two-dimensional separation with the use of the same stationary phase, with different mobile phases (Gadzikowska et al., 2005; Härmäla et al., 1990; Waksmundzka-Hajnos et al., 2006), or by using a stationary phase gradient (Glensk et al., 2002; Waksmundzka-Hajnos et al., 2006). In TLC, there are almost no limits as far as mobile phases are concerned, because they can be easily evaporated from the layer after the

development in the first dimension. Both methods, use of the same layer and different mobile phases or two different layers developed with two mobile phases, make use of different selectivity to achieve complete separation in the two-dimensional process. The largest differences are obtained with a normalphase system, with an adsorption mechanism of separation, and a reversed-phase system, with a partition mechanism of separation, are applied for two-dimensional separations (Nyiredy, 2001). Two-dimensional thin-layer chromatography with adsorbent gradient is an effective method for the separation of large group of substances present in natural mixtures, e.g. plant extracts.

Silica gel is the most popular adsorbent, thus it has been widely used in different chromatographic methods. However, in case of two-dimensional separations of coumarins, it has been rarely applied as it is difficult to select solvent systems which are complementary in selectivity. Härmälä et al. (1990) proposed a very interesting method for the separation of 16 coumarins from the genus *Angelica* with the use of silica gel as an adsorbent. The application of two-dimensional over-pressured layer chromatography enabled complete resolution of the analyzed substances. The authors described a very useful procedure of choosing complementary systems that can be applied in the analysis of complex mixtures. It turned out that the systems, I direction – 100% $CHCl_3$ and II direction – AcOEt/*n*-hexane (30:70, v/v) provided excellent separation of all coumarins, although having only the fourth poorest correlation value.

Due to the possibility of the application of normal- and reversed – phase systems, polar bonded phases have been often a choice for two-dimensional separations. In the case of coumarins, the use of diol- and cyanopropyl-silica have been reported.

Waksmundzka-Hajnos et al. (2006) reported the use of diol-silica for the separation of 10 furanocoumarin standards. Firstly, the compounds were chromatographed with the use of 100% diisopropyl ether (double development), then in the perpendicular direction: 10% MeOH/H_2O (v/v) containing 1% HCOOH. The use of the first direction eluent caused the separation of analyzed substances into three main groups, which is useful for group separation of natural mixtures of coumarins. Chromatography in reversed-phase system enabled the complete resolution of all tested standards. The disadvantage of the applied reversed-phase system is the fact that it has low efficiency, and most of the substances, especially those containing hydroxyl groups are tailing. Diol-silica is similar in its properties to deactivated silica, thus the application of aqueous eluent may be responsible for tailing, which was only slightly reduced after the addition of formic acid.

Better results were obtained after the application of CN-silica. In this case, coumarin standards were firstly chromatographed with the use of normal-phase, then in reversed-phase system. The plate was triple developed in the first direction to improve separation of strongly retained polar coumarins.

The authors also investigated the use of multiphase plates for identification purposes. Coumarins were firstly chromatographed on a RP-18W strip with 55% MeOH/H_2O (v/v), and then in a perpendicular direction they were triple-developed with: 35% AcOEt/*n*-heptane (v/v). The use of reversed-phase system caused the separation of investigated coumarins into two groups: coumarins containing hydroxyl group, and furanocoumarins. The separation, according to the differences in polarity, is even greater than that observed on diol-silica. This system was then applied for separation of the furanocoumarin fraction

from fruits of *H. sibiricum*, where seven compounds were identified in the extract (Cieśla & Waksmundzka-Hajnos, 2009; Waksmundzka-Hajnos et al., 2006).

The use of graft thin-layer chromatography of coumarins was also reported (Cieśla et al., 2008; Cieśla et al., 2008a; Cieśla et al., 2008b). The authors applied two combinations of adsorbents: silica + RP-18W, and CN-silica + silica gel. In the first stage of this experiment, plates pre-coated with CN-silica were developed in one dimension by unidimensional multiple development. The same mobile phase (35% ethyl acetate in n-heptane) was used, over the same distance, and the same direction of the development. Plates were triple-developed with careful drying of the plate after each run. Unidimensional multiple development (UMD) results in increased resolution of neighboring spots (Poole et al., 1989). After chromatography the plates were linearly scanned at 366 nm with slit dimensions 5 mm × 0.2 mm. This chromatographic system was not suitable for separation of structural analogs. Isopimpinellin and byacangelicol are coeluted and phellopterin and bergapten also have very similar retention behavior. The isopimpinellin and byacangelicol molecules have two medium polarity groups in positions 5 and 8, which have similar physicochemical properties. Therefore, it was also easily noticeable that different non-polar substituents did not cause significant difference in retention behavior. Compounds with polar substituents – hydroxyl groups in simple coumarins are more strongly retained on CN-silica layer in normal-phase systems.

When other systems, for example silica with AcOEt–n-heptane and RP 18W with 55% MeOH in water, were used only partial separation of standards was achieved. This results from the similar structures and physicochemical properties of the compounds. On silica layers only polar aesculetin and umbelliferone are more strongly retained. Phellopterin with a long chain in the 8 position (with a shielding effect on neighboring oxygen) is weakly retained. These differences cause the aforementioned coumarins to be completely separated from other standards. Byacangelicol and umbelliferone, and bergapten, isopimpinellin, and xanthotoxin, with only slight differences in number and position of medium-polarity methoxy groups, are eluted together. More significant resolution of the investigated compounds was obtained on RP-18 plates, eluted with aqueous mobile phases. The differences in number, length, and position of medium-polarity and non-polar substituents cause differences in retention behavior of the analytes. These differences result in good separation of bergapten, xanthotoxin, and phellopterin by reversed-phase systems.

In the next step Cieśla et al. (2008b) investigated the search for orthogonal systems, which would ensure better separation selectivity for the coumarins, was conducted. To achieve this, graft TLC, with two distinct layers, was applied. The authors experimentally chose two pairs of orthogonal TLC systems:

- first dimension, CN-silica with 30% ACN + H_2O (three developments); second dimension, SiO_2 with 35% AcOEt + n-heptane (three developments);
- first dimension, SiO_2 with 35% AcOEt + n-heptane (three developments); second dimension, RP-18 with 55% MeOH + H_2O.

An application of multiple development technique (UMD) in the first dimension results in partly separated spots, which are transferred to the second layer with methanol. Use of methanol causes narrowing of starting bands, similarly to the effect of a preconcentrating zone. The preconcentration is responsible for symmetric and well separated spots being

obtained after development of the plate in the second dimension. This makes the densitometric estimation easier.

In the last step of Cieśla and co-authors (2008b) investigations, the separation of furanocoumarin fractions from *Archangelica officinalis, Heracleum sphondylium,* and *Pastinaca sativa* fruits was performed by the use of grafted plates SiO_2 with RP-18W and CN with SiO_2, with appropriate mobile phases. The identity of the extract components was confirmed by comparing retardation factors and UV spectra with the R_f values and spectra obtained for the standards.

Graft TLC in orthogonal systems characterized by different separation selectivity enables complete separation of structural analogs such as furanocoumarins. The use of two different TLC systems enables complete separation and identification of some furanocoumarins present in extracts obtained from *Archangelica officinalis, Heracleum sphondylium*, and *Pastinaca sativa* fruits (Cieśla et al., 2008b).

The graft-TLC system silica + RP-18W were successfully applied for construction of chromatographic fingerprints of different plants from the *Heracleum* genus.

Two-dimensional chromatography has also been applied for quantitative analysis of furanocoumarins in plant extracts (Cieśla et al., 2008b). In order to obtain reproducible results, all investigated compounds should be completely separated. Graft-TLC with the use of adsorbents silica + RP-18W was proven to be the most suitable for quantitative analysis. Resolution of compounds was insufficient in case of 2D-TLC on one adsorbent (CN-silica), as the standards had to be divided into two separate groups for an accurate estimation of peak surface area.

Quantitative analysis is difficult to perform after two-dimensional chromatographic run, as densitometers are not adjusted to scan two-dimensional chromatograms. This problem may be overcome if small steps between scans are used. In the proposed method, the authors scanned the plate with the slit of a dimension 5 mm×0.2 mm, operated at λ= 366 nm, obtaining 36 tracks that were not overlapping. This wavelength was chosen to get rid of intensive baseline noise, observed at lower wavelengths. Peak areas were measured with the use of the method called "peak approximation" (Cieśla et al., 2008b; Cieśla & Waksmundzka-Hajnos, 2009).

5.3.3 High Performance Liquid Chromatography (HPLC)

Furanocoumarins are also examined by means of high performance liquid chromatography (HPLC). This technique has shown to be a very efficient system for separation of this group of compounds. HPLC methods have been reported for the determination of psoralens in callus cultures, vitro culture, serum, dermis, plants, citrus essential oils, phytomedicines, but only the most recently published methods has reported assay validation (Cardoso et al., 2000; Dugo et al., 2000; Markowski & Czapińska, 1997; Pires et al., 2004).

Linear furanocoumarins, such as psoralen, bergapten, xanthotoxin, and isopimpinellin isolated from three varieties of *Apium graveolens* were examined by normal-phase HPLC equipped with a variable wavelength detector set at 250 nm. The mobile phase consisted of a mixture of ethyl acetate (0.1%) and formic acid (0.1%) in chloroform (Waksmundzka-Hajnos & Sherma, 2011).

In most recent applications, reversed-phase HPLC is used to evaluate furanocoumarins quantitatively.

For example, the quantitative analysis of some furanocoumarins from *Pastinaca sativa* fruits was performed by RP-HPLC in system C18/methanol + water in gradient elution. The authors used the following gradient: 0-10 min, 45% MeOH; 10-20 min, 45-55% MeOH; 20-30 min, 55-70% MeOH, and 30-40 min, 70% MeOH in bidistilled water (Waksmundzka-Hajnos et al., 2004).

The determination of two furocoumarins (bergapten and bergamottin) in bergamot fruits, was carried out by the HPLC system equipped with a diode array detector. C18 column and the mobile phase consisted of methanol and 5% (v/v) acetic acid aqueous solution in the following gradient: 5-20% (0-13 min), 20-100% (13-25 min), 100-5% (20-30 min), were used in this investigation (Giannetti et al., 2010).

The optimized HPLC-UV method was used to evaluate the quality of 21 samples of Radix *Angelica dahurica* from different parts of China. Bergapten, imperatorin and cnidilin were separated on C18 column; the mobile phase was 66:34 (v/v) methanol-water (Wang et al., 2007).

The HPLC technique was ensued for analyses of psoralen and bergapten. HPLC separation of the psoralens was performed using a Shimadzu octadecyl Shim-pack CLC-ODS reversed-phase column with a small pre-column containing the same packing. Elution was carried with acetonitrile-water 55:45 (v/v) and detections of the peaks were recording at 223 nm (Cardoso et al., 2002). The same conditions were used for determination of furanocoumarins in three oral solutions by Pires et al. (2004).

A rapid and sensitive reversed-phase HPLC method has been used for the determination of furanocoumarins in methanolic extracts of *Peucedanum tautaricum* Bieb. Compounds were separated on stainless-steel column packed with 5µm particle Hypersil ODS C18. The mobile phase was methanol-water gradient used following: 0-5 mins, isocratic elution with 60% (v/v) methanol; 5–20 mins, linear gradient from 60 to 80% methanol; 20 to 30 mins, linear gradient from 80 to 60% methanol; 30–40 mins isocratic elution with 60% methanol. An acetonitrile–water mobile phase gradient was also used (0–8 mins, isocratic elution with 50% acetonitrile; 8–25 mins, linear gradient from 50 to 70% acetonitrile; 25–28 mins, linear gradient from 70 to 50% acetonitrile; 28–40 mins isocratic elution with 50% acetonitrile) (Bartnik & Głowniak, 2007).

The mobile phase consisted of water with orthophosphoric acid 1:10000 (solvent A), methanol (solvent B) and acetonitrile (solvent C) was used for analysis of coumarins from *Melilotus officinalis* (L.) Pallas. The starting mixture (80% A, 5% B and 15% C) was modified as follows: within 20 mins the mobile phase composition became 65% A, 20% B, 15% C and was kept constant for 10 mins; in the following 10 mins the mixture composition came back to the initial eluting system (Martino et al., 2006).

The search for better conditions for application of HPLC has led to development of UPLC (Ultra Performance Liquid Chromatography), a relatively new liquid chromatography technique enabling faster analysis, consumption of less solvent and better sensitivity. The UPLC method enables a reduction of analysis time by up to a factor of nine compared with conventional HPLC without loss of quality of the analytical data generated. Another very

important advantage is high column efficiency which increases the possibility of compound identification and results in better quantitative analysis. UPLC is more efficient and therefore has greater resolving power than traditional HPLC (Novakova et al., 2006; Skalicka-Woźniak et al., 2009; Wren & Tchelitcheff, 2006).

The quantitative analysis by UPLC was performed for the furocoumarins in *Peucedanum alsaticum* and *P. cervia* (Skalicka-Woźniak et al., 2009). The optimalization of the RP-UPLC separation of the coumarins was achieved by the use of DryLab. The investigation was performed with an Acquity Ultra Performance LC (Waters, Milford, MA, USA) coupled with a DAD detector. Compounds were separated on a stainless-steel column packed with 1.7 μm BEH C18. Two linear mobile phase gradients from 5 to 100% of acetonitrile with gradient times of 10 and 20 min were used. Detection was at 320 nm.

A paper by Desmortreux et al. (2009) reports separation of furocoumarins of essential oils (lemon residue) by supercritical fluid chromatography (SFE). The authors studied many types of stationary phases and the effects of numerous analytical parameters. Amongst the numerous tested columns, good separation of analyzed furanocoumarins was obtained on a pentafluorophenyl (PFP) phase (Discovery HS F5), based on an aromatic ring substituted by five fluorine atoms. The mobile phase used was CO_2-EtOH 90:10 (v/v). Amongst the standard compounds, bergapten was well separated being eluted after the other furocoumarins in the lemon residue sample. The results obtained in this study show that SFC is a perfectly suited method to investigate the psoralens in essential oil composition, because of the great number of compounds separated in a reduced analysis time, and with a very short time for re-equilibration of the system at the end of the gradient analysis. Because of the absence of water in the mobile phase in SFC, the stationary phase can establish more varied interactions than in HPLC, making the stationary phase choice highly significant.

5.3.4 Hyphenated HPLC techniques

A hyphenated, HPLC-TLC procedure for the separation of couamrins, has been proposed by Hawrył et al. (2000). A mixture of 12 coumarins from *Archangelica officinalis* was completely separated as a result of the different selectivities of the two combined chromatographic techniques, RP-HPLC and NP-TLC. Firstly, the analyzed compounds were separated by means of RP-HPLC. The optimal eluent: 60% MeOH in water was chosen with the use of DryLab program. All HPLC fractions were collected, evaporated and finally developed in normal-phase system, on silica gel, with the use of a solvent mixture: 40% AcOEt (v/v) in dichloromethane/heptane (1:1). All fractions were completely separated. The combination of these methods gave successful results, although both methods, if used separately, failed to give good resolution. This procedure may be useful for micropreparative separation of coumarins (Cieśla & Waksmundzka-Hajnos, 2009).

The liquid chromatography coupled with mass spectrometry (LC-MS) technique is becoming increasingly popular, in particular, the introduction of atmospheric pressure chemical ionization (APCI) has dramatically influenced the possibilities for analyzing poorly ionizable compounds. The use of hyphenated techniques such as LC-MS provides great information about the content and nature of constituents of complex natural matrices prior to fractioning and carrying out biological assays. Moreover, MS presents a great advantage not only in its ability to measure accurate ion masses but also in its use in structure elucidation (Chaudhary et al., 1985; Dugo et al., Waksmundzka-Hajnos & Sherma, 2011).

Coumarins can be detected in both positive and negative ion modes. Whereas, the positive ion mode often generates higher yields, the noise level is lower in the negative ion mode, thus improving the quality of the signals. So, preliminary investigations regarding the polarity used are very important.

The main problem of working with LC-MS of natural products is the choice of the ionisation technique. Particle beam (PB) and thermospray (TSP) interfaces are the most commonly used for natural component analysis. Both of them exhibit many drawbacks, such as the difficulty to optimize ionisation conditions and the lack of sensitivity. Electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) techniques, which operate under atmospheric pressure, seem to be very promising. These ionisations differ in the way they generate ions, but show many similarities: both operate at atmospheric pressure, giving molecular weight information and additional structural information. Many classes of compounds can be analyzed by both APCI and ESI. However, ESI is the technique of choice for polar and higher molecular weight compounds, while APCI is suitable for less polar compounds and of lower molecular weight than ESI (Dugo et al., 2000).

A sensitive, specific and rapid LC–MS method has been developed and validated for the simultaneous determination of xanthotoxin (8-methoxypsoralen), psoralen, isoimpinellin (5,8-dimethoxypsoralen) and bergapten (5-methoxypsoralen) in plasma samples from rats after oral administration of *Radix Glehniae* extract using pimpinellin as an internal standard. A chromatographic separation was performed on a C18 column with a mobile phase composed of 1mmol ammonium acetate and methanol (30:70, v/v). The detection was accomplished by multiple-reaction monitoring (MRM), scanning via electrospray ionization (ESI) source operating in the positive ionization mode. The optimized mass transition ion-pairs (m/z) for quantitation were 217.1/202.1 for xanthotoxin, 187.1/131.1 for psoralen, 247.1/217.0 for isoimpinellin, 217.1/202.1 for bergapten, and 247.1/231.1 for pimpinellin (Yang et al., 2010).

A paper by Zheng et al. (2010) reports the quantitation of eleven coumarins including furocoumarins in *Radix Angelicae dahuricae*. By using this HPLC–ESI-MS/MS method, all coumarins were separated and determined within 10 min. These compounds were detected by ESI ionization method and quantified by multiple-reaction monitoring (MRM). The mass spectral conditions were optimized in both positive- and negative-ion modes, and the positive-ion mode was found to be more sensitive. The all coumarins exhibited their quasi-molecular ions $[M^+H]^+$, $[M^+Na]^+$, $[M^+NH_4]^+$, $[M^+K]^+$ and fragment ions $[M^+H-CO]^+$, $[M^+H-C_5H_9O]^+$, $[M^+H-C_5H_8]^+$, $[M^+H-C_5H_8-CO]^+$, $[M^+H-C_5H_8-CO_2]^+$, $[M^+H-CH_3]^+$.

Yang et al. (2010a) proposed a practical method for the characterization of coumarins, i.e. linear furanocoumarins, in *Radix Glehniae* by LC–MS. They described in details over 40 derivatives of psoralens. First, 10 coumarin standards were studied, and mass spectrometry fragmentation patterns and elution time rules for the coumarins were found. Then, an extract of *Radix Glehniae* was analyzed by the combination of two scan modes, i.e., multiple ion monitoring-information-dependent acquisition-enhanced product ionmode (MIM-IDA-EPI) and precursor scan information-dependent acquisition-enhanced product ionmode (PREC-IDA-EPI) on a hybrid triple quadrupole-linear ion trap mass spectrometer. This study has demonstrated the unprecedented advantage of the combination of these two scan modes. The MIM-IDA-EPI mode is sensitive, and no pre-acquisition of MS/MS spectra of

the parent ion is required due to the same precursor ion and product ion. A PREC-IDA-EPI mode was used to provide information on the parent ions, fragment ions and retention times of specified ions so the molecular weights of unknown coumarins and their glycosides could be identified. The information on the fragment ions from the MIM-IDA-EPI mode could be supplemented, and the retention time could be verified. Therefore, the characterization of trace furanocoumarins has become very easy and accurate by the combined use of the two modes and may play an important role in controlling the quality of medicinal herbs.

A high performance liquid chromatography–diode array detection–electrospray ionization tandem mass spectrometry (HPLC/DAD/ESI-MS^n) method was used for the chromatographic fingerprint analysis and characterization of furocoumarins in the roots of *Angelica dahurica* (Kang et al., 2008). The HPLC fingerprint technique has been considered as a useful method in identification and quality evaluation of herbs and their related finished products in recent years, because the HPLC fingerprint could systematically and comprehensively exhibit the types and quantification of the components in the herbal medicines (Drasar & Moravcova, 2004; Kang et al., 2008; Wang et al., 2007). Kang and co-authors (2008) showed that the samples from different batches had similar HPLC fingerprints, and the method could be applied for the quality control of the roots of *Angelica dahurica*. In addition, they identified a total of 20 furocoumarins by HPLC/DAD/ESI-MS^n technique, and their fragmentation patterns in an electrospray ion trap mass spectrometer were also summarized.

Recently, high-speed counter-current chromatography (HSCCC) equipped with a HPLC system for separation and purification of furanocoumarins from crude extracts of plant materials, was also described.

High-speed counter-current chromatography (HSCCC), which was first invented by Y. Ito (1981), is a kind of liquid–liquid partition chromatography. The stationary phase of this method is also a liquid. It is retained in the separation column by centrifugal force. Because no solid support is used in the separation column, HSCCC successfully eliminates irreversible adsorption loss of samples onto the solid support used in conventional chromatographic columns (Ito, 1986). As an advanced separation technique, it offers various advantages including high sample recovery, high-purity of fractions, and high-loading capacity (Ma et al., 1994). In the past 30 years, HSCCC has made great progress in the preparation of various reference standards for pharmacological studies and good manufacturing practice, such as coumarins, alkaloids, flavonoids, hydroxyanthraquiones (Liu et al., 2004b).

Liu and co-authors (2004b) isolated and purified psoralen and isopsoralen from *Psoralea corylifolia* using HSCCC technique. In their investigation, they utilized TBE-300A HSCCC instrument with three multilayer coil separation column connected in series. The two-phase solvent system composed of n-hexane-ethyl acetate-methanol-water was used for HSCCC separation. Each solvent was added to a separatory funnel and roughly equilibrated at room temperature. The upper phase (stationary phase) and the lower phase (mobile phase) of the two-phase solvent system were pumped into the column with the volume ratio of 60:40. When the column was totally filled with the two phases, the lower phase was pumped, and at the same time, the HSCCC apparatus was run at a revolution speed of 900 rmp. After

hydrodynamic equilibrium was reached, the sample solution containing the crude extract was injected into the separation coil tube through the injection valve. Each peak fraction was collected according to the chromatogram and evaporated under reduced pressure. The results of HSCCC tests indicated that n-hexane-ethyl acetate-methanol-water (5:5:4.5:5.5, v/v) was the best solvent system for the separation of psoralen and isopsoralen (Liu et al., 2004b).

In another investigations, the same authors had used the same HSCCC technique to induce preparative isolation and purification of furanocoumarins from *Angelica dahurica* (Fisch. ex Hoffm) Benth, et Hook. f (Liu et al., 2004) and from *Cnidium monnieri* (L.) Cusson (Liu et al., 2004a). The results of the first study (Liu et al., 2004) indicated that the best separation of imperatorin, oxypeucedanin and isoimperatorin was when the lower phase of n-hexane-methanol-water (5:5:5, v/v) and n-hexane-methanol-water (5:7:3, v/v) were used in gradient elution. The following gradient was used: 0-150 min, only the lower phase of n-hexane-methanol-water (5:5:5, v/v); 150-300 min, the volume ratio of the lower phase of n-hexane-methanol-water (5:7:3, v/v) changed from 0 to 100%.

A HSCCC method for separation and purification of psoralens from *C. monnieri* was developed by using with a pair of two-phase solvent system composed of light petroleum-ethyl acetate-methanol-water at volume ratios of 5:5:5:5, 5:5:6:4 and 5:5:6.5:3.5 (Liu et al., 2004a).

In described cases, the crude extracts obtained by HSCCC technique were analyzed by HPLC method. The column used was a reversed-phase symmetry C18 column. The mobile phase was methanol-water (68:32, v/v) (analysis of extract from *A. dahurica*), methanol-water (40:60, v/v) (analysis of extract from *P. corylifolia*), or methanol-acetonitrile-water system in gradient mode as follows: 30:30:40 to 50:30:20 in 30 min (analysis of extract from *C.monnieri*) (Liu et al., 2004, 2004a, 2004b). Bergapten and imperatorin obtained by HSCCC method from *Cnidium monnieri* were also analyzed by high-performance liquid chromatography. The column used was a reversed-phase symmetry C18 column, and the mobile phase adopted was methanol (solvent A) – water (solvent B) in the gradient mode as follows: 0-5 min, 60% A; 5-14 min, 60-80% A; 14-15 min, 80-60% A (Li & Chen, 2004).

5.3.5 Capillary electrophoresis

In some cases, capillary electrophoresis was chosen to determine quantities of furanocoumarins. For example Ochocka et al. (1995) used this method for separating psoralens from roots and aerial parts of *Chrysanthemum segetum* L. The analyses were performed with electrophoresis apparatus with UV detection at 280 nm. The best overall separation was obtained on uncoated silica capillary with 7-s pneumatic injection using a buffer solution of 0.2 M boric acid-0.05 M of borax in water (11:9, v/v) (pH 8.5). In another example, micellar electrokinetic capillary chromatography (MEKC) was used in the separation of coumarins contained in *Angelicae Tuhou Radix* (Chen & Sheu, 1995). In this investigation, the electrolyte was buffer solution [20 mM sodium dodecyl sulfate (SDS) – 15 mM sodium borate – 15 mM sodium dihydrogenphosphate (pH 8.26)] – acetonitrile (24:1).

The pressurized capillary electrochromatography (pCEC) was utilized for the separation and determination of coumarins in *Fructus cnidii* extracts from 12 different regions (Chen et al., 2009). Capillary electrochromatography (CEC), as a novel microcolumn separation

technology, couples the high efficiency of capillary electrophoresis with high selectivity of HPLC. The CEC analytes separation is usually achieved in capillaries containing packed stationary phases by an electroosmotic flow (EOF) generated by a high electric field. The experiments were performed in an in-house packed column with a monolithic outlet frit under the optimal conditions: pH 4.0 ammonium acetate buffer at 10 mM containing 50% acetonitrile at −6 kV applied voltage. This analytical method, with use of the novel column, gives good results in the determination of coumarins.

5.3.6 Gas chromatography

In the recent decade, tasks related to the isolation of furocoumarins and the quality control of related preparations were most frequently solved using GC techniques. Gas chromatography was predominantly used for the identification and quantitative analysis of furocoumarins in preparations and raw plant materials. Investigations of the chromatographic behavior (retention times) of substituted furocoumarins revealed the following general laws: 1) on passage from hydroxy- to methoxycoumarins, the retention time decreases (because of reduced adsorption via hydrogen bonds); 2) furocoumarins with O-alkyl substituents at C5 are eluted after 8-hydroxy isomers; 3) the logarithm of the relative retention time is a linear function of the molecular weight. This GC data can be used for determining the structure and estimating the retention time of analogous coumarins (Lozhkin & Sakanyan, 2006). A number of methods have been described for the analysis of furanocoumarins using capillary gas chromatography (GC) (Beier et al., 1994; Wawrzynowicz & Waksmundzka-Hajnos, 1990).

Gas chromatographic method was used to determine osthole content in *Cnidii Fructus* extract. The analytical conditions are the following: nitrogen as the carrier gas, the flow rate of 40 mL/min; the split ratio of 120:1. The column used was DBTM-5 (30 m × 0.53 mm I.D., 1.5 μm) equipped with a flame ionization detector (FID). The initial oven temperature was programmed to be at 135°C for 12 minutes. The temperature was then raised to 215°C at a rate of 12°C/min for 20 minutes. Caffeine anhydrous was used as the internal standard (Yu et al., 2002).

In another example, GC-FID was used to analyze of psoralen, bergapten, pimpinellin and isopimpinellin present in phytomedicines (creams and pomades) employed in the treatment of vitiligo in Brazil. The GC-FID assay method present here is rapid, sensitive and robust and can be applied to the determination of furanocoumarins in routine analysis of creams, pomades and other lipophilic phytocosmetics. These analyses were performed in a VARIAN 3400 gas chromatograph equipped with a capillary fused silica LM-5 and with a flame ionization detector (FID). H_2 was used as carrier gas at a flow rate 0.8 ml min^{-1} and the injection split ratio was 1:20. The injection temperature was 280°C. Column temperature was programmed from 150 to 240°C with a linear increase of 10°C min^{-1}, then 240–280°C with a linear increase of 5°C min^{-1} and was then held for 15 mins. The detector temperature was 280°C (Cardoso et al., 2000).

5.4 Structural analysis

For the structural identification and characterizing of the psoralen compounds, especially if they are novel, instrumental techniques such as nuclear magnetic resonance (NMR)

spectroscopy and infrared spectroscopy (IR) are used. NMR spectroscopy is an invaluable technique for the structural determination of all furanocoumarins. As well as providing information on the chemical environment of each proton or carbon nucleus in the molecule, the technique can be employed to determine linkages amongst nearby nuclei, often enabling a complete structure to be assembled (Rice-Evans & Packer, 2003).

Dimethyl sulfoxide (DMSO-d_6) and methanol (CD_3OD) are both suitable solvents for furanocoumarins.

The reader is referred to Rojas-Lima et al., 1999; Um et al., 2010; Taniguchi et al., 2011, and Tesso et al., 2005 publications, for details of the principles of NMR and general interpretation of NMR spectra.

6. Conclusions

As furanocoumarins have a lactone structure, they have a wide range of biological activity. Bergapten and the other furanocoumarins are used to treat dermatological diseases (psoriaris, vitiligo). As a result, their photosensitizing properties are playing an important role (Bhatnagar et al., 2007; Trott et al., 2008). Their ability to covalently modify nucleic acids is used in process called "extracorporeal photopheresis" that is medically necessary for either of the following clinical indications: erythrodermic variants of cutaneous T-cell lymphoma (e.g. mycosis fungioides, Sezary's syndrome) or chronic graft-versus-host disease, refractory to standard immunosuppressive therapy (Hotlick et al., 2008; Lee et al., 2007).

The aim of the present chapter was to present an overview of techniques of isolation, separations and identification of furanocoumarins in plant materials. Various analytical approaches exist for detection of coumarins and the analytical techniques should meet the following prerequisites: short time, relatively inexpensive, highly accurate, and precise for a variety of applications. This review may be helpful in the choice of the method of furanocoumarin compounds analysis.

7. References

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4

Analytical Methods and Phytochemistry of the Typical Italian Liquor "Limoncello": A Review

Marcello Locatelli, Giuseppe Carlucci,
Salvatore Genovese and Francesco Epifano
University "G. d'Annunzio" of Chieti-Pescara, Faculty of Pharmacy,
Dipartimento di Scienze del Farmaco,
Italy

1. Introduction

The analyses of food or food-derived products that naturally occur in plants are essential especially related to food safety, food composition, adulterations, and food quality for Protected Geographical Indication (IGP), Controlled Origin Denomination (DOC) or Controlled and Guaranteed Origin Denomination (DOCG) designation.

For these reasons in this field of research a multidisciplinary approach between analytical chemistry, phytochemistry, organic synthetic chemistry, and biochemistry is strongly recommended.

In this contest, the Italian liqueur "Limoncello", obtained by maceration of lemon skin in a hydroalcoholic solution of saccharose, is an explicative example.

Limoncello is produced in Southern Italy, mainly in Campania region (Gulf of Naples, the Sorrentine Peninsula, the coast of Amalfi, and islands of Procida, Ischia, and Capri) but also in Sicily and Sardinia.

Following an ancient tradition, limoncello is a homemade liqueur obtained from peels of Sorrento lemons. However nowadays, due to the fact that its production on industrial scale is widespread and the demand on the international market is increasing, virtually all kinds of lemons are used to make this liqueur. Its main distinctive feature is a bright yellow colour. Limoncello is served especially after-dinner as a digestive after having been stored in a refrigerator at 4 °C. It is very popular and commonly consumed in all Italy and many areas of the Mediterranean region but it is also becoming famous in other countries like United States, Canada, Australia, New Zealand, and northern Europe.

Being so popular, limoncello is also an ingredient for the preparation of several cocktails due to its strong lemon flavour without the sourness or bitterness of lemon juice.

A typical procedure for the preparation of limoncello include as ingredients 8 Sorrento lemons, 1 litre of water, 1 litre of absolute ethanol and 800 g of sugar. Lemons are then peeled in such a way to get only the flavedo (the very external part of the skin coloured in yellow); the resulting raw vegetable material is then macerated in the dark for 15 days. The

alcoholic solution from maceration is then added to the solution of sugar in water and the resulting mixture is filtered. The liqueur so obtained is kept in the dark for 40 days after which period is stored in the refrigerator and consumed as described above.

Resulting from an ethnically ancient tradition and being used as a digestive in several part of the world, it is interesting to investigate the chemical composition and medical properties of Limoncello.
The Council Regulation no. 1576/89 lays (Council Regulation, 1989) down a definition and a description of spirit drinks and also dictates the need for a better comprehension of the chemical nature of limoncello. Besides giving a list of rules on alcoholic beverages, the regulation clearly states that even nature-identical flavouring substances and preparations shall not be authorized in liqueurs derived from *Citrus* fruits.

Apart from some investigations on lemon liquors reported in journals specifically linked to beverage industry (Bonomi et al., 2001; Moio et al., 2000; Naviglio et al., 2001, 2003, 2005a, 2005b; Romano et al., 2004), it can be stated that the increasing interest of the market toward limoncellos is not offset by the number of scientific papers.

The effective problem connected to investigation about phytochemicals is the correct and real identification and quantification in natural sources by means of very sensitive, selective, and validated analytical methodologies.

In 1986, the first study describing the chemical composition of the essential oil derived from Sicilian lemons was reported in the literature by Cotroneo and coworkers (Cotroneo et al., 1986). The same group got further insights on the same topic with a series of manuscript published from the beginning of 90s until 2001 (Dugo G. et al., 1993, 1999, 2001; Dugo P. et al., 1998, 2000; Mondello et al., 1999). These Authors used very sensitive and selective analytical methodologies like high-resolution GC coupled with chiral capillary columns-mass spectrometry (HRGC-MS) and bi-dimensional capillary GC (2D-GC). In 2002 they finally summarized in a review article all the works they made on *Citrus* species (Dugo et al., 2002).

Examining the papers by Dugo and coworkers, the need of using advanced instrumental techniques for the analyses of raw sample extracted from Citrus fruits, mainly due to their chemical complexity, can be pointed out.

In the next paragraph, we'll make a survey of the current reported literature data about extraction procedures and instrumental configuration used for the analyses of this Italian liqueur obtained by maceration of lemon skin.

2. Analytical methods

In the last twenty years a huge progress was made concerning instrumental configuration, sensitivity and selectivity improvement (Locatelli et al., 2011) for analytical methods applied in many fields, especially for food safety.

The main problem consisted in the great complexity of extracted samples and the large number of components. For these reasons the qualitative and quantitative analyses must be very specific and robust enough to isolate, qualificate, and to quantificate the target compound (s).

In this field the best practices and improvements concerns purification and analytes extraction techniques and especially analytical instrument configuration applied to this kind of analyses.

2.1 Extraction procedures

Sample preparation steps are often sensitive to the matrix and, generally, contributed with approx. at 75% to the final error. This is particular true when multiple steps are involved into the procedure, where the final uncertainty is compounded.

The sample preparation has a straight impact on method performances, in particular trueness, precision, limits of quantitation, linearity, reproducibility, and is often the rate-limiting step for several analytical assays.

Sample processing can broadly range in complexity from simple dissolution in an appropriate solvent to a complicated extraction procedure, followed by derivatization and further extraction.

There are three general extraction methods, solvent extraction (or liquid-liquid extraction, LLE), solid phase extraction (SPE), and solid phase micro-extraction (SPME).

The use of these techniques, however, often entails the use of precipitation procedures, crude separation processes, and subsequent concentration methods.

Traditional liquid-liquid extraction procedures employ in a serial of extraction of an aqueous sample with an immiscible organic solvent resulting in a large solvent volume that must be dried and then concentrated prior to analysis, bring to an expensive, and in some cases non-reproducible procedure.

For this reason, further extraction methodologies were developed and validated. In particular, in SPE procedures, a solid sorbent material is packed into a cartridge or imbedded in a disk and performs essentially the same function as the organic solvent in liquid-liquid extraction, with a smaller organic solvent volume consumption.

SPE can be applied in several fields, from bio-analytical to environmental analyses, but it requires a sample volume adequately to extract targets analytes and the possibility to replicate the analysis because SPE is a destructive methodology.

In the early 1990s, the development of a new sampling method, non-destructive, sensitive, reproducible, relatively inexpensive and in particular solventless, allowed the trapping of volatile organic compounds (VOCs) on a silica optical fibre coated with a polymer thin layer followed by their identification by GC.

For the analyses of Limoncello the first extraction procedure used to determine the product volatile components was Liquid-Liquid Extraction (LLE).

In this methodology, extraction organic solvent generally used for this purpose is a hexane/ethyl acetate mixture (Starrantino et al., 1997, Dugo et al., 2000, Versari et al., 2003).

In particular the analyses of lemon-derived products were achieved with a preliminary dilution (1:10)-extraction step with hexane and ethyl acetate (75:25, v/v) (Starrantino et al., 1997) or pure hexane (Mondello et al., 2003) followed by direct analyses of extracts.

Solid-phase micro extraction (SPME) derives principally from SPE technologies and improves the concept of reducing solvent consumption, economic characteristic, and involved through two different extraction methods: headspace SPME (HS-SPME) and contact SPME.

The headspace SPME is based on the absorption of the analytes on a fibre coating placed in the sample's headspace volume and on the partition of the target analytes between the sampling matrix and the fibre. After exposure the fibre bearing the concentred analytes was retracted, removed from the sample vial and VOCs were thermally desorbed by insertion of the fibre into the injector port of the chromatograph.

Recently in the preparative sample scenario appeared several automated and fully independent instrumentation that allow to process high sample number (high-throughput), and especially with an improvement of efficiency and process reproducibility.

When gas-chromatographic assays were used to quantify diluted Limoncello extracts it was also necessary to decrease the concentration of ethanol that tended to immediately saturate the SPME fibres. The best election, and generally, used methodology for the analysis of volatile components was headspace SPME (HS-SPME) on polydimethylsiloxane (PDMS) as thin layer (TL) due to non-polar characteristic of samples compounds.

This technique can be easily automated (Crupi et al., 2007) to improve analyses number (high throughput assay) and to obtain a better control on overall analysis steps, which bring to higher methodology reproducibility.

2.2 Instrumental analysis

Mono-dimensional chromatographic processes (1D) are widely applied in the analysis of food and food-derived products.

Although such methods often provide satisfying analytical results, the complexity of several matrices exceeds the resolution capacity of a single dimension separation system.

In the past years several efforts have been dedicated to the combination of independent techniques with the aim of reinforcement resolving power, until the use of multi-dimensional chromatography (MDC).

A typical comprehensive bi-dimensional chromatographic separation (2D) is achieved, generally, on two distinct columns connected in series with a special transfer system located between them. The type of interface used is connected to the specific methodology. The function of the interface is to cut and then release continuous fractions of the primary column effluent onto a fast separation column. In order to achieve comprehensive analysis and to preserve the 1D separation, the bands injected onto the secondary column must undergo elution before the following re-injection. Secondary retention times must be, at the most, equal or less than the duration of a single modulation period.

Coupled to these chromatographic techniques (both gas chromatography and high-performance liquid chromatography), several detectors are used for a clear and univocal identification and quantification of target analyte(s).

The must used are Flame Ionisation Detector (FID) coupled with GC, Mass Spectrometry Detector (MS) coupled to GC and HPLC, and Tandem Mass Spectrometry (MS/MS) interfaced with HPLC.

Due to the complex chemical composition of Limoncello extracts (comprising several classes of volatile compounds, 85-98%), the election chromatographic techniques are certainly gas-chromatographic (GC) ones.

In mono-dimensional GC the stationary phase is bonded and highly cross-linked; silphenylene polymer (polarity similar to 5% phenyl polymethylsiloxane) in programmable thermal analyses, split/splitless injector in splitless mode. This capillary column is used with both FID and MS detector, with the unique difference in helium flow rate (minor in the MS interfacing).

With this system is possible the analyses of all volatile components fraction derived from SPME extraction, while for the analyses of enantiomeric compounds chiral capillary column characterized by diethyltertbutylsilyl-β-cyclodextrins as stationary phases are generally used.

These analyses are carried out separately to obtain a complete chemical composition of volatile components. Recently Mondello and coworkers (Mondello et al., 2006) coupled these two gas-chromatographic separations in an innovative multi-dimensional GC (MDGC) system, to obtain the complete chemical composition on volatile components including enantiomeric resolution in a single analysis.

The main goal of this configuration is especially due to the possibility of evaluating the enantiomeric ratios as genuineness markers, especially in complex matrices as Limoncellos.

To detect analytes of interest flame ionisation detector (FID) and mass spectrometric detector (MS) are generally chosen.

With the first, that is a "universal" gas-chromatographic detector is possible to obtain, in a single run a complete chemical fingerprint of volatile fraction, with identification of various components by retention index (R_i). This configuration is very useful if are at disposition data bank with R_i or chemical standards. If no pure chemical standards are available, the best choice is gas-chromatography-mass spectrometry interfacing (GC-MS), because is possible to obtain R_i of several volatile components and, in addition, the mass-to-charge ratio for the correct identification of targeted compounds.

Another trend in the last years is the use of MDGC, coupled with mass spectrometry. Gas-chromatographic determination has the disadvantage that by this technique it is difficult to analyse non-volatile components of extracted samples.

High performance liquid chromatography (HPLC) is generally used for the non-volatile components. Is a well-defined technique, robust and reproducible that generally is coupled with "universal" detector as Diode Array Detector (DAD).

In the literature several paper dealing with the analysis of flavones, coumarins, and furanocoumarins composition of lemon-derived products by HPLC using different chromatographic column were reported.

In particular for the analyses of coumarins and furanocoumarins in Limoncellos samples the most widely used column are silica based (generally 300 x 4 mm, 10 µm particle size) to obtain a complete resolution in normal phase mode (Starrantino et al., 1997, Versari et al., 2003), while for the analyses of phenolic compounds Octadecylsilane (ODS) column in reversed phase mode was used (Versari et al., 2003).

Ultraviolet-visible (UV/Vis) detection was carried out at 315-330 nm for coumarins and furanocoumarins and at 280 nm for phenolic compounds.

The analyses, due to the samples matrices complexity, are carried out in gradient elution mode. In particular for the analyses of coumarins and furanocoumarins was used a mobile Phase constituted by hexane: ethyl acetate (92:8 or 88:12, v/v) and hexane: ethyl alcohol (90:10, v/v).

Only recently Crupi and coworkers (Crupi et al., 2007) developed a unique reversed phase method for simultaneous determination at 315 nm of coumarins and furanocoumarins using water and acetonitrile as mobile phase in gradient elution mode.

3. Limoncello chemical composition

It is a matter of fact that the evaluation of the organoleptic properties of limoncello is, although indirectly, connected to the analysis of the essential oil composition. The aroma of the liquor is actually one of the first consumer's perceptions that are crucial in establishing the preference among several products available in the market. For this reason, the analysis of the aromatic fraction of limoncello liquor seems to be an important item in assessing its genuineness and quality, besides ''tracing" the various steps of the preparation procedure.

The current chemical composition of this Italian liqueur is mainly related to the aromatic fraction that was reported by Crupi and coworkers (Crupi et al., 2007) using previously mentioned analytical techniques.

In their comprehensive studies, after SPME extraction of volatile components and chromatographic analyses, both GC and HPLC, they detected several monoterpenes, sesquiterpenes, and oxygenated compounds, as reported in the Table 1.

Versari and coworkers reported similar analyses of several commercial Limoncello samples and chemometric elaboration with Principal Components Analysis (PCA) technique to obtain two main group: the first that showed a composition similar to lemon essential oils (high content of β–pinene, myrcene, *trans*-α-bergamotene, and β-bisabolene, and a low content in neral and geranial) (Versari et al., 2003). The composition of the second group suggested the presence of oxidative phenomena and (or) addition of flavours, in particular the presence of compounds as ethyl acetate, acetaldehyde, 2-methyl-1-propanol and glycerol indicate that fermentation process probably occurred in the sugar syrup during Limoncello dilution step after the extraction process.

The best recurrent molecules of the oil and of Limoncello (both homemade and commercial ones) belonging the volatile compounds family are α-pinene, sabinene, β-pinene, myrcene, *p*-cymene, limonene, γ-terpinene, neral, geraniol, and geranial, and as reported in the Table 1. As results of their investigation, Crupi and coworkers (Crupi et al., 2007) underline that most of analysed Limoncello samples were effectively obtained directly from fruits, but also using terpeneless oils and in some cases synthetic products of reconstituted oils.

From data reported in the Tables 1 and 2 is possible to indicate that the commercial #05 and #02 are of high quality, even if commercial #02, due to high level of *p*-cymene can indicate a long storage time.

Compounds	Oil	Home-made Limoncello	Commercial Limoncello														
			#01	#02	#03	#04	#05	#06	#07	#08	#09	#10	#11	#12	#13	#14	#15	#16
α-Pinene	14.510	21.0	8.8	13.4	8.9	12.6	36.5	11.6	10.6	12.5	9.9	8.9	9.7	9.7	14.6	9.2	9.0	8.9
Sabinene	17.570	211.3	-	14.4	9.0	-	20.8	8.8	-	-	9.7	9.0	-	-	14.9	-	-	9.0
β-Pinene	142.090	-	9.5	66.8	11.6	37.1	247.9	42.4	12.2	44.5	23.4	11.2	18.4	9.3	78.5	13.6	11.0	11.6
Myrcene	11.900	18.8	8.9	16.1	9.0	13.0	35.7	12.1	11.0	11.0	9.2	9.1	10.5	10.2	15.0	9.5	9.3	9.1
p-Cymene	340	4.5	9.2	84.9	13.3	17.4	11.8	17.2	17.6	28.7	12.7	10.9	14.5	15.3	58.5	13.2	9.5	11.3
Limonene	626.290	837.2	26.5	634.6	43.6	353.3	1671.0	272.1	212.1	288.78	42.6	45.3	179.3	141.4	475.7	50.2	60.6	57.9
γ-Terpinene	104.670	143.9	9.5	11.9	10.3	47.8	281.9	31.5	30.2	34.0	12.3	10.1	13.8	21.6	25.1	8.9	10.9	15.9
Neral	13.520	390.0	30.4	10.8	-	23.6	11.7	13.5	10.2	11.0	10.1	24.8	64.0	26.5	9.9	44.6	26.3	85.0
Geraniol	270	3.2	3.3	4.9	1.9	64.7	10.9	10.9	296.1	106.2	4.6	2.5	6.2	177.5	4.6	21.8	2.8	3.6
Geranial	20.960	581.6	40.2	10.1	-	24.5	17.9	-	9.7	13.7	10.2	32.1	94.0	19.4	11.3	65.5	40.4	133.5

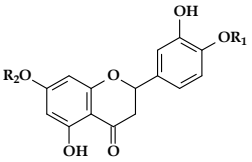
Table 1. Amount (mg/L) of major representative volatile components in several lemon samples-derivatives in oil, homemade Limoncello, and commercial Limoncello products (Crupi et al., 2007).

Compound	Commercial Limoncello											
	#01	#02	#03	#04	#05	#06	#07	#08	#09	#10	#11	#12
Bergamottin	1.7	4.7	21.5	1.1	0.9	3.8	2.3	19.6	3.7	1.2	1.0	3.6
5-geranyloxy-7-methoxycoumarine	0.7	1.6	0.8	0.5	0.5	1.3	0.8	7.0	1.2	0.4	0.2	1.2
Citroptene derivate	-	-	0.1	0.1	-	-	-	0.3	-	-	-	-
5-isopentenyloxy-7-methoxycoumarine	-	-	0.4	0.2	-	-	-	1.0	-	-	-	-
Citroptene	2.0	-	1.5	1.0	1.3	0.3	1.8	4.0	0.4	1.1	0.4	1.0
Imperatorin	4.4	0.8	-	0.6	-	-	-	1.6	-	-	-	-
Eriocitrin	16.9	-	27.8	21.7	65.9	-	-	71.2	-	29.1	-	22.3
Sinapinic acid	0.4	-	0.5	-	1.0	-	-	Trace	-	1.5	-	0.5
Narirutin	5.2	14.5	1.8	0.8	2.2	-	-	2.2	-	-	-	5.9
Naringin	-	1.5	-	1.2	Trace	-	-	Trace	Trace	Trace	1.4	1.3
Hesperidin	16.7	-	25.3	12.0	32.1	-	-	42.3	-	6.7	-	7.6
Neohesperidin	0.6	-	Trace	Trace	Trace	-	-	1.2	-	Trace	-	0.4
Diosmin	1.9	13.3	7.6	Trace	7.9	-	5.3	-	0.8	4.0	Trace	8.6

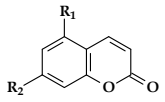
Table 2. Identificated and quantificated coumarins, psoralem, and phenolics composition (mg/L) of several commercial Limoncello samples (Versari et al., 2003).

Terpenes are photosensitive compounds; in particular limonene and terpinenes are involved in this irreversible process that negatively affects the organoleptic properties of the beverage. The terpenes fraction and/or the presence of oxidized by-products can predict the origin and the quality of a Limoncello.

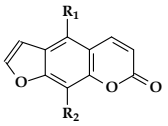
Versari and coworkers (Versari et al., 2003) reported that other mainly present compounds, analysed by HPLC-DAD, were phenolics, coumarins, and psoralem derivatives. These compounds are mainly flavones, coumarins, and furanocoumarins, as represented in Figure 1.



R_1	-H	R_2	-Rutinose	Eriocitrin
R_1	-H	R_2	-Neohesperidose	Neoriocitrin
R_1	$-CH_3$	R_2	-Rutinose	Hesperidin
R_1	$-CH_3$	R_2	-Neohesperidose	Neohesperidin
R_1	-H	R_2	-Rutinose	Narirutin
R_1	-H	R_2	-Neohesperidose	Naringin
R_1	$-CH_3$	R_2	-Rutinose	Diosmin



R_1	$-OCH_3$	R_2	$-OCH_3$	Citroptene
R_1	-Geranyloxy chain	R_2	$-OCH_3$	5-geranyloxy-7-methoxycoumarine
R_1	-Isopentenyloxy chain	R_2	$-OCH_3$	5-isopentenyloxy-7-methoxycoumarine



R_1	-H	R_2	-Isopentenyloxy chain	Imperatorin
R_1	-Geranyloxy chain	R_2	-H	Bergamottin

Fig. 1. Chemical structures of flavones, coumarins, and furanocoumarins identified and quantified in Limoncello by Versari and co-worker (Versari et al., 2003).

Problems connected to storage time were reported also by Da Costa and Theodore (Da Costa and Theodore, 2010) in a paper that report a complete study of over two-hundred volatile components in Limoncello and their changes that occurred with aging.

Another well-recognized question investigated by Poiana and coworkers (Poiana et al., 2006) regarded the differentiation from sample-to-sample about chemical and physical-chemical parameters of alcoholic extracts of lemons deriving from the Amalfi and Sorrento areas. These discrepancies are probably correlated with the different cultivars and growing conditions.

In particular this research underlined the evolution during the season for some classes of components. The carbonyl-to-oxygenated, alcohols-to-oxygenated and esters-to-oxygenated compounds ratios are indices of flavouring quality. These showed similar trends for both lemon types. In comparison to other lemon productions a higher amount of minor classes of components were also recorded.

4. *Citrus* essential oil heavy metal composition – food quality

Citrus essential oil is a very complex mixture of several classes of volatile (85-98%) and non-volatile (2-15%) compounds, as reported in the previous paragraphs. In this classification,

the main present compounds are terpenes, hydrocarbons, esters, aldehydes, and ketones. The key difference between essential oils from different raw plant varieties is especially related to the composition of the volatile fraction (Steuer et al., 2001). *Citrus* essential oil is generally used as aromatising agent and additive for food and food-derived products, beverages, cosmetics and in some pharmaceuticals. In this sample extract is possible to incur into the presence of organic and inorganic contaminants, as well documented by the presence of pesticide residues (Saitta et al., 2000, Dugo et al., 1997; Verzera et al., 2004) and plasticizers (Di Bella et al., 1999, 2000, 2001; Saitta et al., 1997); however, there is a needs of available data regarding the presence of heavy metals in this products. Some reports concerning the microelements composition of *Citrus* peel extracts were published (Gorinstein et al., 2001; Simpkins et al., 2000). Metals, such as iron, copper, zinc, and cobalt, are non-toxic at modest concentrations, while cadmium, lead, mercury, and arsenic are toxic even in very low concentration level and constitute a significant health hazard (Rojas et al., 1999).

Metals levels in *citrus* essential oils mainly depend on the type of soil and treatment, but are also affected by the extraction procedures to manufacture food-derived products, such as scraping or pressing because the fruits inevitably come in contact with metallic surfaces.

In this field the best instrumental analysis concerns especially the use of electrochemical techniques, such as derivative potentiometric stripping analysis (dPSA) and Atomic Absorption Spectroscopic methods (AAS) in Graphite Furnace Atomic Absorption Spectroscopy Analysis (GFAAS) configuration to determine trace metals concentrations in a variety of matrices, such as alloys, food, biological materials, and environmental samples.

Recently La Pera and coworkers (La Pera et al., 2003) published an interesting work inherent the use of these two techniques for the simultaneous determination of cadmium, copper, lead, and zinc in *Citrus* essential oils with high recoveries values from real samples extracts.

In the Table 3 were reported the mean concentration determined by dPSA and AAS both on acid and methanol extracts.

		Cd		Cu	
Sample	**Treatment**	**dPSA**	**AAS**	**dPSA**	**AAS**
Lemon	Acid extracts	1.57±0.03	1.43±0.03	16.94±0.21	16.00±0.20
Lemon	Methanol extracts	1.63±0.03	1.55±0.05	21.65±1.59	18.10±2.65
		Pb		**Zn**	
Sample		**dPSA**	**AAS**	**dPSA**	**AAS**
Lemon	Acid extracts	111.24±0.81	103.60±1.10	802.55±2.48	799.60±3.00
Lemon	Methanol extracts	113.24±0.72	103.30±7.15	809.62±2.26	788.99±9.10

Table 3. Heavy metal mean concentration (ng/g; n=3) and standard deviation (n=3) detected by dPSA and AAS in Lemon samples (La Pera et al., 2003).

These results indicates that *Citrus* lemon essential oils contains several heavy metals and that a deep control on food-derived products is required to obviate to any significant health hazard.

In particular, Verzera and coworkers (Verzera et al., 2004) showed that there are great differences between lemon biological oils and traditional ones inherent the organophosphorus pesticide content.

In particular these Authors reports that Parathion methyl, Parathion ethyl, Quinalphos, Methidathion, Clorpyphos methyl, and Azinphos ethyl are the must representative organophoshorus pesticides founded in lemon oils samples, both in biological and traditional agricultural methods.

Traditional oils were found to contain from 3.52 to 3.85 ppm of previously cited compounds, while biological ones were found to contain from 0.17 to 0.74 ppm.

Only in lemon oils obtained from fruits deriving from traditional agricultural methods were found Dicofol as organochlorine pesticides at 1.0 ppm level.

Previously reported values inherent organophoshorus pesticides were obtained by gas chromatography coupled with Flame Photometric Detector (FPD) that is similar to FID in that the sample exits the analytical column into a hydrogen diffusion flame. Where the FID measures ions produced by organic compounds during combustion, the FPD analyzes the spectrum of light emitted by the compounds as they luminesce in the flame.

Organochlorine pesticides were also obtained by gas chromatography coupled with Electron Capture Detector (ECD). The ECD measures electron-capturing compounds (frequently halogenated compounds) by creating an electrical field in which molecules exiting a GC column can be detected by the drop in current in the field.

5. Conclusion

A deep knowledge of all the chemical aspects of a Limoncello, in this contest, could greatly help with assessing its authenticity and genuineness. The analysis of food products may be directed to the assessment of food quality and authenticity, the control of a technological and production process, the determination of nutritional values, and the detection of molecules and secondary metabolite eventually present in food-derived products with a possible advantageous effect on human health and safety.

Consequently, a main aim in food chemistry regards especially the continuous improvement, development, and in particular, validation of increasingly sensitive and selective analytical techniques.

The availability of hyphenated analytical methods and 2D chromatographic methods capable of revealing the origin, the authenticity and the quality of a Limoncello may encourage the producers to prepare high quality products, appreciated by the consumer not because of the massive advertisement, but for the characteristics of their composition.

Further investigations must be devoted especially to the improvement of the instrumental performance and high-throughputs analyses, to the implementation of supplementary

options such as cryo-trapping and to the enhancement of MS and MS/MS detection in both conventional gas and high performance liquid chromatography, and multi-dimensional gas-chromatography (MDGC) applications.

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5

The Effects of Non-Thermal Technologies on Phytochemicals

Gemma Oms-Oliu, Isabel Odriozola-Serrano and Olga Martín-Belloso
University of Lleida,
Spain

1. Introduction

Phytochemicals are non-nutritive plant chemicals that possess protective roles in the human body, against disease. These phytochemicals are considered to be biologically active secondary metabolites that also provide color and flavor, and are commonly referred to as nutraceuticals (Kalt, 2001). There are thousands of known phytochemicals, which have been found to be derived mainly from phenylalanine and tyrosine, and which perform a variety of functions such as pigmentation, antioxidation, protection against UV light, etc. (Shahidi & Naczk, 2004). Evidences of the benefits to human-health associated with the consumption of plant-derived phytochemicals have caused an increase in the demand for fresh-like fruits and vegetables., where are present in different forms as alkaloids (eg., caffeine and threbromine), carotenoids (e.g. lycopene), flavonoids (e.g., flavon-3-ols), isoflavones (e.g. genistein), phenolic acids (e.g., capsaicin, gallic acid and tannic acid), etc., depending on plant species.

The health-promoting effects of many phytochemicals are attributed mainly to their antioxidant activity, although there could also be other modes of action. Fruit and vegetables are known to contain significant amounts of phytochemicals with free-radical and nonradical scavenging capacity towards reactive oxygen species. These deleterious substances have been identified as toxic against cell tissues, thus causing oxidative damage to proteins, membrane lipids and DNA, inhibiting enzymatic pathways, and inducing gene mutation. It is believed that these processes are underpinning several chronic human diseases such as diabetes, certain forms of cancer as well as some cardiovascular and degenerative diseases (Eberhardt et al., 2000; Arab & Steck, 2000).

Changes in the concentration of phytochemicals through processing and storage can greatly compromise the quality, and eventually the acceptance of a food. Despite most of these compounds, to some extent, may be affected by abusive temperatures, thermal processing remain the most commonly used technology for inactivating microorganisms and enzymes in processed food. However, during thermal processing, in addition to the inactivation of microorganism, sensory and nutritional compounds of plant-based foods are negative affected. The consumers demand towards products that keep their original nutritious values and, in this context, non-thermal technologies are preservation treatments that are effective at mild temperatures (up to 40 ^{o}C), thereby minimising negative thermal effects on nutritional and quality of products. These non-thermal processes ensure microbial and

enzyme inactivation with reduced effects on nutritional and quality parameters. Non-thermal technologies as, pulsed electric fields (PEF), high pressure (HP), irradiation or ultrasounds have been studied and developed during the last decades with the final aim of implementing them at an industrial level. The impact of non thermal technologies on microorganisms, enzymes and quality-related parameters has been extensively reviewed. However, many efforts in the last years have been made to evaluate the impact of PEF, HP, irradiation and ultrasounds on the stability of phytochemicals. The present review aims at reviewing the effects of non thermal technologies on health-related compounds in plant based products.

2. Non-thermal technologies to preserve phytochemicals

2.1 Non-thermal processing basics

Non-thermal technologies may allow obtaining safe and shelf-stable plant-based products with minor changes or increased content in phytochemicals. Most differences between non-thermal and thermal treatments can be explained through the temperatures reached through processing. In general, temperature during processing and storage are important factors affecting the phytochemical content of the processed product. PEF processing involves the application of a high intensity electric field (20–80 kV/cm) in the form of short pulses to a food placed between two electrodes. PEF technology provides fresh-like and safe foods while reducing quality losses that can be triggered after thermal processing (Morris et al., 2007). Application of continuous PEF processing is not suitable for solid food products that do not allow pumping and is restricted to low-conductive food products without air bubbles. Liquid food products are susceptible to be treated by PEF because they are primarily composed by water and nutrients, which are electrical conductors due of the presence of large concentrations of salts and dipolar molecules. Hence, PEF technology has been suggested for the pasteurization of foods such as juices, milk, yogurt, soups, and liquid eggs (Vega-Mercado et al., 1997; Evrendilek et al., 2004; Elez-Martínez et al., 2005; Monfort et al., 2010). In general, a continuous PEF treatment system is composed of treatment chambers, a pulse generator, a fluid-handling system, and monitoring systems (Elez-Martínez et al., 2006). Temperature- and pulse-monitoring systems are used to supervise the process. The effectiveness of PEF technology not only depends on the type of equipment used but also on the treatment parameters and media to be processed (Barbosa-Cánovas et al., 1999). In addition, processing parameters such as electric field strength, total treatment time, pulse shape, pulse frequency, pulse width, polarity and temperature are involved in the degradation or generation of phytochemicals.

HP processing uses water as a medium to transmit pressures from 300 to 700 MPa to foods resulting in a reduction on microbial loads and thus extending shelf life (Patras et al., 2009). Pressure is nearly transmitted uniformly throughout the food, thus leading to very different applications in many different food products. Although pressure results are uniform, the HP technique cannot completely avoid the well-known classical limitation of heat transfer especially during pressure build up and decompression. An increase or a decrease of pressure is associated with a proportional temperature (T) change of the vessel contents, respectively, due to adiabatic heating or cooling temperature gradient. The effectiveness of HP treatment on the overall food quality and safety is not only influenced by extrinsic (process) factors such as treatment time, pressurisation/decompression rate, pressure/ temperature levels and the

number of pulses, but also by intrinsic factors of the treated food product such as food composition and the physiological states of microorganisms (Knorr, 2001; Smelt et al., 2002). Effects of pressure and temperature on food constituents are governed by activation volume and activation energy. Differences in sensitivity of reactions towards pressure (activation volume) and temperature (activation energy) lead to the possibility of retaining or even diminishing some desired natural food quality attributes such as vitamins, pigments and flavour or modifying the structure of food system and food functionality, while optimizing the microbial food safety or minimizing the undesired food quality related enzymes (Barbosa-Cánovas et al., 1997; Messens et al., 1997; Hendrickx et al., 1998).

Exposure to radiation, either ionizing or non ionizing, is being regarded as one of the non thermal methods for food preservation with the best potential, especially for the decontamination of raw material for food production. The ionizing radiation source could be high-energy electrons, X-rays (machine generated), or gamma rays (from Cobalt-60 or cesium- 137), while the non-ionizing radiation is electromagnetic radiation that does not carry enough energy/quanta to ionize atoms or molecules, represented mainly by ultraviolet rays (UV-A, UV-B, and UV-C), visible light, microwaves, and infrared. Decontamination though ionizing radiation consists of the application of doses of 2-7 kGy. It has been proven that radiation can safely and effectively eliminate pathogenic nonsporeforming bacteria in foods (Alothman et al., 2009). However, radiation is not being widely used because of some misconceptions by consumers about its role in causing cancer. Radiation can influence the levels of phytochemicals and the capacity of a specific plant to produce them at different levels. Radiation treatments have been shown to either increase or decrease the antioxidant content of fresh plant produce, which is dependent on the dose delivered (usually low and medium doses have insignificant effects on antioxidants), exposure time, and the raw material used. The enhanced antioxidant capacity/activity of a plant after radiation is mainly attributed either to increased enzyme activity (e.g., phenylalanine ammonia-lyase (PAL) and peroxidase activity) or to the increased extractability from the tissues (Alothman et al., 2009).

Although ultrasound is unlike to become a commercial technology on its own, it can be applied in combination with other technologies with preservation purposes. Its lethal microbial effects have been related to cavitation, a phenomenon that generates high temperatures and pressures at a microscopic level that are responsible for the formation of highly reactive free radicals and for the mechanical damage of microorganisms (Raso et al., 1998). Ultrasound processing of juices is reported to have minimal effect on the degradation of key quality parameters such as colour and ascorbic acid in orange juice during storage at 10 ^{o}C (Tiwari et al., 2009a). This positive effect of ultrasound is assumed to be due to the effective removal of occluded oxygen from the juice (Knorr et al., 2004).

2.2 Plant-based foods preserved by non-thermal technologies

2.2.1 Tomato

Vitamin C has received much attention when aiming at evaluating the effect of non thermal processing technologies on the phytochemicals. Vitamin C retention in PEF-treated juices depended on processing factors and thus, the lower the electric field strength, the treatment time, the pulse frequency or the pulse width, the higher the vitamin C retention in tomato juices (Odriozola-Serrano et al., 2007). According to these authors, maximal relative vitamin

C content (90.2%), was attained with PEF treatments of 1 μs pulse duration applied at 250 Hz in bipolar mode at 35 kV/cm for 1000 μs. Regarding the stability of vitamin C through the storage, the concentration of vitamin C decreased over time in both heat (90ºC for 60s) and PEF-treated (35 kV/cm for 1500 μs with 4 μs bipolar pulses at 100 Hz) tomato juices following an exponential trend. In addition, these works demonstrated vitamin C is better retained in PEF treated juices than in those thermally processed after 56 days at 4 ºC. Oxidation of ascorbic acid occurs mainly during the processing of juices and depends upon many factors such as oxygen presence, heat and light. Most differences between PEF and heat treatments can be explained through the temperatures reached through processing. Ascorbic acid is a heat-sensitive bioactive compound in the presence of oxygen. Thus, high temperatures during processing can greatly affect the rates of its degradation through an aerobic pathway (Odriozola-Serrano et al., 2008a). Studies in tomato juice showed that HP processing (300 and 500 MPa) could not preserve vitamin C and the depletion of vitamin C after HP treatment is dependent mainly on temperature intensity and treating time (Hsu et al., 2008). A long exposure (up to 6 h) to extreme pressure/temperature combinations (e.g., 850 MPa combined with temperatures from 65 to 80 ºC) degraded AA to a large extent (Oey et al., 2008). In tomato puree, a 40% and 30% decrease, respectively, in the content of ascorbic acid (AA) and total AA was observed after HP treatment of 400 MPa/25 ºC/15 min (Sánchez-Moreno et al., 2006).

Processing by using non thermal technologies may be advantageous regarding the amount and stability of carotenoids. For instance, lycopene concentrations in PEF-processed tomato juice have been found to be higher than those found in untreated juices (Odriozola-Serrano et al., 2008b). Consistently, Sánchez-Moreno, et al. (2005a) observed that the content of total carotenoids in a tomato-based cold soup 'gazpacho', increased roughly a 62% after applying bipolar 4-μs pulses of 35 kV/cm for 750 μs at 800 Hz. Odriozola-Serrano et al. (2009) have observed that β-carotene in treated tomato juice undergo a significant increase (31%-38%), whereas γ-carotene content is depleted (3%-6%) after a PEF treatment (35 kV/cm for 1500 μs with 4 μs bipolar pulses at 100 Hz). Authors suggested that a plausible explanation for this fact is that γ-carotene may undergo cyclization to form six membered rings at one end of the molecule, giving β-carotene as a product. During storage, PEF-processed tomato juices better maintained the individual carotenoid content (lycopene, neurosporene and γ-carotene) than thermally-treated and untreated juices for 54 days at 4 ºC (Odriozola-Serrano et al., 2009). Individual carotenoids with antioxidant activity (β-carotene, β-cryptoxanthin, zeaxanthin and lutein) appeared to be resistant to a HP treatment of 400 MPa at 40 °C for 1 min, thus resulting into a better preservation of the antioxidant activity of a tomato-based soup with respect to the thermally pasteurized (Sánchez-Moreno et al., 2005b). It seems that high pressure influences the extraction yield of carotenoids. A significant increase in the measured carotenoid content of pressurized (400 MPa/25 ºC/15 min) compared to either thermal treated or untreated tomato purée was observed by Sánchez-Moreno et al. (2006). Due to this potential, HP technology has also been studied to extract lycopene from tomato paste waste (Jun, 2006). In contrast, other studies have not reported major effects of HP treatments on tomato products. García et al. (2001) treated a tomato homogenate for 5 min with 500 and 800 MPa and did not find any influence on the total lycopene and β-carotene concentration. Barba et al. (2010) reported that total carotenoids are particularly affected by HP, having the unprocessed vegetable beverages made with tomato, green pepper, green celery, onion, carrot, lemon and olive oil, a higher total carotenoids content than HP-

processed samples. The apparently inconsistent results may be explained through the combined effect of pressure and temperature.

Tomato juices have been found to be a rich source of flavonoids, containing as the main flavonols quercetin and kaempferol, and minor phenolic acids such as ferulic, *p*-coumaric, caffeic acid, etc. PEF processing (35 kV/cm for 1500 μs with 4 μs bipolar pulses at 100 Hz) and thermal treatments (90 ºC 30 s and 90 ºC 60 s) did not affect phenolic content of tomato juices. Both PEF- and heat-treated tomato juices undergo a substantial loss of phenolic acids (chlorogenic and ferulic) and flavonols (quercetin and kaempferol) during 56 days of storage at 4ºC. Caffeic acid content was slightly enhanced over time, regardless the kind of processing, whereas PEF and heat treated tomato juices underwent a substantial depletion of *p*-coumaric acid during storage (Table 1). The increase of caffeic acid in tomato juices after 28 days of storage could be directly associated with residual hydroxylase activities, which convert coumaric acid in caffeic acid. Total phenolics in tomato based beverages and tomato purées appeared to be relatively resistant to the effect of HP (Patras et al., 2009; Barba et al., 2010).The effect of ionizing radiation on the phenolic content of tomatoes has also been studied. The gamma-radiation treatment (2, 4, and 6 kGy) markedly reduced the concentration of the phenolic compounds (*p*-hydroxybenzaldehyde, *p*-coumaric acid, ferulic acid, rutin and naringenin) in tomatoes (Schindler et al., 2005).

	Phenolic compound	Process	Phenolic retention (%) after 56 days of storage at 4 ºC
Phenolic acids	Chlorogenic	HIPEF	86
		TT	79
	Ferulic	HIPEF	67
		TT	69
	p-coumaric	HIPEF	53
		TT	53
	Caffeic	HIPEF	132
		TT	118
Flavonols	Quercetin	HIPEF	80
		TT	64
	Kaempferol	HIPEF	82
		TT	75

HIPEF: High intensity pulsed electric fields treatment at 35 kV/cm for 1000 μs; bipolar 4-μs pulses at 100 Hz; TT: thermal treatment at 90 ºC for 60 s

Table 1. Phenolic acid and flavonols retention during storage for 56 days at 4 ºC of tomato juices stabilized by heat or HIPEF treatments. Adapted from Odriozola-Serrano et al. (2009)

2.2.2 Orange

Ascorbic acid is a heat-sensitive bioactive compound in the presence of oxygen. Thus, high temperatures during processing can greatly affect the rates of its degradation through an aerobic pathway. Storage conditions such as storage temperature or oxygen concentration may have a significant influence on the rates of vitamin C degradation. Vitamin C is usually degraded by oxidative processes which are stimulated in the presence of light, oxygen, heat

peroxides and enzymes (especially ascorbate oxidase and peroxidase). Many authors have reported that vitamin C in different fruit and vegetable products is not significantly affected by HP processing (Sánchez-Moreno et al., 2009). Sánchez-Moreno et al. (2006) reported 91% retention of ascorbic acid in orange juice after HP processing at 400 MPa/40 ºC/1min. In addition, HP orange juices (400 MPa/ 40 ºC/ 1 min) maintained better the vitamin C during more days of refrigerated storage than low pasteurized treated juice (70 ºC/30 s) (Plaza et al., 2006). However, differences in vitamin C pressure stability during storage could be explained by the initial oxygen content and possible endogenous pro-oxidative enzyme activity. The effects of ultrasonication on the vitamin C content of orange juice have also been studied. Degradation of vitamin C in sonicated orange juices was observed and the degradation level depended on the wave amplitude and treatment time (Tiwari et al., 2009a). Increased shelf life based on ascorbic acid retention was found for sonicated orange juice compared to thermal processed samples at 98 ºC for 21 s due to higher processing temperature (Tiwari et al., 2009a).

Some studies have demonstrated that carotenoid content is increased significantly after PEF processing compared to the untreated orange juices. Cortés et al. (2006) observed that the carotenoid concentration in orange juice rose slightly after applying intense PEF treatments of 35 and 40 kV/cm for 30-240 μs. Carotenoid concentration rose as treatment time increased when HIPEF treatments of 25 or 30 kV/cm were applied to orange-carrot juice (Torregrosa et al., 2005). It has been reported that thermal treatment may imply an increase in some individual carotenoids owing to greater stability, enzymatic degradation, and unaccounted losses of moisture, which concentrate the sample (Rodriguez-Amaya, 1997). However, carotenoids are highly unsaturated compounds with an extensive conjugated double-bonds system and they are susceptible to oxidation, isomerisation and other chemical changes during processing and storage. Cortés et al. (2006) reported a significant decrease in total carotenoids of orange juice when applying bipolar treatments of 30 kV/cm for 100 μs. This decrease in provitamin A carotenoids could be correlated with a significant decrease in vitamin A by 7.52% in high-intensity PEF-treated orange juice (30 kV/cm, 100 μs) and by 15.62% in a pasteurized orange juice (90ºC, 20 s). Moreover, PEF processing (35 kV/cm, 750 μs) or thermal treatments (70 ºC, 30 s and 90 ºC, 30 s) did not exert any effect on vitamin A content of an orange juice (Sánchez-Moreno et al., 2005a). Research efforts have been made to obtain fruit and vegetable juices by HP processing without the quality and nutritional damage caused by heat treatments (Sánchez-Moreno et al., 2009). Individual carotenoids with antioxidant activity (β-carotene, β-cryptoxanthin, zeaxanthin and lutein) appeared to be resistant to a HP treatment of 400 MPa at 40 °C for 1 min, thus resulting into a better preservation of the antioxidant activity of orange juices with respect to those thermally pasteurized (Sánchez-Moreno et al. 2005a). Interestingly, an orange juice treated at 350 MPa/30ºC/5 min exhibited a higher carotenoid content (α-carotene, 60%; β-carotene, 50%; α-cryptoxanthin, 63%; β-cryptoxanthin, 42%) than a freshly squeezed juice (De Ancos et al., 2002), which was attributed to the desnaturation of the carotenoid-binding protein induced by pressure. Regarding the stability of carotenoids through storage of HP-pasteurized juices, non significant changes have been reported for at least 10 days of refrigerated storage in an orange juice treated at 100MPa/60ºC/5 min, whereas substantial losses were found at the end of the storage period of samples processed at 350MPa/30ºC/2.5 min or 400MPa/40ºC/1 min (20.56% and 9.16%, respectively). Plaza et al. (2010) reported that HP-treated orange juice showed a higher content in carotenoids than heat pasteurized juice during refrigerated

storage at 4 ºC. In consequence, vitamin A values showed an increase above 40% the value of the untreated sample. The inactivation of enzymes that caused losses of carotenoids during storage and the improvement of the extraction caused by HP treatments are the reasons exposed by some authors to explain that results (De Ancos et al., 2002).

Flavonoids are the most common and widely distributed group of plant phenolics. Among them, flavones, flavonols, flavanols, flavanones, anthocyanins, and isoflavones are particularly common in fruits. In this way, Sánchez-Moreno et al. (2005a) evaluated the effect of a PEF treatment at 35 kV/cm for 750 μs with 4-μs bipolar pulses at 800 Hz on the flavanone content of orange juice. No changes in the total flavanones were observed, nor in the individual flavanone glycosides and their aglycons hesperetin and naringenin. Recent results show that HP processed orange juice (400 MPa, 40 ºC, 1 min) presented a significant increased on the extractability of each individual flavanone with regard untreated juice and hence on total flavanone content whereas mild pasteurization (70 ºC, 30 s) treatments retained similar levels to those found in untreated juices (Plaza et al., 2010). Regarding the main flavanones identified in orange juice, HP treatments (400 MPa/40 ºC/1 min) increased the content of naringenin by 20% and by 40% the content of hesperetin in comparison with an untreated orange juice (Sánchez-Moreno et al., 2005a). The increase in the extractability of flavanones by Plaza et al. (2010) in HP orange juice happened at beginning due to treatment. Thus, during refrigerated storage at 4 ºC, flavanone content in HP juice decreased around 50% during the first 20 days of storage at 4 ºC. The degradation of phenolic compounds during storage has been mainly related to the residual activity of polyphenol oxidase (PPO) and peroxidase (POD) (Odriozola-Serrano et al., 2009).

2.2.3 Berries

Different studies have proven the effectiveness of PEF in achieving higher vitamin C content in comparison with heat treatments in berries. Odriozola-Serrano et al. (2008a) reported that vitamin C retention just after treatment in heat-processed (90ºC, 60s) strawberry juice was significantly lower (94%) than that found in a juice treated at 35 kV/cm for 1700 μs in bipolar 4 μs pulses at 100 Hz (98%). Low processing temperatures reached through PEF-processing (T<40 ºC) would explain the higher retention of vitamin C in HIPEF-treated strawberry juice compared to the thermally processed samples. The concentration of vitamin C in thermally and PEF-processed juices decreased gradually with storage time. Although during 21 days of storage the concentration of vitamin C was similar among processed strawberry juices, beyond this day, juices subjected to thermal treatment at 90 ºC for 60 s exhibited lower vitamin C content compared to PEF-treated juices (Odriozola et al., 2008c). Recommended daily intake (RDI) of vitamin C is currently revised but should be never below 60 mg, as established by the U.S. Food and Drug Administration (FDA, 1999). According to this recommendation, a strawberry juice 250 mL serving size should contain 24 mg/100 mL in order to contribute to the 100% of the RDI. Vitamin C content of juice processed with either PEF or heat at 90 ºC for 30 s felt below the RDI at 35 days of storage. The concentration of vitamin C in juices treated at 90 ºC for 60 s was reduced below 24 mg/mL after 28 days of storage at 4 ºC. As compared to fruit based products, a high residual ascorbic acid concentration after HP treatment is mostly found. In berries, a high retention of AA in strawberry nectar was observed after HP treatment at 500 MPa/room temperature/3 min (Rovere et al., 1996). Changes of vitamin C content in pressure treated

food products during storage have been followed. It is suggested that further vitamin C degradation after HP processing during storage could be reduced by lowering storage temperature, for example, in pressurized (500 MPa/room temperature/3 min) strawberry nectar (Rovere et al., 1996). A kinetic study on degradation of vitamin C in pressure treated strawberry coulis has shown that a pressure treatment neither accelerated nor slowed down the kinetic degradation of ascorbic acid during subsequent storage. Sancho et al. (1999), observed identical kinetics of vitamin C degradation in pressurized (400 MPa/20 ^{o}C/30 min) and untreated coulis during storage at 4 ^{o}C. In general, it can be concluded that ascorbic acid is unstable at high pressure levels combined with high temperatures (above 65 ^{o}C) and the major degradation is caused by oxidation especially during adiabatic heating. Therefore, eliminating the oxygen content in packaging can decrease the ascorbic acid degradation during processing and subsequent storage (Oey et al., 2008). The effects of ultrasonication on the vitamin C content of juices have been also studied. Degradation of vitamin C in sonicated strawberry juices was observed and the degradation level depended on the wave amplitude and treatment time (Tiwari et al, 2009b). Ascorbic acid degradation during sonication may be due to free radical formation and production of oxidative products on the surface of bubbles (Tiwari et al., 2009a). During refrigerated storage, similar vitamin C depletion in strawberry juice was observed in both sonicated and untreated juices (Tiwari et al., 2009b).

Flavonoids are the most common and widely distributed group of plant phenolics. Among them, flavones, flavonols, flavanols, flavanones, anthocyanins, and isoflavones are particularly common in fruits. In strawberry juices, *p*-hydroxybenzoic content was enhanced slightly but significantly after PEF processing (35 kV/cm for 1700 μs in bipolar 4-μs pulses at 100 Hz) compared to the untreated juice, whereas ellagic acid was substantially reduced when the heat treatment was conducted at 90^{o}C for 60 s. No significant differences in flavonol (kaempferol, quercetin and myricetin) content were obtained between fresh and treated strawberry juices; thus, these phenolic compounds were not affected by processing (Odriozola-Serrano et al., 2008c). It is well known that anthocyanins are unstable pigments and can be decolorized and degraded by many factors such as temperature, pH, oxygen, enzymes, light, the presence of copigments and metallic ions, ascorbic acid, sulphur dioxide and sugars. Numerous studies have evaluated the stability of anthocyanins to different kinds of nonthermal processing. Zhang et al. (2007) observed that after processing a cyanidin-3-glucoside methanolic solution by PEF (300 pulses of 1.2-3 kV/cm, $T^{a} \leq 47$ ^{o}C), the anthocyanin was degraded and the formation of the colorless anthocyanin species, particularly chalcones took place. In real foods such as strawberry juice, it was suggested that the lower the treatment time and the higher the electric field strength, the greater the anthocyanin retention in strawberry juice (Odriozola-Serrrano et al., 2008b). Contrarily, Zhang et al. (2007) reported that the degradation of cyanidin-3-glucoside in blackberry increased as the electric field strength rose. Jin and Zhang (1999) indicated that the losses of anthocyanins in PEF-treated cranberry juice increased during the storage period at 4 ^{o}C. However, the content over time was higher that of thermally-treated samples, which contained the lowest amount of anthocyanins after two weeks of storage. Pelargonidin-3-glucoside and pelargonidine-3-rutinoside content and, in turn, total anthocyanins of strawberry juices, depleted with storage time after PEF (35 kV/cm for 1,700 μs in bipolar 4-μs pulses at 100 Hz) and thermal treatments (90^{o}C for 60 or 30 s) (Odriozola-Serrano et al., 2008b) (Figure 1). Changes in anthocyanins content throughout storage of PEF-treated juices

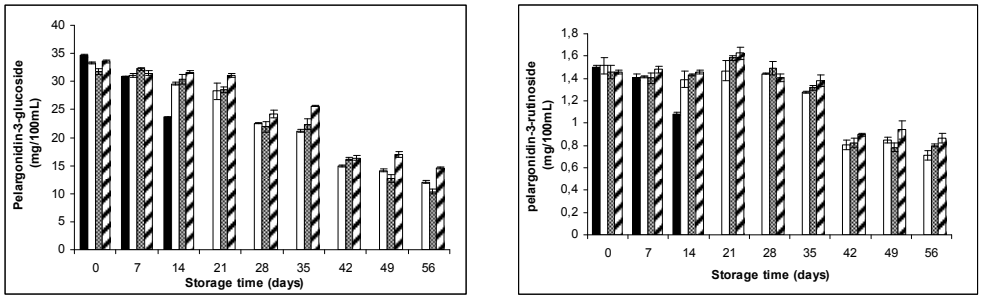

Fig. 1. Effect of HIPEF and heat processing on pelargonidin-3-glucoside (a) and cyaniding-3-glucoside of strawberry juices throughout storage at 4 ºC. Strawberry juices: (■) untreated, (□) heat treated at 90 ºC for 30 s, (▩) heat treated at 90 ºC for 60 s and (◪) HIPEF treated at 35 kV/cm for 1700 µs in bipolar 4-µs pulses and 100 Hz. Data shown are mean ± standard deviation. Adapted from Odriozola-Serrano et al. (2008c)

were associated to the presence of residual enzyme activities such as β-glucosidase (Aguiló-Aguayo et al., 2008). HP treatment at ambient temperature has been reported to have minimal effects on the anthocyanins content of various food products (Oey et al., 2008). Anthocyanins have been reported to be stable to HP treatments in different products such as strawberry juice (Zabetakis et al., 2000), blackcurrant juice (Kouniaki et al., 2004) and raspberry juice (Suthanthangjai et al., 2004). No significant changes in anthocyanin content of strawberry and blackberry purées after 15 min treatments at 500-600 MPa was also reported by Patras et al. (2009). Similarly, cyanidin-3-glucoside, a predominant anthocyanin in berries, was found to be stable in a model solution at 600 MPa for 30 min at 20 ºC. However, 30 min application of 600 MPa at 70 ºC reduced cyanidin-3-glucoside by 25%, whereas only 5% was lost after 30 min heating at the same temperature and ambient pressure (Corrales et al., 2008). Combined pressure and temperature treatment of blueberry pasteurized juice led to a slightly faster degradation of total anthocyanins during storage compared to heat treatments at ambient pressure (Buckow et al., 2010). Thus, pressure seems to accelerate anthocyanin degradation at elevated temperatures. This could be related to condensation reactions involving covalent association of anthocyanins with other flavanols present in fruit juices. During refrigerated storage, the stability of anthocyanins in HP-treated fruit juices at moderate temperatures has been related to the residual PPO and POD activities, a sufficient activity for rapid oxidation of anthocyanins and other polyphenols in the presence of oxygen. Ultrasonication may be also considered a potential technology for processing of red juices because of its minimal effect on anthocyanins. Tiwari et al. (2009b) reported a slight increase (1-2%) in the pelargonidin- 3-glucoside content of the juice at low acoustic energy density (0,33 W/mL) and treatment time (3 min) which may be due to the extraction of bound anthocyanins from the suspended pulp. Some authors have also studied the effects of non ionizing radiation in phenolic content of berries. UV-C doses at 0.25 and 1.0 kJ/m^2 increased anthocyanins concentrations in the fresh strawberries (Baka et al., 1999). Also, UV-C treatment for different durations (1, 5, and 10 min) increased the antioxidant capacity and the concentrations of anthocyanins and phenolic compounds of strawberries (Erkan et al., 2008). Related to ionizing radiation, gamma-radiation (1-10 kGy) led to the degradation of cinnamic, *p*-coumaric, gallic, and hydroxybenzoic acids

(Breitfellner et al., 2002a). The hydroxylation (decomposition) of these phenolic acids has been attributed to the formation of free hydroxyl (OH·) radicals during the treatment. Catechin and kaempferol components also diminished noticeably due to gamma-radiation (1-6 kGy), whereas ellagic acid derivatives and quercetin concentrations were not affected by the treatment in strawberries (Breitfellner et al., 2002b).

2.2.4 Fruit juice-milk beverage

Fruit juice and milk beverage is a product in which the antioxidant capacity of fruit constituents can be delivered in combination with the health benefits of milk. Morales et al. (2010a) did not found significant differences in vitamin C retention (87–90%) between (35 kV/cm, 4 μs bipolar pulses at 200 Hz for 800 or 1400 μs) and thermally treated (90 ºC, 60 s) blend fruit juice-soymilk beverages. In addition, the vitamin C content of the beverages decreased gradually during storage, regardless of the treatment applied. However, throughout the first 31 days, vitamin C was better maintained in the 800 μs-PEF treated fruit juice-soymilk beverages (46.4%) than in those treated for 1400 μs (22.6%); whereas, those that where thermally treated showed the lowest retention of vitamin C (6.7%) (Morales et al., 2011). These results showed that the shorter the PEF treatment time, the higher the vitamin C retention, as previously found in other studies focused on individual fruit juices treated by PEF. The higher retention of vitamin C of PEF treated fruit juice-soymilk beverages compared to those thermally treated might be due to the lower processing temperatures achieved during PEF treatments (<32 ºC). Currently, few studies have been carried out about the effect of HP processing on phytochemicals of fruit juice–milk beverages. High ascorbic acid retention (91%) in the orange juice-milk beverage after HP (100-400 MPa/120-540 s) treatment was reported by Barba et al. (2011).

Initial degradation of ascorbic acid was less in orange juice-milk beverages treated by PEF (25 kV/cm and 280 μs) than in a heat-treated (90 ºC, 20 s) juice (Zulueta et al., 2010a). During storage, total carotenoid content of untreated and treated blend of fruit juice-soymilk beverage tended to decrease as the storage time increased (Morales et al., 2011). Moreover, thermally treated juices showed higher rate of degradation than those PEF-treated. Although the pathways of carotenoids degradation have not been well established, oxidation is the main cause of carotenoid loss, which is a spontaneous free-radical chain reaction in the presence of oxygen (Sánchez-Moreno et al., 2003). During autooxidation of carotenoids, alkylperoxyl radicals are formed and these radicals attack the double bounds resulting in formation of epoxides (Odriozola-Serrano et al., 2009). Total carotenoid content was significantly enhanced in orange juice-milk beverage treated by HP (100-400 MPa) when treatment time was 420 and 540 s in comparison with the unprocessed samples (Barba et al., 2011). According to these authors, this may be because when pressures of 100 MPa are applied, they are sufficient to cause breakage of the intracellular vacuoles and the cell walls of the plant or they also suggested that an increase in free carotenoids in juices after HP might be because there is probably an alteration in the structure of the proteins that are linked to the carotenoids. With regard to individual carotenoid concentrations, there were no significant differences for any of them (Neoxanthin + 9-cis-violaxanthin, Mutatoxanthin, Lutein, Zeaxanthin, β-Cryptoxanthin, α-Carotene, Phytoene+phytofluene, β-Carotene, ζ-Carotene, 15-cis-β-Carotene), and only in the case of the electric field strength of 35 kV/cm there was significant increase in ζ-carotene in comparison with the untreated beverage after

60 μs of treatment. Although the reductions in carotenoids with provitamin A activity are very small after pasteurization, the decreases in the concentrations of lutein (22.8%) and zeaxanthin (22.5%) after pasteurizing are considerable and must be taken into account, because these two xanthophylls play a fundamental part in sight, prevent degenerative eye diseases, and are antioxidant compounds that give quality to food products (Zulueta et al., 2010b).

Coumaric acid, narirutin and hesperidin were the most abundant phenolic compounds in a blend fruit juice-soymilk beverage. Immediately after PEF (35 kV/cm with 4 μs bipolar pulses at 200 Hz for 800 or 1400 μs) or thermal (90 ºC, 60 s) treatments, hesperidin content of the beverage showed a huge rise, resulting in a significant increase on the total phenolic concentration. In addition, total phenolic concentration seemed to be highly stable during refrigerated storage (Morales et al., 2011). According to these authors, changes observed on the phenolic content of the fruit juice-soymilk beverage after PEF or thermal treatments could be due to some of the followed reasons: (i) biochemical reactions could have occurred during the PEF or heat processing, which led to the formation of new phenolic compounds; (ii) PEF or thermal processing might have caused significant effects on cell membranes or in phenolic complexes with other compounds, releasing some free phenolic acids or flavonoids; (iii) PEF and thermal process may inactivate PPO, preventing further loss of phenolic compounds; and (iv) PEF treatment might have induced favorable conditions to increase PAL activity, resulting in an enhancement of phenolic concentration in the beverage. Levels of total phenolic compounds also increased significantly by HP in processed orange juice–milk; reaching a maximum at 100 MPa/420 s, when there was a significant increase of 22% in comparison with unprocessed samples (Barba et al., 2011). As it was mentioned before, the increase in total phenolic content may be related to an increased extractability of some of the antioxidant components following high-pressure processing.

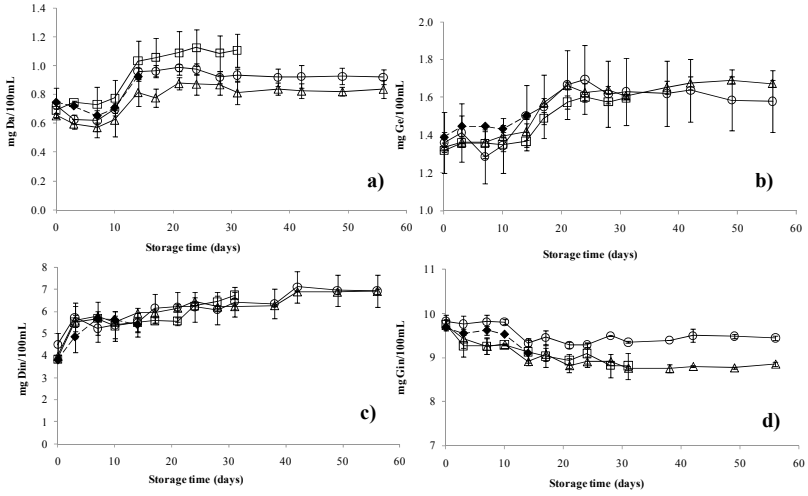

Fig. 2. Individual isoflavone profile: a) daidzein, b) genistein, c) daidzin and d) genisitin of untreated (dotted line, ♦), high intensity pulsed electric field (35 kV/cm with 4 μs bipolar pulses at 200 Hz for 800 (continue line, □) or 1400 μs (continue line, △)) and thermal (continue line, ○) (90 ºC, 60 s) treated fruit juice-soymilk beverages throughout storage at 4ºC (Morales et al., 2010b)

During the last years, soy beverages consumption has gradually increased due to their significant concentration of health-promoting compounds, such as isoflavones. PEF seems to be a good technology in order to obtain fruit juice-soymilk beverage with a high content of isoflavones and fresh-like characteristics. In a blend fruit juice-soymilk beverage, PEF treatment (35 kV/cm with 4 μs bipolar pulses at 200 Hz for 800 or 1400 μs) did not caused significant changes on the total isoflavone content and, during the storage period, total isoflavone content tended to increase throughout the time. Genistein, daidzein and daidzin content increased; while genistin showed a slight decrease, irrespective of the treatment applied (Figure 2) (Morales et al., 2010b). These authors suggested that the concentration of some isoflavones in the fruit juice-soymilk beverage might increase during storage from the flavonoids (mainly naringenin from orange) present in the fruits used for the elaboration of the beverage. Nevertheless, there is a need for more in-depth research to provide biochemical evidence of the observed changes.

2.2.5 Broccoli

Isothiocyanates are organosulfur compounds formed by enzyme-catalysed hydrolysis of glucosinolates, which are largely found in vegetables of the Brassicaceae family. Some isothiocyanates have shown anticarcinogenic potential (Conaway et al., 2002). Knowledge about the impact of non thermal technologies on the stability of these compounds is really scarce. Van Eylen et al. (2007) studied the pressure (600-800 MPa) and temperature (30-60ºC) stabilities of sulforaphane and phenylethyl isothiocyanate in broccoli juice. Authors concluded that isothiocyanates are relatively thermolabile and pressure stable. At the same time, mild pressure treatments where suggested as the most advantageous, because myrosinase activity is stabilized, thus leading to products with increased isothiocyanate content. In a subsequent study, Van Eylen en al. (2008) observed that the composition of glucosinolate hydrolysis products may greatly differ between different HP process conditions. Upon this base, HP treatments can be selected to optimise the health beneficial properties of plant foods.

The non ionizing radiation treatments have been shown to increase the antioxidant capacity of broccoli, which could be useful from the nutritional point of view. UV-C (4-14 kJm^{-2}) treated broccoli florets displayed lower total phenolic and flavonoid content along with higher antioxidant capacity compared to the control samples (Costa et al., 2006). On the other hand, exposure to UV-C (8 kJm^{-2}) increased total phenolic and ascorbic acid contents, as well as the antioxidant capacity of minimally processed broccoli (Lemoine et al., 2007) These authors related, an increment in the activity of PAL after treatment with UV-C to an increase in the content of phenolic compounds in treated samples since PAL is one of the key enzymes in phenolic synthesis.

2.2.6 Others

As it was mentioned before, PEF processing may allow obtaining juices with higher antioxidant potential and extended shelf-life, thus becoming a feasible alternative to heat processing. PEF processing may help to achieve fresh-like carrot juices with increased amounts of health-related phytochemicals. PEF processing (35 kV/cm for 1500 μs with 6-μs bipolar pulses at 200 Hz) resulted into a carrot juices with significantly greater vitamin C retention of 95.1% than thermal processing (90 ºC, 30 s and 90 ºC, 60 s), which exhibited a

retention of 86.6–89.0% (Quitao-Teixeira et al., 2009). Watermelon juice was subjected to high-intensity pulsed electric fields (HIPEF). The effects of process parameters including electric field strength (30–35 kV/cm), pulse frequency (50–250 Hz), treatment time (50–2050 μs), pulse width (1–7 μs) and pulse polarity (monopolar/bipolar) on lycopene, vitamin C and antioxidant capacity were studied using a response surface methodology (Oms-Oliu et al., 2009). Watermelon juices treated at 25 kV/cm for 50 μs at 50 Hz using mono- or bipolar 1-μs pulses exhibited the highest vitamin C retention (96.4–99.9%). On the other hand, vitamin C loss was higher than 50% when PEF treatment was set up at 35 kV/cm for 2050 (s at 250 Hz applying mono- or bipolar 7-μs pulses. Such severe conditions seem to greater affect vitamin C retention in watermelon juice than in other juices such as orange, orange–carrot or strawberry juices, which exhibited retention of vitamin C above 80%, because more acidic conditions are known to stabilise vitamin C (Oms-Oliu et al., 2009). During storage, vitamin C was better retained in the PEF-treated carrot juice than in the thermally processed juices for 56 days at 4 ^{o}C. Differences in vitamin C reduction between PEF and heat treated juices throughout the storage might be due to the activity of enzymes such as ascorbate oxidase. A first-order kinetic model adequately fitted vitamin C depletion (R^2 = 0.9680–0.838; Af = 1.039–1.068) as a function of the storage time (Quitao-Teixeira et al., 2009). Storage conditions such as temperature or the oxygen concentration may also have a significant influence on the rates of vitamin C degradation. Sonication also showed to increase vitamin C content in sonicated (ficar les condicions) guava juice compared to untreated sample, the most likely reason being the elimination of dissolved oxygen that is essential for ascorbic acid degradation during cavitation (Cheng et al., 2007). Research reporting on the impact of ionizing radiation on vitamins of plant-derived products is restricted to the effect of gamma-radiation on vitamin C. In general, most fresh-cut vegetables (iceberg, romaine, green and red leaf lettuce, spinach, tomato, cilantro, parsley, green onion, carrot, broccoli, red cabbage, and celery) can tolerate up to 1 kGy radiation without significant losses in vitamin C content (Fan and Sokorai, 2008). In the case of minimally processed irradiated cucumber and carrot, no significant differences between the vitamin C content of control and treated samples were reported through refrigerated storage (Khamat et al., 2005; Hajare et al., 2006). Minor vitamin C losses were reported for minimally processed refrigerated capsicum after gamma-radiation (1-3 kGy) during storage (Ramamurthy et al., 2004). Vitamin C content of irradiated fresh-cut celery (0.5-1.5 kGy) or lettuce (1 kGy) during refrigerated storage was higher than in non-irradiated products (Lu et al., 2005; Zhang et al., 2006). Regarding fresh-cut fruits, Fan et al. (2006), reported that vitamin C of cantaloupe was not substantially affected by treatment with non ionizing radiation. No differences have been reported between treated and untreated products regarding the stability of vitamin C during storage. On the other hand, light treatments applied as short pulses with a total fluence of 4.8 and 12 J cm^{-2} maintained amounts of vitamin C similar to those found in untreated fresh-cut mushrooms during 7 days of refrigerated storage under modified atmosphere packaging (Oms-Oliu et al., 2010).

An increase in lycopene (114%) was observed in watermelon juice treated with 7-μs bipolar pulses for 1050 μs at 35 kV/cm and frequencies ranging from 200 to 250 Hz (Oms-Oliu et al., 2009). The increase in lycopene has been related to the conversion of some carotenoids to lycopene as a result of an intense PEF treatment. β-Carotene concentration substantially increased in processed carrot juices compared to the untreated juice and thermally treated juices. PEF-treated carrot juice maintained β-carotene content better than heat treatments

during 56 days of storage at 4 ◦C. The major cause of carotenoid losses in vegetable products is the oxidation of the highly unsaturated carotenoid structure. The severity of oxidation depends on the structure of carotenoids and the environmental conditions, and the compounds being formed may vary upon the oxidation process and the carotenoids structure (Quitao-Teixeira et al., 2009). Therefore, the higher depletion of β-carotene throughout the storage in thermally treated carrot juice compared to those PEF-processed might be due to the greater changes in carotenes structure as a consequence of high temperature. In addition, a better retention of carotenoids in HP treated carrot pureés compared to thermally processed samples was observed (Patras et al., 2009). Such effect has been well documented elsewhere and would appear to be related to an increase in extractability of antioxidant components following high pressure treatment rather than an absolute increase.

No significant differences in the amount of total phenolics were observed between untreated and PEF-treated products such as spinach puree (Yin et al., 2007) and carrot juice (Quitão-Teixeira et al., 2009) just after processing. It has been also reported that radiation treatments can generate free radicals, thus leading to an induction of stress responses in plant foods, which in turn may lead to an increase in the antioxidant synthesis. Results by Song et al. (2006) are consistent with this idea. These authors observed that total phenolic content of carrot and kale juices was substantially increased by applying a radiation treatment. However, reductions in the total phenolic content have been reported for treatments of more than 10 kGy in some irradiated products (Villavicencio et al., 2000; Ahn et al., 2005).

3. Conclusion

Non thermal technologies may allow obtaining safe and shelf-stable plant-based products with minor changes or increased content in health-related phytochemicals. Most differences between non thermal and heat treatments can be explained through the temperatures reached through processing. In general, temperature during processing and storage are important factors affecting the phytochemicals of the processed product. The stability of these compounds through storage is dependent in each case on the residual amounts of enzymes involved in their degradation. In addition, processing parameters are involved in the degradation or generation of bioactive compounds. In-depth research is needed in order to elucidate the mechanisms involved in the destruction or generation of these compounds in a food matrix processed by these novel technologies.

Few studies assessing the impact of non thermal technologies on the bioavailability of bioactive compounds reported an increase of plasma vitamin C and a decrease of the oxidative stress and inflammation biomarkers in healthy humans. Thus, new applications of non thermal processing technologies should be further explored not only to stabilize the content of health-related phytochemicals but also their bioavailability and biological activity in humans.

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6

Phytochemical Constituents and Activities of *Morinda citrifolia* L.

Duduku Krishnaiah*, Rajesh Nithyanandam and Rosalam Sarbatly
Universiti Malaysia Sabah,
Malaysia

1. Introduction

Both artificial and naturally occurring antioxidants have been reported to play major roles in protecting membranes and tissues from free radical and xenobiotic-induced oxidative damage (Burton, 1989; Carini et al., 1990). Most living organisms harbour both enzymatic and non-enzymatic systems that protect them against excessive reactive oxygen species. However, various external factors (smoke, diet, alcohol and some drugs) and aging decrease the efficiency of these protective systems, thereby disrupting the redox equilibrium that is established under healthy conditions. Thus, antioxidants that scavenge reactive oxygen species may be of great value in preventing the onset and propagation of oxidative diseases (Willet, 1994). Recently, more attention has been paid to the role of natural antioxidants, mainly phenolic compounds, which may have higher antioxidant activity than do conventional antioxidants, such as vitamins C, E and β-carotene (Vinson et al., 1995). The anti-oxidative effects of natural phenolic compounds, in pure form or in extracts from various plant sources (vegetables, fruits and medicinal plants), have been studied *in vitro* using a variety of model systems (Meyer et al., 1998; Pietta et al., 1998; Yen & Hsieh, 1998). Therefore, antioxidants, which can neutralize free radicals, may be of central importance in the prevention of carcinogenicity, cardiovascular disease and neurodegenerative changes associated with aging (Halliwell, 1994; Yu, 1994). Epidemiological studies have shown that the consumption of vegetables and fruits can protect humans against oxidative damage by inhibiting and/or quenching free radicals and reactive oxygen species (Ames et al., 1993).

Oxidative stress occurs commonly in living organisms, and it is involved in the pathology of cancer, arteriosclerosis, malaria, and rheumatoid arthritis. Moreover, it may play a role in neurodegenerative diseases and ageing processes. It has been demonstrated that many vegetables, fruits, medicinal plants and other foods contain compounds with bioactivity against oxidative stress, and this activity has been attributed to vitamin C, vitamin E, α-tocopherol, β-carotene, and polyphenolic compounds (Krishnaiah et al., 2011; Moure et al., 2001). Therefore, research regarding natural antioxidants from foods and plants, particularly from folk medicinal plants, is receiving increasing attention throughout the world.

2. *Morinda citrifolia* L.

The ancestors of the Polynesians are believed to have brought many plants with them, as food and medicine, when they migrated from Southeast Asia 2000 years ago. Of the 12 most

common medicinal plants they brought, *Morinda citrifolia* L. was the second most popular plant used in herbal remedies to treat various common diseases and to maintain overall good health. Other names of *Morinda citrifolia* L. include M. bracteata Roxb.; M. litoralis Blanco; Indian mulberry, Bengkudu, Mengkudu (Malay). It has been reported to have a broad range of health benefits for subjects with cancer, infections, arthritis, diabetes, asthma, hypertension and pain. The Polynesians utilised the whole *Morinda citrifolia* L. plant in their medicinal remedies and as a dye for some traditional clothing. The roots, stems, bark, leaves, flowers, and fruits of the *Morinda citrifolia* L. plant are all involved in various combinations in almost 40 known and recorded herbal remedies. Additionally, the roots were used to produce a yellow or red dye for tapa cloth and fala (mats), and the fruit was eaten for health and nutrition. There are numerous Polynesian stories of heroes and heroines that used *Morinda citrifolia* L. to survive famine. *Morinda citrifolia* has a long history of use as a food in tropical regions throughout the world.

It has also been reported to have broad therapeutic effects, including anti-cancer activity, in both humans and laboratory animal models. However, the mechanisms underlying these effects remain unknown. *M. citrifolia* is unique in view of the large number of medicinal claims that have been made for its efficacy and its rapidly evolving commercial success; nevertheless, little is known about its pharmacological potential compared with other popularly used botanicals.

M. citrifolia L., a shrub originating in tropical Asia or Polynesia, has been extensively used in folk medicine and as a dye in Asian countries. In the tropics, it seems to have been greatly valued medicinally, and the plant is normally cultivated for its roots, leaves and fruits. *M. citrifolia*, which grows prevalently in tropical regions, has recently gained a great deal of interest from scientists and medical professionals due to its pharmaceutical value. Wang et al. (2002) have published a review of *M. citrifolia* L. research that summarises the therapeutic effects of various compounds in this plant.

The *M. citrifolia* L. plant is a small evergreen tree found growing in open coastal regions at sea level and in forest areas up to approximately 1300 feet above sea level. The plant is often found growing along lava flows. Polynesians are reported to have successfully used *M. citrifolia* L. to treat breast cancer and eye problems. *M. citrifolia* has been tested for a number of biological activities in animal and anti-microbial studies and found that the dried fruit has smooth muscle stimulatory activity and histaminergic effects.

The roots of these plants are reported to be good sources of anthraquinones, which are usually present as aglycones and, to lesser extent, in the form of glycosides (Thomson, 1971; Zenk, El-Shagy & Schulte, 1975). Most parts of the tree have been widely used medicinally to relieve rheumatism and other pains and for their healing effects (Perry & Metzger, 1980).

Traditionally, the roots of *M. citrifolia* L. plants were used by Polynesians to produce yellow or red dye, but more importantly, they are now known to contain medicinally active components, such as anthraquinones, which due to their anti-oxidative activity, possess various therapeutic properties. These properties include anti-bacterial, anti-viral, and anti-cancer activities, as well as analgesic effects. These factors make the compounds potentially useful in several medical applications. An increasing number of studies are focusing on finding efficient methods for producing and extracting anthraquinones from these plants. Much of the literature also involves producing the compound in root cultures of *M. citrifolia*.

Nevertheless, extraction of anthraquinones directly from plant roots is still more widely conducted and is conventionally performed by solvent extraction. Other techniques, which include super critical carbon dioxide extraction, subcritical water extraction, ultrasonic-assisted extraction (UAE), and microwave-assisted extraction (MAE) have also become of interest as alternatives to the conventional methods.

2.1 Plant description

The genus Morinda (*Rubiaceae*), which includes the species *M. Citrifolia* L., is made up of around 80 species. *M. Citrifolia* is a bush or small tree, 3-10 m tall, with abundant broad elliptical leaves (5-17 cm length, 10-40 cm width).

Fig. 1. Unripe fruit

The small tubular white flowers are grouped together and inserted on the peduncle. The petioles leave ring-like marks on the stalks and the corolla is greenish-white (Morton, 1992; Elkins, 1998; Dixon et al., 1999; Ross, 2001; Cardon, 2003). The *M. citrifolia* L. fruit (3-10 cm length, 3-6 cm width) is ovular and fleshy with an embossed appearance (Fig. 1). It is slightly wrinkly, semi-translucent, and ranges in colour from green to yellow to almost white at the time of picking. It is covered with small reddish-brown buds containing the seeds. The ripe fruit emits a strong butyric acid-like, rancid smell (Morton, 1992; Dixon et al., 1999). The pulp is juicy and bitter, a light dull yellow or whitish colour, and gelatinous when the fruit is ripe; numerous hard triangular reddish-brown pits are found, each containing four seeds (approximately 3.5 mm) (Dittmar, 1993).

Moreover, *M. citrifolia* L. leaves are well known for their strong antioxidant activity, and they have been shown to be safe in acute, subacute, and subchronic oral toxicity tests on mice (West et al., 2007). Inspired by ancient Polynesian legends, *M. citrifolia* L. leaves have been developed into therapeutic teas. The leaves are also the source for a variety of other health-promoting commercial products. Commercial *M. citrifolia* L. leaf products have been available in Japan and United States for more than seven years, used mainly for making infusions. However, some manufacturers produce capsules containing powdered *M.*

citrifolia L. leaves. The major world-wide source of *M. citrifolia* L. leaves is French Polynesia because leaves from this nation having undergone a safety evaluation (West et al., 2007). Other sources include Panama, Fiji and Hawaii.

Recently, *M. citrifolia* L. fruit juice is in high demand as an alternative medicine due to its potential anti-microbial, anti-cancer, anti-inflammatory, and antioxidant effects (Wang et al., 2002). However, scientific evidence for the benefits of *M. citrifolia* L. fruit juice is still limited. In the past decade, *M. citrifolia* L. fruit juice has emerged on the worldwide market as a safe and popular health product due to its phytochemicals and nutrients. Written documentation of the consumption of this fruit as a food source precedes the twentieth century. Captain James Cook of the British Navy noted in the late 1700s that the fruit was eaten in Tahiti. An 1866 publication in London explained that *M. citrifolia* L. fruit was consumed as a food in the Fiji islands.

Later publications describe the use of this fruit as a food throughout the Pacific islands, Southeast Asia, Australia, and India. In Roratonga, "the fruit was often eaten by the natives". Australian Aborigines were reported to be "very fond" of the fruit. In Samoa, *M. citrifolia* L. fruit was common fare, and in Burma, the fruit was cooked in curries or eaten raw with salt. In 1943, Merrill described *M. citrifolia* L. as an edible plant in a technical manual of edible and poisonous plants of the Pacific islands, in which the leaves and fruits could be used as emergency food. Abbott also reported that *M. citrifolia* L. had been used as a food, drink, medicine, and colourful dye. The medicinal history and accumulated scientific studies have revealed and confirmed the Polynesians' claims regarding the health benefits of *M. citrifolia* L.

M. citrifolia L. has identifiable leaves, white tubular flowers, and a distinctive, ovoid, "grenade-like" yellow fruit. The fruit can grow in size up to 12 cm or more and has a lumpy surface covered by polygonal-shaped sections. The seeds, which are triangular and reddish brown, have an air sac contained at one end, making the seeds buoyant. This could explain, in part, the wide distribution of the plant throughout the Polynesian islands. The mature *M. citrifolia* L .fruit has a foul taste and odour. *M. citrifolia* L. is not considered to be at risk in the wild.

The fruit juice of *M. citrifolia* L. is in high demand in alternative medicine for various illnesses, such as arthritis, diabetes, high blood pressure, muscle aches and pains, menstrual difficulties, headaches, heart disease, Acquired Immune Deficiency Syndrome (AIDS), cancer, gastric ulcers, sprains, mental depression, senility, poor digestion, atherosclerosis, blood vessel problems and drug addiction (Kamiya et al., 2004; Wang et al., 2002). Therefore, one of the challenges in recent years has been to process fruit juice so as to make a more modern drug from a traditional product (Chunhieng et al., 2003). A number of *in vitro* biological activities have been reported, such as angiogenesis inhibition, antioxidant activity, inhibition of cycloxygenases-1 and -2, and tyrosine kinase inhibition. However, most of these have only been tested with crude extracts or fractions of *M. citrifolia* L., and the compound(s) responsible for these biological activities have not been fully determined, except for two compounds, neolignan and americanin A, which were identified in an n-butanol-soluble partition of the methanol extract of *M. citrifolia* L. fruits (Su et al., 2005; Zin et al., 2002).

M. citrifolia L. has recently been the object of many claims concerning its nutraceutical properties. Various publications have shown that *M. citrifolia* L. can be used to relieve multiple diseases, and its registered uses span the Pacific, Asia, and Africa. Two clinical studies have reported that relief from arthritis and diabetes are associated with *M. citrifolia* L. consumption (Elkins, 1998; Solomon, 1999). These beneficial effects may derive from certain compounds, such as scopoletin, nitric oxide, alkaloids and/or sterols, and they also may be due to the antioxidant potential of *M. citrifolia* L. As a result of this reputation, consumption of this fruit is currently high not only in the producing countries but also in the United States, Japan and Europe.

In response to this demand, some countries (such as Costa Rica and Cambodia) have increased their cultivation of *M. citrifolia* L. In these countries, the fruit is often commercialised fresh or as juice in both formal and informal markets, but it is also found as pasteurised juice, either in pure form or in combination with other juices (usually grape or blackberry juice). Commercial interest in *M. citrifolia* L. has increased tremendously in recent years, as indicated by the number of patents registered. In the United States alone, 19 patents have been registered by the US patent and Trademark Office since 1976 (USPTO, 2005). *M. citrifolia* L. juice has been accepted by the European Union as a novel food (European Commission, Scientific Committee for Food, 2002). Nevertheless, despite the real market opportunities, there has been little scientific research addressing the actual nutritional and functional properties of *M. citrifolia* L. products.

Several classes of compounds have been isolated from *M. citrifolia* L., including amino acids, anthraquinones, coumarins, fatty acids, flavonoids, iridoids, lignans and polysaccharides (Chan-Blan-co et al., 2006). Among these, scopoletin, a coumarin derivative, is one of the representative ingredients in *M. citrifolia* L. Its contribution to anti-microbial, anti-inflammatory, and antioxidative activities has been well elucidated (Deng et al., 2007). Samoylenko et al. (2006) recommended scopoletin as a constituent marker for *M. citrifolia* L. quality control. This compound has been ubiquitously found in *M. citrifolia* L. collected from Atlantic and Pacific regions and in all examined squeezed fruit juices. Determination of scopoletin in *M. citrifolia* L. might help to control the quality of *M. citrifolia* L. products. However, no reports have evaluated the antioxidative activity of scopoletin and other coumarin derivatives in *M. citrifolia* L.

Many reports on the antioxidative activity of *M. citrifolia* L. itself have been published. Assays of free-radical-scavenging activity with 1,1-diphenyl-2-picrylhydrazyl (Su et al., 2005; Yang et al., 2007), inhibition of copper-induced low-density lipoprotein oxidation (Kamiya et al., 2004), nitric oxide scavenging activity (Basu & Hazra, 2006) and quenching of H_2O_2 (Chong et al., 2004; Jeffers et al., 2007) have been performed to evaluate the antioxidative effects of *M. citrifolia* L. and its products. Polyphenols, reducing glycosides (Calzuola et al., 2006), lignin derivatives (Su et al., 2005) and anthraquinones (Chong et al., 2004) have been suggested as sources of antioxidative activity in *M. citrifolia* L. However, there is little available information with which to quantitatively evaluate the antioxidative activity of *M. citrifolia* L ingredients. Recently, a correlation between total phenol and free-radical-scavenging activity was reported ($r=0.41$, Yang et al., 2007).

The chemical components of *M. citrifolia* L. have not been well studied, and several anthraquinones and asperuloside are all that have been previously isolated (Levand &

Larson, 1979; Srivastava & Singh, 1993). For centuries, scientists and medical professionals have been investigating the chemical constituents in all parts of *M. citrifolia* (Noni or Yor), including leaves, fruit, bark and roots. The plants contain several medicinally active components that exhibit various therapeutic effects. These include anti-bacterial, anti-viral and anti-cancer activities as well as analgesic effects. Critical reviews of the therapeutic properties of the plants are given by Chan-Blanco et al. (2006) and Wang et al. (2002). Anthraquinones have been shown to be responsible for the therapeutic properties of *M. citrifolia* L., and among this group of compounds, damnacanthal, which is present mainly in the roots, is of particular interest due to its important anticancer activity (Hiramatsu et al., 1993).

Previous phytochemical studies revealed that *M. citrifolia* L. leaves contain a variety of phytochemical constituents, including terpenoids (Ahmad & Bano, 1980; Saludes et al., 2002; Takashima et al., 2007) phytosterols, fatty acids and their glycosides (Takashima et al., 2007) iridoids and their glycosides (Sang et al., 2001 a,b,c,d; Sang et al., 2003) and flavonol glycosides (Sang et al., 2001a).

Flavonol glycosides appear to predominate in *M. citrifolia* L. leaves; rutin and other flavonol glycosides have previously been identified in raw *M. citrifolia* L. leaves (Sang et al., 2001a). However, the presence of flavonol aglycones in *M. citrifolia* L. leaves has not been previously reported. Flavonoids have been indicated to possess a variety of biological activities (Garcia-Mediavilla et al., 2006; Kampkotter et al., 2007), and they may play an important role in *M. citrifolia* L. leaves. To date, there has been no validated analytical method for determining the flavonol constituents of *M. citrifolia* L. leaves.

2.2 Yield

M. citrifolia L. is a perennial bush, and it is possible to find fruits at different stages of maturity on the same plant at the same time. The species is generally found from sea level to 400 m, although it adapts better to coastal regions (Luberck & Hannes, 2001). Under favourable conditions, the plant bears fruit approximately nine months to one year after planting. At this stage, the fruits can be harvested, but they are generally small, and the yield per tree is low. Some producers choose not to harvest in the first year, and they prune in order to let the bush grow stronger. In Hawaii, *M. citrifolia* L. fruits are harvested throughout the year, although there are seasonal patterns in flowering and fruit bearing (meteorological factors, fumigation, and irrigation) (Nelson, 2001, 2003).

In Hawaii, *M. citrifolia* L. plots are usually harvested two or three times per month, although fruit production is lower during winter. With a density of 638 plants per hectare; good soil fertility, drainage, and irrigation; appropriate pest, disease and weed control; and an appropriate fertilisation plan, it is possible to obtain yields of 7 tonnes/ha/year after the fifth year (Nelson, 2001, 2003). With a juice extraction rate of approximately 50% (w/w), one hectare can thus yield around 35 tons of juice. However, many factors may affect these yields, and most producers do not obtain such good results because of diseases and/or poor agricultural practices (growing wide plants). In Hawaii, an average annual yield of 50 tonnes/ha is generally attained (Nelson, 2001, 2003).

Maturity stage	Colour	Firmness
1	Dark green	Very hard
2	Green-yellow	Very hard
3	Pale yellow	Very hard
4	Pale yellow	Fairly hard
5	Translucent-greyish	Soft

Table 1. Evolution of fruit skin colour and firmness in the course of ripening (adapted from Chan-Blanco et al., 2006)

Depending on the post-harvest technology programme adopted, the fruits may be harvested at different stages of development and continue to mature. The evolution of the colour and firmness of fruits left to ripen naturally on the tree is reported in Table 1. Nonetheless, most processors buy *M. citrifolia* L. harvested at the "hard white" stage for juice production as the fruits become soft too quickly once this stage is reached (Nelson, 2001, 2003). The change from stage 4 to 5 occurs very quickly (within a few hours), and the pulp practically liquefies and turns from green to white, as well as develops the characteristic butyric smell.

The fruits are individually selected on the tree and harvested by hand. At the "hard white" stage, they are well able to withstand being transported in baskets or containers, and exposure of the fruits to light or high temperatures immediately after harvest does not affect their overall quality. Before processing, fruits are ripened at room temperature for one day or more, depending on the end product (such as tea, juice, pulp, dietetic products) (Nelson, 2003).

2.3 Chemical composition of *M. citrifolia* L.

Approximately 160 phytochemical compounds have already been identified in the *M. citrifolia* L. plant, and the major micronutrients are phenolic compounds, organic acids and alkaloids (Wang & Su, 2001). Of the reported phenolic compounds, the most important are anthraquinones (such as damnacanthal, morindone, morindin.) and also aucubin, asperuloside, and scopoletin (Wang & Su, 2001). The main organic acids are caproic and caprylic acids (Dittmar, 1993), whereas the principal reported alkaloid is xeronine (Heinicke, 1985). Chan-Blanco et al. (2006) reviewed the chemical constituents of different parts of the plant (Table 2).

However, chemical composition differs significantly according to the part of the plant. The complete physico-chemical composition of the fruit has not yet been reported, and only partial information is available on *M. citrifolia* L. juice. The fruit contains 90% water, and the main components of the dry matter appear to be soluble solids, dietary fibre and proteins (Chunhieng, 2003). The fruit's protein content is surprisingly high, representing 11.3% of the juice dry matter, and the main amino acids are aspartic acid, glutamic acid and isoleucine (Chunhieng, 2003).

According to a book on Malaysian medicinal plants, the chemical constituents of *M. citrifolia* L. are 5,7-Acacetin-7-O-β-D(+)-glycopyranoside, ajmalicine isomers, alizarin, asperuloside, asperulosidic acid, chrysophanol (1,8-dihydroxy-3-methylanthraquinone), damnacanthol, digoxin, 5,6-dihydroxylucidin, 5,6-dihydroxylucidin-3-β-primeveroside, 5,7-dimethylapigenin-4'-O-β-D(+)-galacto pyranoside, lucidin, lucidin-3-β-primeveroside, 2-methyl-3,5,6-trihydroxy

Source (plant part)	Chemical constituent	References
Flower	2-methyl-4-hydroxy-5,7-dimethoxyanthraquinone 4-O-β-D-glucopyranosyl-(1-4)-α-L-rhamnopyranoside	Sang et al. (2002)
Flower	5,8-dimethyl-apigenin 4′-O-β-D-galacatopyranoside	Sang et al. (2002), Elkins (1998)
Flower	Aracetin 7-O-β-D-glucopyranoside	
Fruit	β-D-glucopyranose pentaacetate	Sang et al. (2002), Elkins (1998)
Fruit	2,6-di-O-(β-D-glucopyranosyl-1-O-octanoyl-β-D-glucopyranose	Dittmar (1993)
Fruit	6-O-(β-D-glucopyranosyl-1-O-octanoyl-β-D-glucopyranose	Wang et al. (1999)
Fruit	Ascorbic acid	Liu et al. (2001)
Fruit	Asperulosidic acid	Morton (1992), Elkins (1998), Wang et al. (2002), McClatchey (2002)
Fruit	Asperuloside tetraacetate	Wang et al. (1999), Liu et al. (2001), Cardon (2003)
Fruit	Caproic acid	Dittmar (1993)
Fruit	Caprylic acid	Sang et al. (2002), Dittmar (1993), Elkins (1998), Wang et al. (2002), Levend and Larson (1979)
Fruit	Ethyl caprylate	Solomon (1999), Dittmar (1993), Cardon (2003), Elkins (1998), Wang et al. (2002), Levand and Larson (1979)
Fruit	Ethyl caproate	Dittmar (1993)
Fruit	Hexanoic acid	Dittmar (1993)
Fruit	Octanoic acid	Farine et al. (1996), Sang et al. (2002)
Fruit	Quercetin 3-O-α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranoside	Farine et al. (1996), Sang et al. (2002), Cardon (2003), Wang & Su (2001)
Heartwood	Physcion 8-O-α-L-arabinopyranosyl-(1-3)-β-D-galactopyranosyl-(1-6)-β-D-galactopyranoside	Wang & Su (2001), Wang et al. (2002)
Leaves	Alanine	Sang et al. (2002), Srivastava & Singh (1993), Cardon (2003)
Leaves	Quercetin 3-O-α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranoside	Sang et al. (2002)
Leaves	Serine	Dittmar (1993), Elkins (1998)
Leaves	Threonine	Dittmar (1993), Elkins (1998)
Leaves	Tryptophan	Dittmar (1993), Elkins (1998)

Source (plant part)	Chemical constituent	References
Leaves	Tyrosine	Dittmar (1993), Elkins (1998)
Leaves	Ursolic acid	Sang et al. (2002), Cardon (2003), Elkins (1998), Wang et al. (2002)
Leaves	Valine	Dittmar (1993), Elkins (1998)
Plant	2-methyl-3,5,6-trihydroxyanthraquinone	Cardon (2003), Inoue et al. (1981)
Plant	2-methyl-3,5,6-trihydroxyanthraquinone 6-O-β-D-xylopyranosyl-(1-6)-β-D-glucopyranoside	Cardon (2003), Inoue et al. (1981)
Plant	3-hydroxymorindone	Cardon (2003), Inoue et al. (1981)
Plant	3-hydroxymorindone 6-O-β-D-xylopyranosyl-(1-6)-β-D-glucopyranoside	Cardon (2003), Inoue et al. (1981)
Plant	5,6-dihydroxylucidin 3-O-β-D-xylopyranosyl-(1-6)-β-D-glucopyranoside	Cardon (2003), Inoue et al. (1981)
Plant	5,6-dihydroxylucidin	Cardon (2003), Inoue et al. (1981)
Plant	Aucubin	Elkins (1998), Wang et al. (2002)
Plant	Linoleic acid	Wang et al. (2002)
Plant	Lucidin	Cardon (2003), Inoue et al. (1981), Ross (2001)
Plant	Lucidin 3-O-β-D-xylopyranosyl-(1-6)-β-D-glucopyranoside	Cardon (2003), Inoue et al. (1981)
Plant	Scopoletin	Farine et al. (1996), Wang et al. (2002)
Leaves	Arginine	Dittmar (1993)
Leaves	Aspartic acid	Dittmar (1993)
Leaves	β-sitosterol	Sang et al. (2002), Chunhieng (2003), Elkins (1998), Wang et al. (2002)
Leaves	Citrifolinoside B	Sang et al. (2002)
Leaves	Cysteine	Dittmar (1993), Elkins (1998)
Leaves	Cystine	Dittmar (1993), Elkins (1998)
Leaves	Glutamic acid	Dittmar (1993)
Leaves	Glycine	Dittmar (1993), Elkins (1998)
Leaves	Histidine	Dittmar (1993), Elkins (1998)
Leaves	Isoleucine	Dittmar (1993), Elkins (1998)
Leaves	Kaempferol 3-O-α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranoside	Sang et al. (2002)

Source (plant part)	Chemical constituent	References
Leaves	Kaempferol 3-O-β-D-glucopyranosyl-(1-2)- α-L-rhamnopyranosyl-(1-6)-β-D-galactopyranoside	Sang et al. (2002)
Leaves	Leucine	Dittmar (1993), Elkins (1998)
Leaves	Methionine	Dittmar (1993), Elkins (1998)
Leaves	Phenylalanine	Dittmar (1993), Elkins (1998)
Leaves	Proline	Dittmar (1993), Elkins (1998)
Leaves	Quercetin 3-O-β-D-glucopyranoside	Sang et al. (2002)
Root, heartwood, root bark	Morindone	Sang et al. (2002), Inoue et al. (1981), Dittmar (1993), Ross (2001), Cardon (2003), Wang et al. (2002)
Root, heartwood, seeds	Damnacanthal	Sang et al. (2002), Cardon (2003)
Leaves	Quercetin 3-O-β-D-glucopyranosyl-(1-2)- α-L-rhamnopyranosyl-(1-6)-β-D-galactopyranoside	Sang et al. (2002)
Root	8-hydroxy-8-methoxy-2-methyl-anthraquinone	Cardon (2003), Solomon (1999)
Root	Rubichloric acid	Elkins (1998), Morton (1992)
Root	1,3-dihydroxy-6-methyl anthraquinone	Morton (1992)
Root	Morenone 1	Solomon (1999)
Root	Morenone 2	Solomon (1999)
Root	Ruberythric acid	Cardon (2003)
Root	Rubiadin	Cardon (2003), Elkins (1998), Inoue et al. (1981), Ross (2001)
Root bark	Chlororubin	Dittmar (1993), Elkins (1998)
Root bark	Hexose	Dittmar (1993)
Root bark	Morindadiol	Dittmar (1993)
Root bark	Morindanidrine	Dittmar (1993)
Root bark	Morindine	Cardon (2003), Dittmar (1993), Elkins (1998), Morton (1992)
Root bark	Pentose	Dittmar (1993)
Root bark	Physcion	Solomon (1999)
Root bark	Rubiadin monomethyl ether	Dittmar (1993)
Root bark	Soranjidiol	Dittmar (1993), Elkins (1998), Ross (2001)
Root bark	Trioxymethylanthraquinone monoethyl ether	Dittmar (1993)
Root, root bark, fruit	Alizarin	Cardon (2003), Dittmar (1993), Elkins (1998), Ross (2001), Wang et al. (2002)
Seeds	Ricinoleic acid	Solomon (1999)

Table 2. Chemical compounds of *M. citrifolia* L. (adapted from Chan-Blanco et al., 2006)

anthraquinone, 3-hydroxymorindone, 3-hydroxymorindone-6-β-primereroside, α-methoxyalizarin, 2-methyl-3,5,6-trihydroxyanthraquinone-6-β-primeveroside, monoethoxyrubiadin, morindadiol, morindin, morindone (1,5,6-trihydroxy-2-methylanthraquinone), morindone-6-β-primeveroside, nordamnacanthal, quinoline, rubiadin, rubiadin 1-methyl ether, saronjidiol, ursolic acid, alkaloids, anthraquinones and their glycosides, caproic acid, caprylic acid, fatty acids and alcohols (C_{5-9}), flavone glycosides, flavonoids, glucose (β-D-glucopyranose), indoles, purines, and β-sitosterol.

Minerals account for 8.4% of the dry matter, and these minerals are mainly potassium, sulphur, calcium and phosphorus; traces of selenium have been reported in the juice (Chunhieng, 2003, Table 3). Vitamins have been reported in the fruit, mainly ascorbic acid (24-158 mg/100 g dry matter) (Morton, 1992; Shovie & Whistler, 2001) and provitamin A (Dixon et al., 1999). Phenolic compounds have been found to be the major group of functional micronutrients in *M. citrifolia* L. juice: damnacanthal, scopoletin, morindone, alizarin, aucubin, nordamnacanthal, rubiadin, rubiadin-1-methyl ether and other anthraquinone glycosides have been identified in *M. citrifolia* L. (Morton, 1992; Dittmar, 1993; Dixon et al., 1999; Wang & Su, 2001). Damnacanthal is an anthraquinone that has been characterised and has some important functional properties (mainly anti-carcinogenic) (Solomon, 1999). Scopoletin is a coumarin that was isolated in 1993 at the University of Hawaii and has been found to have analgesic properties as well as a significant ability to control serotonin levels in the body (Levand & Larson, 1979). Other researchers have shown that scopoletin may also have anti-microbial (Duncan et al., 1998) and anti-hypertensive effects (Solomon, 1999).

Multiple Hawaiian teams (Heinicke, 1985; Solomon, 1999) have reported the presence of a novel component, proxeronine, which is an alkaloid that is claimed to combine with human proteins and improve their functionality. The above authors attribute most of the beneficial effects of *M. citrifolia* L. to xeronine. Nonetheless, neither the chemical characterisation of this alkaloid nor the method used to assess its levels has been published to date.

Characteristic	Chunhieng (2003)[a]	Shovic and Whistler (2001)[a]	European commission (2002)[b]
pH	3.72	-	3.4-3.6
Dry matter	9.8±0.4%	-	10-11%
Total soluble solids (°Brix)	8	-	-
Protein content	2.5%	0.4 g/100 g	0.2-0.5%
Lipids	0.15%	0.3 g/100 g	0.1-0.2%
Glucose	11.9±0.2 g/l	-	3-4 g/100 g
Fructose	8.2±0.2 g/l	-	3-4 g/100 g
Potassium	3900 mg/l	188 mg/100 g	30-150 mg/100 g
Sodium	214 mg/l	21 mg/100 g	15-40 mg/100 g
Magnesium	14 mg/l	14.5 mg/100 g	3-12 mg/100 g
Calcium	28 mg/l	41.7 mg/100 g	20-25 mg/100 g
Vitamin C	-	155 mg/100 g	3-25 mg/100 g

Table 3. Physico-chemical composition of *M. citrifolia* L. juice (adapted from Chan-Blanco et al., 2006)

Approximately 51 volatile compounds have been identified in the ripe fruit (Sang et al., 2001), including organic acids (mainly octanoic and hexanoic acids), alcohols (3-methyl-3-butene-1-ol), esters (methyl octanoate, methyl decanoate), ketones (2-heptanone) and lactones ((E)-6-dodeceno-γ-lactone) (Farine et al., 1996).

Major components

A number of major components have been identified in the *M. citrifolia* L. plant, such as scopoletin, octanoic acid, potassium, vitamin C, terpenoids, alkaloids, anthraquinones (such as nordamnacanthal, morindone, rubiadin, rubiadin-1-methyl ether and anthraquinone glycoside), β-sitosterol, carotene, vitamin A, flavones glycosides, linoleic acid, alizarin, amino acids, acubin, L-asperuloside, caproic acid, caprylic acid, ursolic acid, rutin and a putative proxeronine.

A research group led by Chi-Tang Ho at Rutgers University in the United States (US) is searching for new novel compounds in the *M. citrifolia* L. plant. They have successfully identified several new flavonol glycosides, an iridoid glycoside from *M. citrifolia* L. leaves, a trisaccharide fatty acid ester, rutin, and an asperulosidic acid from the fruit. Two novel glycosides and a new unusual iridoid named citrifolinoside have been shown to have an inhibitory effect on AP-1 transactivation and cell transformation in the mouse epidermal JB6 cell line. James Duke listed 23 different phytochemicals found in *M. citrifolia* L. as well as 5 vitamins and 3 minerals in an authoritative handbook of phytochemicals.

Xeronine system

Retired biochemist Ralph Heinicke states that *M. citrifolia* L. fruit contains a natural precursor of xeronine that he named Proxeronine. Proxeronine is converted to the alkaloid xeronine in the body by an enzyme he named proxeroninase. His hypothesis is that xeronine is able to modify the molecular structure of proteins. Thus, xeronine has a wide range of biological activities. When a protein such as an enzyme, receptor, or signal transducer is not in the appropriate conformation, it will not work properly. Xeronine will interact with the protein and make it fold into its proper conformation. The result is a properly functioning protein. Whenever a problem arises in the cell due to a structural problem with a protein, xeronine's presence would be beneficial. His hypothesis may explain why Tahitian Noni @ juice (TNJ) can help in many health problems in different ways. He has obtained several patents for xeronine. He states that the active ingredient in many of the effective folklore drugs is xeronine. This alkaloid is a critical normal metabolic coregulator. The ailments that he believes are helped by *M. citrifolia* L. include high blood pressure, menstrual cramps, arthritis, gastric ulcers, sprains, injuries, mental depression, senility, poor digestion, drug addiction, and pain. "I have devoted much of my life to the study of this unique substance that I have named 'xeronine'. I am convinced of the tremendous benefits achieved by furnishing the body with a proper supply of this material" (Heinicke, 2001).

2.4 Biological activity of *M. Citrifolia* L.

2.4.1 Anti-microbial effects

The anti-microbial activity of *M. citrifolia* L. may have been its first observed property; indeed, the fruit contains relatively large amounts of sugars that do not ferment even when fruits are stored in closed containers at ambient temperature. This property is used to

transport the fruit by boat from the scattered Pacific islands to processing plants without specific treatment.

It has been reported that *M. citrifolia* L. inhibits the growth of certain bacteria, such as staphylococcus aureus, Pseudomonas aeruginosa, Proteus morgaii, Bacillus subtilis, Escherichia coli, Helicobacter pylori, Salmonella and Shigella (Atkinson, 1956). The same author claims that the anti-microbial effect observed may be due to the presence of phenolic compounds such as acubin, l-asperuloside, alizarin, scopoletin and other anthraquinones. Another study showed that an acetonitrile extract of the dried fruit inhibits the growth of Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli, and Streptococcus pyrogene (Locher et al., 1995).

It has also been found that ethanol and hexane extracts of *M. citrifolia* L. have an antitubercular effect as they inhibit the growth of Mycobacterium tuberculosis by 89-95% (Saludes et al., 2002). The major components identified in the hexane extract are E-phytol, cycloartenol, stigmasterol, β-sitosterol, campesta-5,7,22-trien-3-β-ol, and the ketosteroids stigmasta-4-en-3-one and stigmasta-4-22-dien-3-one.

Other studies have reported a significant antimicrobial effect on various strains of Salmonella, Shigella, and E. coli (Bushnell et al., 1950; Dittmar, 1993). Furthermore, they showed that the anti-microbial effect is highly dependent on the stage of ripeness and on processing, being greater when the fruit is ripe and undried.

2.4.2 Anti-cancer activity

The immunomodulatory properties (the capacity to enhance the host immune system) of *M. citrifolia* L. juice have been studied by a Japanese research team (Hirazumi et al., 1996; Hirazumi & Furusawa, 1999). The ethanol precipitable fraction (ppt) of *M. citrifolia* L. juice, corresponding to a polysaccharide-rich substance composed of glucuronoic acid, galactose, arabinose and rhamnose, has been found to have immunomodulatory and anti-tumour effects against Lewis lung carcinoma (LLC). In cell models, *M. citrifolia* L.-ppt seems to stimulate the production of T-cells, thymocytes and macrophages that produce cytokines, which are important mediators of tumour cytostasis and cytotoxicity.

M. citrifolia L.-ppt also appears to stimulate murine effector cells to release several mediators such as cytokines. These mediators slow down the cell cycle in tumours, increase the response of cells to other immunised cells that fight tumour growth and have potent macrophage activator activity suspected of playing a role in the death of tumours (Hirazumi et al., 1996; Hirazumi & Furusawa, 1999).

In the same study, mice were inoculated with LLC, and those ingesting a daily dose of 15 mg of *M. citrifolia* L. juice showed a significant increase (119%) in lifespan. Nine out of 22 mice with terminal cancer survived for more than 50 days. In addition, the ingestion of *M. citrifolia* L.-ppt combined with conventional chemotherapy proved to increase the lifespan of mice with cancer, (Hirazumi et al., 1994).

Another Japanese team studied the influence of damnacanthal, an anthraquinone extracted from a chloroform extract of *M. citrifolia* L. fruits. Surprisingly, the researchers found that damnacanthal induces normal morphology in a particular type of cell found in human neoplasias (K-ras-NKR cells) that multiply uncontrollably and are highly malignant (Hiramatsu et al., 1993).

Another study showed that commercial *M. citrifolia* L. juice (Tahitian Noni juice) prevents the formation of chemical carcinogen-DNA-adducts. In the above study, rats with artificially induced cancer in specific organs were fed for one week with 10% *M. citrifolia* L. juice in their drinking water and rat food (rat chow) ad libitum. They showed reduced DNA-adduct formation depending on sex and organ. The reduction rates were: in female rats, heart 30%, liver 42%, lungs 41% and kidneys 80%; in male rats, heart 60%, liver 70%, lungs 50% and kidneys 90% (Wang & Su, 2001).

2.4.3 Anti-oxidant properties

The antioxidant properties of ethanol and ethyl acetate extracts of *M. citrifolia* L. fruit have been assessed using the ferric thiocyanate method (FTC) and thiobarbituric acid test (TBA). The authors found that ethyl acetate extract strong inhibited lipid oxidation, comparably to the same weight of pure α-tocopherol and butylated hydroxyl toluene (BHT) (Mohd et al., 2001).

Radical scavenging activity was also measured *in vitro* by the tetrazolium nitroblue (TNB) assay in commercial juice by assessing the capacity of the juice to protect cells and lipids from oxidative alteration promoted by superoxide anion radicals (SARs). The SAR scavenging activity of *M. citrifolia* L. juice was shown to be 2.8 times higher than that of vitamin C, 1.4 times that of pycnogenol (PYC) and almost the same magnitude as that of grape seed powder (Wang & Su, 2001).

2.4.4 Anti-inflammatory activity

The anti-inflammatory activity of an aqueous extract from *M. citrifolia* L.-juice was observed by inducing a locally acute inflammatory response with the help of a pro-inflammatory agent (bradykinin). It was found that the oral administration of *M. citrifolia* L. juice extract (200 mg) rapidly inhibits the formation of rat paw oedema. This effect may have resulted from interference with the B2 receptor-mediated mechanism by which bradykinin induces rat paw oedema (Mckoy et al., 2002).

Another study showed that commercial *M. citrifolia* L. juice selectively inhibits cyclo-oxygenase enzymes (COX-1 and COX-2) involved in breast, colon and lung cancer and also has anti-inflammatory activity (Su et al., 2001). The ability of noni juice to inhibit these enzymes was compared to that of traditional commercial non-steroidal inflammatory drugs, such as aspirin, Indomethacin and Celebrex. *M. citrifolia* L. juice showed selective inhibition of COX activity *in vitro* and a strong anti-inflammatory effect comparable to that of Celebrex, and presumably, this juice lacks side effects.

2.4.5 Analgesic activity

Recent research has examined the analgesic properties of commercial juice in rats. The results showed that rats fed 10% or 20% *M. citrifolia* L. juice had greater pain tolerance (162% or 212%, respectively) compared with the placebo group (Wang et al., 2002). A French research team has also studied the analgesic and sedative effects of *M. citrifolia* L. on mice through the writhing and hotplate tests. *M. citrifolia* L. root extract (1600 mg/kg) showed significant analgesic activity in the animals, similar to the effect of morphine (75% and 81%

protection using *M. citrifolia* L. extract and morphine, respectively), and it also proved to be non-toxic (Younos et al., 1990).

2.4.6 Cardiovascular activity

Recent research has demonstrated the ability of *M. citrifolia* L. fruit to prevent arteriosclerosis, a disease related to the oxidation of low density lipoproteins (LDLs). Methanol and ethyl acetate extracts showed 88% and 96% inhibition, respectively, of copper-induced LDL oxidation by the thiobarbituric acid relative substance method. This beneficial effect could be due to the presence of lignans, which are phenylpropanoid dimers (Kamiya et al., 2004).

2.5 Biological activities of *M. citrifolia* L. products

2.5.1 Antibacterial activity

Acubin, l-asperuloside, alizarin in *M. citrifolia* L. fruit, and certain other anthraquinone compounds in *M. citrifolia* L. roots, are all proven antibacterial agents. These compounds have been shown to fight infectious bacteria such as Pseudomonas aeruginosa, Proteus morgaii, Staphylococcus aureus, Bacillis subtilis, Escherichia coli, Salmonella, and Shigela. These antibacterial compounds in *M. citrifolia* L. are responsible for the treatment of skin infections, colds, fevers, and other bacteria-caused health problems. Bushnell reported on the antibacterial properties of certain plants found in Hawaii, including *M. citrifolia* L. He further reported that *M. citrifolia* L. was traditionally used to treat broken bones, deep cuts, bruises, sores and wounds. Extracts from the ripe *M. citrifolia* L. fruit exhibit moderate antibacterial properties against Salmonella typhosa. Salmonella montevideo, Salmonella schottmuelleri, Shigella paradys, BH and Shigella paradys, III-Z. Leach demonstrated that acetone extracts obtained from Cycas circinalis, *M. citrifolia*, Bridelia penangiana, Tridax Procumbens, Hibiscus tiliaceus, and Hypericum papuanun show antibacterial activity. The widespread medicinal use of these plants would suggest that they do contain pharmacologically active substances, and alternative methods of extraction and screening should be utilised to find the major bioactive component in the plants for the purpose of new drug development. Locher reported that selected plants including *M. citrifolia* have a history of use in Polynesian traditional medicine for the treatment of infectious diseases. These plants have been investigated for anti-viral, anti-fungal, and anti-bacterial activity *in vitro*. Their study using *in vitro* biological assays confirmed that some of the Hawaiian medicinal plants in ethnobotanical reports have curative properties against infectious diseases.

Duncan demonstrated that scopoletin, a health promoter in *M. citrifolia* L., inhibits the activity of E. coli, which is associated with recent outbreaks resulting in hundreds of serious infections, even death. *M. citrifolia* L. also helps stomach ulcers by inhibiting H. pylori bacteria.

2.5.2 Antiviral activity

Umezawa and coworkers found that a compound isolated from *M. citrifolia* L. roots named 1-methoxy-2-formyl-3-hydroxyanthraquinone suppresses the cytopathic effect of HIV-infected MT-4 cells without inhibiting cell growth.

2.5.3 Anti-tubercular effects

At the International Chemical Congress of the Pacific Basin Societies meeting in Honolulu, Saludes and colleagues from the Philippines reported that *M. citrifolia* L. kills Mycobacterium tuberculosis. A concentration of extracts from *M. citrifolia* L. leaves killed 89% of the bacteria in a test tube, almost as effectively as the leading anti-TB drug Rifampicin, which has an inhibitory rate of 97% at the same concentration. Although there have been anecdotal reports of native use of *M. citrifolia* L. in Polynesia as a medicine against tuberculosis, this is the first report demonstrating the antimycobacterial potential of compounds obtained from *M. citrifolia* L. leaves. "I hope that pharmaceutical companies will pay attention to this research and explore the *M. citrifolia* L. plant as a potential source of drugs", said Saludes in Manila.

2.5.4 Antitumor activity

At the 83rd Annual meeting of the American Association for Cancer Research in 1992, Hirazumi, a researcher at the University of Hawaii, reported that the alcohol-precipitate of *M. citrifolia* L. fruit juice (noni-ppt) has anticancer activity on lung cancer in C57 B1/6 mice. This *M. citrifolia* L.-ppt was shown to significantly prolong (by up to 75%) the life of mice with implanted Lewis lung carcinoma. It was concluded that the *M. citrifolia* L.-ppt seems to suppress tumour growth indirectly by stimulating the immune system. Improved survival time and curative effects occurred when *M. citrifolia* L.-ppt was combined with sub-optimal doses of the standard chemotherapeutic agents, such as adriamycin (Adria), cisplatin (CDDP), 5-fluorouracil (5-FU) and vincristine (VCR), suggesting that *M. citrifolia* L.-ppt has important clinical utility as a supplemental agent in cancer treatment. These results indicate that *M. citrifolia* L.-ppt may enhance the therapeutic effect of anticancer drugs. Therefore, it may be of benefit to cancer patients by enabling them to use lower doses of anti-cancer drugs to achieve the same or even better results.

Dr. Wang and coworkers demonstrated a cytotoxic effect of TNJ on a cultured leukaemia cell line at various concentrations. TNJ showed dose-dependent cytotoxicity on cultured cancer cells by inducing cancer cell necrosis at high doses and apoptosis at lower doses. Synergistic effects of TNJ with known anticancer drugs have been found. At sub-optimal doses, both prednisolone and TNJ can induce apoptosis. When the dose of prednisolone is fixed and the dose of TNJ increases, apoptotic cells significantly increase. Therefore, TNJ is able to enhance the efficacy of anticancer drugs such as prednisolone. When a single dose of Taxol induces a lower percentage of apoptosis in leukaemia cells, TNJ enhances the rate of apoptosis to 100%. These results indicate that TNJ is able to enhance the therapeutic effects of anticancer drugs such as Taxol. These findings regarding the combination of anticancer drugs with TNJ may be significant. This approach may allow lower doses of synthetic anticancer drugs to be used, increase the tolerance of patients to the toxicity of anticancer drugs, and increase immune function, thus creating a new method for treating cancer patients.

In 1993, Hiramatsu and colleagues reported in Cancer Letters the effects of over 500 extracts from tropical plants on K-Ras-NRK cells. Damnacanthal, isolated from *M. citrifolia* L. roots, is an inhibitor of Ras function. The ras oncogene is believed to be associated with signal transduction in several human cancers such as lung, colon, and pancreatic cancer and leukaemia.

Hiwasa and coworkers demonstrated that damnacanthal, an anthraquinone compound isolated from the *M. citrifolia* L. roots, has potent inhibitory activity towards tyrosine kinases such as Lck, Src, Lyn and EGF receptors. In his research, he examined the effects of damnacanthal on ultraviolet ray-induced apoptosis in ultraviolet-resistant human UVr-1 cells. Consequently, the ultraviolet light induced a concurrent increase in both phosphorylated extracellular signal-regulated kinases and stress-activated protein kinases. After pretreatment with damnacanthal, there was a stimulatory effect on ultraviolet-induced apoptosis.

Dong reported that two glycosides extracted from *M. citrifolia* L.-ppt were effective at inhibiting cell transformation induced by TPA or EGF in the mouse epidermal JB6 cell line. This inhibition was found to be associated with the inhibitory effects of these compounds on AP-1 activity. The compounds also blocked the phosphorylation of c-jun, a substrate of JNKs, suggesting that JNKs are a critical target for the compounds in mediating Ap-1 activity and cell transformation.

2.5.5 Antihelmintic activity

An ethanol extract of tender *M. citrifolia* L. leaves was found to induce paralysis and death in the human parasitic nematode Ascaris Lumbricoides within a day. A botanist via Morton reported that *M. citrifolia* L. has been used in the Philippines and Hawaii as an effective insecticide.

2.5.6 Hypotensive activity

Dang Van Ho of Vietnam demonstrated that total extract of *M. citrifolia* L. roots has a hypotensive effect. Moorthy and coworkers found that an ethanol extract of *M. citrifolia* L. roots lowers blood pressure in anesthetised dogs. Youngken's research team determined that a hot water extract of *M. citrifolia* L. roots lowers blood pressure in anesthetised dogs. A Hawaiian physician reported that *M. citrifolia* L. fruit juice has a diuretic effect.

2.5.7 Immunological activity

Asahina found that an alcohol extract of *M. citrifolia* L. fruit at various concentrations inhibits the production of tumour necrosis factor-alpha (TNF-α), which is an endogenous tumour promoter. Therefore, the alcohol extract may inhibit the tumour promoting effect of TNF- α. Hirazumi found that *M. citrifolia* L.-ppt contains a polysaccharide-rich substance that inhibits tumour growth. It does not cause significant cytotoxicity in adopted cultures of lung cancer cells, but it can activate peritoneal exudate cells to impart profound toxicity when co-cultured with tumour cells. This suggests the possibility that *M. citrifolia* L.-ppt may suppress tumour growth through activation of the host immune system. *M. citrifolia* L.-ppt is also capable of stimulating the release of several mediators from murine effector cells, including TNF- α, interleukin-1 beta (IL-1β), IL-10, IL-12, interferon-gamma (IFN- γ) and nitric oxide (NO). Hokama separated ripe *M. citrifolia* L. fruit juice into 50% aqueous alcohol and precipitate fractions, which was found to stimulate BALB/c thymus cells in (^{3}H)thymidine analysis. It has been suggested that inhibition of Lewis lung tumours in mice, in part, may be due to the stimulation of the T-cell immune response.

Wang and coworkers at the University of Illinois college of Medicine observed that the thymus is enlarged in animals treated with TNJ. The wet weight of the thymus was 1.7 times that of control animals on the seventh day after receiving 10% TNJ in drinking water. The thymus is an important immune organ in the body that generates T cells and is involved in the aging process and cellular immune fractions. TNJ may enhance immune function by stimulating thymus growth and thereby exerting anti-aging and anticancer activities and protecting people from other degenerative diseases.

2.5.8 Mental health and improved high frequency hearing

A small human clinical trial of the effects of TNJ on auditory function and quality of life in patients with decreased bone mineral density and auditory function was conducted at the UIC College of Medicine, Rockford, IL. This study showed that TNJ improves both mental health and high frequency hearing. The results suggest that increased amounts or extended duration of TNJ intake may be required to influence this disorder.

2.6 Study of TNJ for cancer prevention

"To take medicine only when you are sick is like digging a well only when you are thirsty – is it not already too late?" (Chi Po, c 2500 B.C.). This proverb suggests that prevention is more important than treatment.

Cancer is the second leading cause of death in the US. According to the American Cancer society, 1500 people per day die from cancer in the United States. Fighting against cancer is a great task for scientists engaged in this field. The aetiology of most cases of human cancer remains unknown. Exposure to environmental carcinogens accounts for more than 90% of human cancers. Cigarette smoke is the number one high-risk environmental factor. Although some cancers are preventable, a means to prevent most cancers is not yet known. Seeking a natural way to prevent human cancer is an urgent task for cancer prevention investigators.

Studies of food, diet, and cancer have indicated that lifestyle changes such as eating more fruits and vegetables and quitting smoking will help prevent cancer. "A new plate" for America (75% vegetables, 25% meat) appeared at the 2001 annual conference of the American Institute for Cancer Research. Although TNJ possesses a broad range of therapeutic effects, its ability to prevent cancer remains unclear. Recently, new hypothesis has been investigated: whether or not TNJ can help prevent cancer during the early stages of chemical carcinogenesis.

This hypothesis was examined using two carcinogenic animal models and one human clinical study of a group of current smokers. The animal models included the following: the DMBA-induced mammary gland tumourigenesis model and an acute liver injury model induced by the liver carcinogen carbon tetrachloride (CCl_4). These are classical extrinsic carcinogenic models. DMBA-induced DNA adduct formation and histological examination by light and electron microscopy were used as sensitive biomarkers to evaluate the preventive effects of TNJ at the initiation stage of multiple-step carcinogenesis. In the mammary breast carcinogenic model, to monitor the mechanisms of carcinogenesis and DMBA DNA-adduct formation in mammary tissue, the focus was on the pathogenic

changes after DMBA administration. In the acute liver injury model, the histopathological changes in liver tissue and levels of both super-oxide anion free radicals (SAR) and lipid hydroperoxide (LPO) after CCl_4 administration were the focus.

DMBA DNA-adduct formation was used as a marker to examine whether TNJ is able to prevent carcinogen-induced DNA damage. Most chemical carcinogens need to be activated by endogenous enzymes to be transformed into a form that readily binds to genetic DNA to form DNA-adducts. Carcinogen-DNA adduct formation is an important DNA damage marker that predicts the possibility of cancer development. Most scientists agree that carcinogen-induced DNA adduct formation is an early critical step in the multiple stages of carcinogenesis. Carcinogen-DNA adducts can be repaired by endogenous enzymes. Unrepaired adducts are fixed after one cell cycle. Unrepaired DNA damage is responsible for mutations and subsequent cancer development. Therefore, preventing carcinogen-DNA adduct formation is a key aspect of preventing the initial steps of carcinogenesis. If TNJ can prevent and/or block the formation of carcinogen-induced DNA adducts, it may prevent cancer at the initiation of multiple-stage carcinogenesis.

In recent years, increasing demand for higher quality and safer foods and medicines, as well as concern for environmental pollution during their commercial production, have triggered stringent regulations on toxin levels in foods and medicines as well as on the discharge of pollutants to the environment. In addition, there has been increasing consumer preference for natural substances. All these factors have provided strong motivation for the development of cost-effective new technologies, such as the eco-friendly extraction of natural substances employing green and safe solvents. In recent years, supercritical fluid extraction (SFE) has emerged as a highly promising environmentally benign technology for the production of natural extracts such as flavours, fragrances, spice oils, and oleoresins; natural anti-oxidants; natural colours; nutraceuticals and biologically active compounds. The state of a substance is called supercritical when both temperature and pressure exceed their critical point values. A supercritical fluid combines two beneficial properties, namely high density (which imparts high solvent power) and high compressibility (which permits high selectivity due to large changes in solvent power in response to small changes in temperature and pressure). In addition, SFE offers very attractive extraction characteristics owing to its favourable diffusivity, viscosity, surface tension, and other thermo-physical properties.

Since the 1980s, several potential applications of SFE have been reported. So far, the most popular SF has been carbon dioxide (CO_2), owing to its easy availability, low cost, nonflammability, nontoxicity, and its possession of a wide spectrum of solvent properties. Its critical temperature is 31.1 ˚C and its critical pressure is 73.8 bar. Dense or supercritical carbon dioxide could very well be the most commonly used solvent in this century due to its wide-ranging applications. Its near-ambient critical temperature makes it ideally suitable for processing thermally labile natural substances. It is generally regarded as safe (GRAS), and it yields microbial-inactivated, contaminant-free, tailor-made extracts of superior organoleptic profile and longer shelf life with highly potent active ingredients. The SFE technique ensures high consistency and reliability in the quality and safety of bioactive heat-sensitive botanical products as it does not alter the delicate balance of bioactivity of natural molecules. All of these advantages are almost impossible with conventional processes. Therefore, SFE technology using SC-CO_2 as the solvent is an ideal alternative to the conventional techniques for the extraction of bioactive ingredients from spices.

3. Conclusion

Morinda citrifolia L., commonly known as noni, has a long history of widespread use as a food in tropical regions from Indonesia to the Hawaiian Islands, and it is used as an herbal remedy for multiple diseases. Its fruit, leaves, seeds, bark and roots have been traditionally used for the prevention or improvement of various diseases, including arthritis, infections, colds, cancer, and diabetes. It has been found that *Morinda citrifolia L.*, has antioxidant potential equivalent or similar to that of synthetic antioxidants, such as BHT and BHA, which are currently used as food additives. The antioxidants which are found in *Morinda citrifolia L.*, have no side effects, and thus they could replace synthetic antioxidants in the food processing industry and have potential for use in preventive medicine. Thus, this fruit can be used as an antioxidant additive in the food processing industry.

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7

Flavonoids in some Iranian Angiosperms

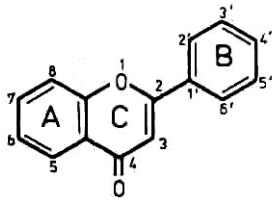
Mitra Noori
Department of Biology, Faculty of Science, Arak University, Arak, Iran

1. Introduction

Flavonoids are as one set of the polyphenolic compounds among secondary metabolites in different organs of plants that possess a wide range of biological activities [Parr and Bolwell 2000, Noori 2002, Noori et al 2009]. Their distribution in plants, synthesis and mode of action have been extensively studied [Shirley 1996].

1.1 Structure, biosynthesis and variety

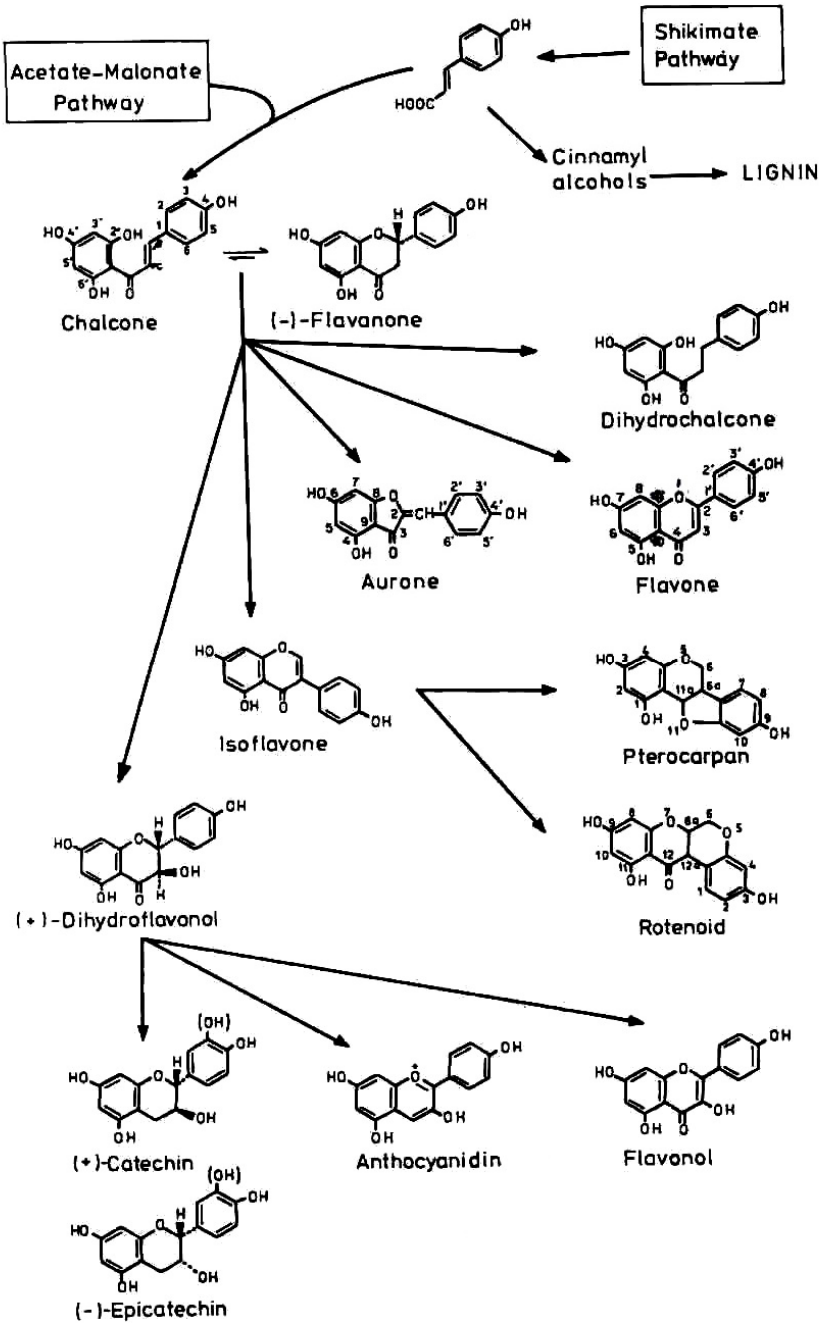
All flavonoids contain fifteen carbon atoms in their basic nucleus and these are arranged in a C_6-C_3-C_6 configuration, that is, two aromatic rings linked by a three carbon unit which may or may not form a third ring. They are divided into different groups depending on the configuration of the rings and substitutions on these rings of a variety of side-groups which characterize the individual compounds [Stace 1980] (Scheme 1).



Scheme 1. The basic nucleus of flavonoids (Stace 1980)

The flavonoid variants are all related by a common biosynthetic pathway which incorporates precursors from both the "Shikimate" and "Acetate-Malonate" pathways [Hahlbrock and Grisebach 1975; Wong 1976], the first flavonoid produced immediately following confluence of the two pathways (Scheme 2).

The flavonoid initially formed in the biosynthesis is now thought to be the chalcone and all other forms are derived from this by a variety of routes [Hahlbrock 1981] (Scheme 2). More than 4000 varieties of flavonoids have been identified in different higher and lower plant species (De Groot and Rauen 1998). The main flavonoid groups are flavones (e.g. luteolin), flavanone (e.g. naringenin), flavonols (e.g. kaempferol), anthocyanidins (e.g. pelargonidin) and chalcones (e.g. butein) [Harborne et al 1975].



Scheme 2. Showing two biosynthetic pathways of flavonoids (Hahlbrock and Grisebach 1975; Wong 1976).

1.2 Occurance

Flavonoids are found in fruit, vegetables, grains, bark, roots, stems, leaves, flowers, tea and wine [Middleton 1998, Robles et al 2003]. The flavonoid nucleus is normally linked to a sugar moiety to form a water-soluble glycoside. Most flavonoids are stored in the plant cell vacuoles, although they also occur on the surfaces of leaves and stems (Farman 1990). In contrast to earlier studies, all these compounds are no longer judged as waste products, nor as evolutionary remnants without current function, nor as mere metabolic end products that are toxic to the plant and are therefore to be stored away in vacuoles [Parr and Bolwell 2000].

1.3 Biological activities and their usages

Flavonoids possess a wide range of biological activities, medicinal and pharmacological effects [Parr and Bolwell 2000, Noori 2002, Noori et al 2009].

1.3.1 Biological activities

A large variety of colours such as orange, scarlet, crimson, mauve, violet, blue and purple that we encounter in different part of plants, especially flowers and fruits, are caused by anthocyanins (=anthocyanidin glycosides). Chalcones and some flavones and flavonols also absorb light in the visible region and are associated with bright yellow or cream coloured flowers. Other flavones account for the whiteness in most white flowers, without which they would perhaps appear translucent. Even some of the brown and black pigments found in plants are either due to oxidative products of flavonoids or related phenolic compounds. [Farman 1990]. They are beneficial for the plant itself as physiological active compounds, as stress protecting agents, as attractants or as feeding deterrents, and, in general, by their significant role in plant resistance [Treutter 2006]. Also these compounds serve essential functions in plant reproduction by recruiting pollinators and seed disperses. They are also responsible for the beautiful display of fall color in many plant species, which has recently been suggested to protect leaf cells from photo-oxidative damage, thereby enhancing the efficiency of nutrient retrieval during senescence [Field et al 2001].

1.3.2 Medicinal and pharmacological effects

Flavonoids medicinal and pharmacological effects are their contributions to human health which has made them prominent in the past 10 years (Parr and Bolwell 2000). Many flavonoids are active principles of medicinal plants and exhibit pharmacological effects [Yilmaz and Toledo 2004].

1.4 Chemotaxonomy

Flavonoid compounds are taxonomically important. They are popular characters for chemosystematic studies because: the almost universal presence of flavonoids in vascular plants; 2. Their structural diversity; 3. The fact that each species usually contains several flavonoids; 4. The chemical stability of many flavonoids in dried plant material enabling herbarium material to be used; 5. Flavonoid profiles using different chromatographic techniques are easily obtained. 6. Flavonoids are reasonably easy to identify using published

UV spectra data and available standards; 7. Flavonoids often show correlations with existing classifications at the family, genus and species level, and support revisions of existing classifications at the family, genus and species level. However, flavonoids rarely provide "key" characters (the flavonoid may be absent in one or more members of the taxon, and the same flavonoid may occur in an unrelated taxon, e.g. isoflavonoids occur in the Leguminosae and Iridaceae and biflavonyls in the Gymnospermae and some Angiospermae) [Harborne and Turner 1984].

1.5 Flavonoids in Leguminosae

The Leguminosae is economically the single most important family in the dicotyledonae, and also of major significance in nature The family is especially rich in flavonoids, producing about 28% of all known flavonoids and 95% of all isoflavonoid aglycones (Hegnauer and Grayer-Barkmeijer 1993). The importance of the phenolic constituents in the family has been stressed by Bate-Smith (Bath-Smith 1962). As Gomes et al (1981a) showed in "Advances in Legume Systematics" the Leguminosae are especially well endowed with flavonoid constituents, many of which are only known in these plants. Whithin the Leguminosae, some 850 compounds, including 362 isoflavones, are known [Dewick 1993]. There is a basic structures, Such as genistein (4´, 5, 7-trihydroxyisoflavone), 5-dexy derivatives (some 66% of structures), prenylated derivatives (some 51% of structures) and compounds with extra hydroxylation (e. g. at the 6-, 8- or 2´-positions). Isoflavonoids usually occur in the free state, and are obtained from root, wood, bark or seed rather than leaf or flower [Ingham 1981, 1983]. Flavonoids, as distinct from isoflavonoids and neoflavonoids, are widespread in the *Papilionoidaea* and there is little doubt that they occur not only in the species of the some tribes, but will eventually be found in all tribes [Gomes et al 1981b]. Harborn (1965) obtained quercetagetin from hydrolyzed petal of *Coronilla glauca* L.. He also found halogenin, 3-*O* rutinoside and limocitrin, 3- *O* rutinoside from C. glauca flower (Harborn 1981). Catechin, epigallocatechin, leucodelphinidin and 3, 3´, 4´, 5, 5´, 7-hexahydroxyflavan have been identified from *Alhaji maurorum* Medikus ground parts [Islambeko et al 1982]. Malvidin from hydrolyzed flower, myricitrin from flower and laef of *Cercis siliquastrum* L. have been isolated [Torck et al 1969, Sagareishvili and Ananiya 1990].

1.6 Polygonaceae flavonoids

Based on Isobe and Noda (1987) flavonoids and flavonol glycosides are of wide-spread occurrence in the genus *Polygonum*. Among them, glycosylation at C-3 of the quercetin nucleus has been found to be the most common trend, and present in all species of this genus [Park, 1987]. While rhamnose, gloucose, arabinose and rhamnosyl-rhamnose are the most common sugars found as aglycones of the flavonol glycosides [Mun and Park 1995], galactosylation is rather uncommon in the genus *Polygonum* or in the family polygonaceae [Collins et al 1975]. Kawasaki et al (1986) isolated thirty-three kinds of flavonoids from Polygonaceae species leaves. Quercetin glycosides were commonly found in the family. In the quercetin glycosides, 3-*O*-rhamnoside was most frequently found, 3-*O*-glucuronide is also distributed widely. Myricetin glycosides were rare. Methylated flavonols were found in some species of the section *Echinocaulon* and *Persicaria* [Kawasaki et al 1986]. The aerial exudate of *Polygonum senegalense* has been reported to contain 12 flavonoids of the chalcone

and flavanone types, and they are distinctly different from internal tissue aglycones [Midiwo et al 2007]. Also Hsu (2006) studies revealed that *Polygonum aviculare* L. extract has high phenolics and flavonoid contents.

Trichopoulou et al (2000) showed that some wild edible species of *Rumex* such as *R. acetosa* L. and *R. japonicas* Houttuyn have a very high flavonol content. Hasan et al (1995) studies showed besides rutin, quercetin 3-rhamnoside and kaempferol 3-rhamnosyl (1 -> 6) galactoside, a new flavonol glycoside, quercetin 3-glucosyl (1 -> 4) galactoside, and 1, 6, 8-trihydroxy-3-methyl anthraquinone (emodin) have been characterized from leaves of *R. chalepensis*.

1.7 Euphorbiaceae flavonoids

Several studies indicated that flavonoids occurred in various species of *Euphorbia*. Nagase reported the isolation of 5, 7, 4'-trihydroxy-flavone-7-glucoside from the leaves and stems of *E. thymifolia* [Nagase et al 1942]. Ten years later, quercetin was isolated from ethyl acetate extract of aqueous solution of hydroalcoholic extract of *E. pilulifera* [Hallett and Parks 1951]. Sotnikova and his coworkers identified 3′, 4′-pentahydroxyflavone 3β-D-galactopyranoside, steppogenin stepposide, isomyricitrin and nineteen other flavonoids in *E. stepposa* [Sotnikova and Litvinenko 1968, Sotnikova et al. 1968]. Muller and Pohl (1970) isolated six new flavonoids all being glycosides of rhamnetin from *E. amygdaloides*. The qualitative composition of flavonoids in alcoholic extract of *E. helioscopia* indicated fifteen substances with flavonoidal nature, by two-dimentional paper chromatography. Acid hydrolysis of *E. helioscopia* alcoholic extract by Volobueva (1970) yielded quercetin and kaempferol. Quercetin-3-xylosidoglucoside has been identified as one of the two flavonoids found in methanolic extract of *E. chamaesyce* and quercetin-3β-D-galactopyranoside gallate has been reported in *E. verrucosa* and *E. platiphyllos* [Singla and Pathak 1990]. Chromatography on cellulose of methanolic extract of *E. lucida* yielded quercetin and its derivatives, viz., isoquercetin, avicularoside, hyperoside and rutoside [Burzanska 1975]. The hypotensive principales of *E. maddeni* were found to be kaempferol-4'-*O*-glucose and hyperin (Sahai et al 1981). In an effort to identify the constituents responsible for the antiviral activity of *E. grantii*. Van Hoof et al. (1984) isolated derivatives of 3-methylquercetin. Polyphenolic components from the aerial parts of *E. soongarica* and *E. alatavica* have been identified as the esters of gallic acid, luteolin-3-rhamnoside and luteoline-3-galactoside [Omurkhamzinova and Erzhanova 1985]. Gautam and Mukhraya (1981) have isolated quercetin-3-O-β-D-glucopyranosyl (1-4)-O-α-L-rhamnopyranoside from the leaves of *E. dracuncoloides*. Kaempferol 3-O-glucoside and quercetin 3-O-glucoside were obtained from *E. larica*, *E. virgata*, *E. chamaesyce* and *E. magalanta*. Also all these taxa, except *E. chamaesyce* contained kaempferol 3-rutinoside and rutin. *E. larica* also yielded 6-methoxyapigenin while *E. virgata* and *E. magalanta* yielded kaempferol. There is an unknown acytylated kaempferol derivative in *E. chamaesyce* (Ulubelen et al 1983). Murillo and Jakupovic (1998) identified myricetin-3-rhamnoside and one flavonoid glycosides in *E. aucherii* which was collected in Iran. Aimova et al (1999) found quercetin 3-(2‴-Galloylglucosyl) (1→2)-α-L-arabinofuranoside in *E. pachyrrhiza*. Studies of Halaweish et al (2003) are the first report of quercetin-3-O-β glucuronic acid in *E. esula*. They separated and identified kaempferol-3-O-β glucuronic acid and quercetin-3-O-β glucuronic acid from the species. Papp et al (2005) studies showed arial

parts of *Euphorbia cyparissias* had 2 main flavonoids: kamfpherol-3-glucuronide and quercetin-3-glucuronide. Adedapo et al (2005) showed arial branch extract of *E. hirta* contains kaempferol, quercitol and quercitrin. The phytochemical studies of Falodun et al (2006) on *E. heterophylla* extract revealed the presence of flavonoids in the extract.

Abdel-Sattar (1985) reported existing flavonoids in *Chrozophora*. Then Hashim et al (1990) found kampferol, acacetin, luteolin and apigenin glycosides in *Chrozophora* species. Isorhamnetin and quercetin glycosides were separated from *C. oblique* [Mohamed 2001]. Talischi et al (2005) reported apigenin and quercetin glycosides from metanolic extract of *C. tinctoria* aerial parts. Also Delazar et al (2006) separated 5-flavonoid glycosid from aerial parts metanolic extract of *C. tinctoria*. Shi et al (2006) has reported 7-flavonoid glycoside from *C. sabulosa* species. Vassallo et al (2006) were separated three new flavon glycosids from *C. senegalensis* leaf metabolic extract. Apigenin and loteulin glycosids were reported from leaf aqueous-etanolic extract of *C. brocchiana* species [Hawas 2007].

1.8 Resedaceae flavonoids

Several studies indicated that flavonoids occurred in various species of *Reseda*. Eight flavone, 15 flavonols and one isoflavone have been reported from the *Reseda. Reseda luteola* contains 40% flavonoids, primarily luteolin, but also luteolin-7-O glucoside and apigenin [Woelfle et al 2009]. Moiteiro et al (2008) found luteolin 4́-O-glucoside in *Reseda luteola* for first time. Berrahal et al (2006) reported five flavonoid glycosides, quercetin-7-*O*-α-L-rhamnosyl-3-*O*-β-D-glucoside, isorhamnetin-3 *O*-β-D-glycosyl-7-*O*-α-L-rhamnoside, kaempferol-7-*O*-α-L-rhamnoside, kaempferol-7-*O*-α-L-rhamnosyl-3-*O*-β-D-glucoside and kaempferol-3, 7-*O*-α-L-dirhamnoside from aerial parts of *R. villosa* for first time. El-Sayad et al (2001) isolated aglycone flavonols, kaempferol and quercetin from the Mediterranean *Reseda* species. Also Yuldashev et al (1996) reported flavonol diglycosides of kaempferol, quercetin and isorhamnetin from four other *Reseda* species. Rzadkowska (1969) isolated four 3-*O*-glycosides from *R. lutea*.

1.9 Cyperaceae flavonoids

Clifford and Harborne (1969) studies showed identification of the flavonoid pigment aureusidin from *Scirpus nodosus*. Quercetin, kaempferol, apigenin and luteolin were reported from *S. wichurai* [Ahmed et al 1984]. Naser et al (2000) identified lupeol betulin, betulinalaldehyde and apigenin from *Scirpus tuberosus*. Also β-sitosterol, quercetin 3- β-glucoside, quercetin 3, 7- β-diglucoside and isorhamnetin 3, 7- β-glucoside were identified from *Scirpus litoralis* using spectroscopic analyses [Naser et al 2000]. Yang et al (2010) used a developed capillary electrophoresis with amperometric detection method for the determination of some phenolic compounds in the rhizome of *Scirpus yagara* Ohwi. Their work determined existing four phenolic compounds: transresveratrol, scirpusin A, scirpusin B, and p-hydroxycinnamic acid in the species rhizome.

1.10 Aim

The aim of this study was to compare the leaf flavonoids profiles of some Iranian Angiosperm species from Leguminosae, Polygonaceae, Euphorbiaceae, Resedaceae and Cyperaceae.

2. Materiales and methodes

2.1 Collection of plant material and praperation

Mature fresh leaves of eight Legumes, seven *Polygonum*, seven *Rumex*, seventeen *Euphorbia*, two *Chrozophora*, four *Reseda* and five *Scirpus* species from different parts of Iran were collected during 2006-2010 as described in Table 1. Plants identified using available references [Rechinger 1964, Mobayen 1979, 1980, Ghahreman 1979-2006]. Specimens of each sample were prepared for reference as herbarium vouchers that were deposited at the Arak University herbarium. Samples were air dried for detection and identification of flavonoids.

2.2 Extraction of the plant material

For a comparative analysis of the flavonoids, small extracts of all the accessions were prepared by boiling 200 mg of powdered air dried leaf material for 2 min in 5 ml of 70% EtOH. The mixture was cooled and left to extract for 24 h. The extract was then filtered, evaporated to dryness by rotary evaporation at 40°, and taken up in 2 ml of 80% MeOH for analysis by 2-Dimensional Paper Chromatography (2-D PC).

2.3 Flavonoid analysis by 2-Dimensional Paper Chromatography (2-D PC)

For the detection of flavonoids, ca 20 µl of each of the small extracts was applied to the corner of a quarter sheet of Whatman No 1 chromatography paper as a concentrated spot (10 applications of 2µl). The chromatogram for each sample was developed in BAW (n-BuOH-HOAc-H_2O=4:1:5; V/V; upper layer), 1st direction, and HOAc (=15% aqueous acetic acid), 2nd direction, with rutin (= quercetin 3-*O*-rutinoside) as a standard. After development, the chromatograms were viewed in longwave UV light (366 nm) and any dark absorbing and fluorescent spots were marked. R_f-values in BAW and 15% HOAc were calculated.

2.4 Methods of identification of the flavonoids

When sufficient amounts of purified flavonoids had been obtained, as in the cases of the flavonoids from studied samples, they were identified by means of UV spectroscopy using shift reagents to investigate the substitution patterns of the flavonoids [Mabry et al. 1970, Markham 1982] and by acid hydrolysis to identify the aglycone and sugar moieties. Cochromatography with standards was also performed where possible. Flavonoid standards available for comparison during the study obtained commercially from Merck, Sigma and Fluka.

2.5 Acid hydrolysis and identification of flavonoid aglycones

A small amount of each purified flavonoid (ca 0.5 mg) was dissolved in 0.5 ml of 80% MeOH in a test tube. To this sample 2 ml of 2M HCl were added and the mixture was heated in a water bath at 100°C for 0.5 h. The solution was cooled, 2 ml of EtOAc were added and thoroughly mixed with the aqueous layer using a whirley mixer. The upper EtOAc layer was removed with a pipette, evaporated to dryness, dissolved in 0.5 ml of MeOH and applied as spots on thin layer chromatograms (cellulose). The TLC plates were run in three solvents alongside standards to identify the aglycone moiety [Harborne 1998].

Voucher data	Taxon	Number of total flavonoids	Number of flavonoid sulphates	Number of flavone C-and C-/O-glucosides	Number of aglycones
	Chrozophora				
*CAM2	*C. tinctoria*	3	3	2	8
CAM22	*C. hierosolymitana*	3	2	3	8
	Euphorbia				
*CMK 23	*E. bungei* Boiss.	5	2	3	0
CMK 65	*E. chamaesyce* L.	8	4	4	0
CMK 57	*E. cheiradenia* Boiss. et Hohen.	7	5	2	0
CMK 63	*E. cordifolia* Ell.	9	3	6	0
CMK 60	*E. esula* L.	7	3	4	0
CMK 59	*E. falcata* L.	7	3	4	0
CMK 32	*E. helioscopia* L.	5	3	2	0
CMK 26	*E. heteradena* Jaub,et Spach.	9	3	6	0
CMK 54	*E. macroclada* Boiss.	6	1	5	0
CMK 70	*E. microsciadeae* Boiss.	9	5	4	0
CMK 69	*E. ozyridiforma* Parsa.	7	4	3	0
CMK 62	*E. peplus* L.	8	4	4	0
CMK 74	*E. petiolata* Banks et Soland	9	6	3	0
CMK 16	*E. seguieriana* Necker.	8	5	3	0
CMK 10	*E. splendida Mobayen.*	7	3	4	0
CMK 48	*E. szovitsii* Fisch.& Mey.	8	5	3	0
CMK 34	*E. tehranica* Boiss.	8	6	2	0
	Papilionoideae				
*CMJ148	*Alhagi camelorum* Fisch.	2	1	1	0
CMJ149	*Cercis siliquastrum* L.	2	1	1	0
CMF1	*Coronilla varia* L.	5	4	1	0
CMJ150	*Glycyrrhiza glabra* L.	2	1	1	0
CMJ151	*Medicago sativa* L.	0	0	0	0
CMJ152	*Robina peseudo-acacia L.*	4	3	1	0
CMJ153	*Sophora alopecuroides ssp. alopecuroides*	0	0	0	0
CMN1	*Sophora alopecuroides ssp. tomentosa*	0	0	0	0
	Polygonum				
*CEM1	*P. aviculare*	4	0	4	0
CEM2	*P. convolvolus*	1	0	1	0
CEM3	*P. hyrcanicum*	4	0	4	0
CEM4	*P. patulum*	3	0	3	0
CEM5	*P. alpestre*	3	0	3	0
CAM6	*P. arenastrum*	4	0	4	0
CAM7	*P. persicaria*	3	1	2	0
	Reseda				
*CMG27	*Reseda aucheri*	8	5	3	0
CMG21	*R. buhseana* Mull-Arg.	10	5	4	1
CMG22	*R. bungei* Boiss.	8	7	1	0
CMG11	*R. lutea* L.	7	6	1	0
	Rumex				
*CMR2	*R. chalpensis*	10	4	6	0
CMR4	*R. crispus*	6	2	4	0
CMR6	*R. obtusifolius*	10	4	6	0
CMR7	*R. tuberosus*	10	5	5	0
CMR9	*R .pulcher*	8	2	6	0
CMR12	*R. acetosella*	8	2	6	0
CMR14	*R.conglomeratus*	9	2	6	1
	Scirpus				
*CNM4	*S. holoschenus* L.	8	4	1	3
CMN23	*S. lacustris* L.	6	5	0	1
CMN8	*S. littoralis* Kuntze	10	8	1	1
CMN6	*S. maritimus* L.	9	5	3	1
CMN18	*S. multicaule*	12	5	2	5

Table 1. The sampling and also two-dimensional paper and thin layer chromatographically data of 48 studied plant samples from Markazi Province, Iran.

Voucher data	Identification									
	Apigenin	Chrycin	Kaempferol	Luteolin	Myricetin	Naringenin	Quercetin	Rhamnetin	Rutin	Vitexin
Chrozophora										
*CAM2	++++	-	-	-	-	-	+++	-	++	-
CAM22	++++	-	-	-	-	-	++++	-	-	-
Euphorbia										-
*CMK 23	-	-	++	-	-	-	+++	-	++	-
CMK 65	-	-	-	-	-	-	+++	-	+++	-
CMK 57	-	-	-	-	-	-	+++	-	+++	-
CMK 63	-	-	-	-	-	-	-	-	++	-
CMK 60	-	-	+++	-	-	-	+++	-	+	-
CMK 59	-	-	+++	-	-	-	++	-	+++	-
CMK 32	-	-	-	-	-	-	+++	-	+	-
CMK 26	-	-	+++	-	-	-	++	-	++	-
CMK 54	-	-	-	-	+++	-	++	-	-	-
CMK 70	-	-	-	-	-	-	+++	-	+++	-
CMK 69	-	-	+++	-	-	-	++	-	+	-
CMK 62	-	-	++	-	-	-	+	-	++	-
CMK 74	-	-	-	-	+++	-	+++	-	-	-
CMK 16	-	-	+++	-	-	-	+++	-	+++	-
CMK 10	-	-	-	-	-	-	+	-	+	-
CMK 48	-	-	++	-	-	-	+++	-	++	-
CMK 34	-	-	-	-	-	-	-		+	-
Papilionoideae										
*CMJ148	-	-	++	-	-	-	--	-	++	-
CMJ149	-	-	++		+++		+++		++	-
CMF1	-	-	-	-	±	-	+	+	±	-
CMJ150	-	-	--	-	-	-	++	-	++	-
CMJ151	-	-	-	-	-	-	-	-	-	-
CMJ152	++		++		-	-	-	-	++-	-
CMJ153	±	-	-	-	-	-	-	-	-	-
CMN1	±	-	-	-	-	-	-	-	-	-
Polygonum										
*CEM1	-	-	+	-	+	-	+	-	+	-
CEM2	-	-	-	-	-	-	+	-	-	-
CEM3	-	-	+	-	+	-	+	-	-	-
CEM4	-	-	+	-	-	-	+	-	-	-
CEM5	-	-	+	-	-	-	+	-	+	-
CAM6	-	-	+	-	+	-	+	-	+	-
CAM7	-	-	-	-	+	-	+	-	-	-
Reseda										
*CMG27	-	-	+	-	+	-	+++	++	++	-
CMG21	-	-	+++	+	++	-	-	-	-	-
CMG22	-	-	+++	+	++	-	-	-	-	-
CMG11	-	-	+++	++	-	-	+++	±	++	
Rumex									+	
*CMR2	+	-	+	+	-	-	+	+		
CMR4	-	-	-	±	-	++	++	+	-	-
CMR6	-	-	±	++	-	±	±	+	+	-
CMR7	++	-	±	-	-	±	±	++	-	-
CMR9	+	-	+	+	-	-	++	+	+	-
CMR12	-	-	+	-	-	±	+++	-	-	-
CMR14	+	-	-	±	±	±	++	+	-	-
Scirpus										
*CNM4	++	+	-	+	-	+++	+	+	+	+
CMN23	-	±	-	+	±	±	+++	+	++	+++
CMN8	++	+	-	+	-	+++	-	+++	+	+
CMN6	-	+	-	+	-	++	-	++	+	+
CMN18	++	+	-	++	-	+++	++	++	+	++

*C=Collection number

-(non flavonoid), ± (non or a few flavonoid), + (few flavonoid), ++ (middle concentration of flavonoid), +++ (high concentration of flavonoid)

Table 1. Continued

3. Results

All studied plant species exceptional two subspecies *Sophora alopecuroides* and *Medicago sativa* contained flavonoid compounds in their leaves. Their flavonoid profiles show a wide variety between the species. Data in Table 1 shows the sampling and also two-dimensional paper and thin layer chromatographical data of 48 studied *plant samples* from Markazi Province, Iran.

4. Discussion

Studies of leaf flavonoids showed some phytochemical characters such as total number of flavonoids, flavonoid group such as aglycone, flavones *C*- and *C-/O* glycoside and flavonoid sulphate and kind of flavonoids such as kaempherol, quercetin, myricetin are valuable for chemotaxonomy and their usage.

Chemical study of two *Chrozophora* species using two dimentional paper chromatography (2-DPC) and thin layer chromatography (TLC) showed both *Chrozophora* species contain flavonoid sulphates, flavone *C* and *C-/O*-glycosides and aglycon. Also all of studied species have apigenin and quercetin while rutin was just found in *C. tinctoria* species that is recorded first time for Markazi Province. All of studied species have flavonoid compounds that have variation in their flavonoid type and number (Table 1).

Phytochemical studies of the Euphorbiaceae have been extremely useful in clarifying systematic relationships within the family (Simpson and Levin 1994). Flavonoids occur widely in plants and are a biologically major and chemically diverse group of secondary metabolites that are popular compounds for chemotaxonomic surveys of plant genera and families [Harborne 1994]. There are some studies in this connection. Mues and Zinsmeister (1988) have discussed about variation of occurrence phenolic compounds in mosses and liverworts. Also they showed there is a clear flavonoid distinction between the subclasses Marchantiidae and Jungermanniidae. Another important chemotaxonomic programme has concerned the ferns and fern allies [Harborne 1986]. The phenolic patterns appear to be more useful for studying relationships within relatively narrow taxonomic limits, e. g. at the species and genus level. Turning to the angiosperms, a chemotaxonomic survey of 255 species of the family Iridaceae has been carried out by Williams et al (1986), who found that flavone *C*-glycosides were present in 66% of the samples [Harborne 1986]. Another family survey has been carried out in the *Polygonaceae*, in which 28 species were analysed for their flavonoid pattern [Harborne 1986]. Studying flavonoid pattern can be used for chemosystematic and lower taxonomic levels. 25 *Avena* species (Poaceae) were investigated for the flavonoid content of leaf tissue [Saleh et al 1988]. Diploid *triticum* species could be divided into two groups depending on the presence or absence of two major di-*C*-glycosylflavones (Harborne et al 1986). Flavonoid data of the genus *Vitis* indicate three chemical groups [Moore and Giannasi 1994]. Several studies indicated that flavonoids occurred in various species of *Euphorbia*. They may be useful taxonomic markers within the genus. Also *Euphorbia* flavonoids are very important for their toxicity and some different potential clinical applications such as their antiatherosclerotic, antiinflammatory, antitumor, antithrombogenic, antiosteoporotic and antiviral effects [Nijveldt et al 2001]. Papp et al (2005) showed populations of *Euphorbia cyparissias* can be separated clearly from each other according to their morphology and flavonoid pattern.Our results showed all studied

Euphorbia species contained flavonoid compounds in their leaves that their flavonoid profiles show a wide variety between the taxa. There are flavonoid sulphate and flavone *C* and *C*-/*O*-glycosides in all species, but *E. bungei*, *E. heteradena* and *E. microsciadea* in addition these two flavonoid types have dihydroflavonol 3-*O*-monoglycosides. *E. cordifoila*, *E. heteradena*, *E. microsciadeae* and *E. petiolata* have the highest number of total flavonoid compounds (9) and *E. bungei* and *E. helioscopia* have the lowest number of flavonoid compounds (2) in their leaves (Table 1). Identification of flavonoids by standards showed all of studied *Euphorbia* species contain rutin with the exception of *E. macroclada* and *E. petiolata*. Also all taxa studied, except 2 species (*E. cordifolia* and *E. tehranica*) have quercetin. Harborne and Baxter (1999) reported that quercetin is widely distributed in various plant families. Kaempferol found in 8 species and myricetin was found just in *E. macroclada* and *E. petiolata* (Table 1). As Volobueva (1970) showed two-dimentional paper chromatography and acid hydrolysis of *E. helioscopia* alcoholic extract yielded quercetin and kaempferol. Also Gautam and Mukhraya (1981) isolated quercetin 3-*O* glucoside and kaempferol 3-*O* glucoside from *E. larica*, *E. virgata*, *E. chamaesyce* and *E. magalanta* and rutin obtained with the exception *E. chamaesyce*. Both quercetin and kaempferol are flavonols. The flavonols may be among the most important flavonoids, they are the most ancient and widespread of the flavonoids, synthesized even in mosses and ferns, and have a wide range of potent physiological activities [Stafford 1991]. Chemical study of 17 *Euphorbia* species using two dimentional paper chromatography (2-DPC) and thin layer chromatography (TLC) showed rutin, quercetin and kaempferol are the most representative compounds for the genus and the presence of myricetin is a taxonomic character for separation of some *Euphorbia* species. It is believed that *Euphorbia* species can be separated from each other according to their flavonoid pattern.

Application of plant flavonoides data can revealed similarity and relationship between plants and inferring phylogeny and used in their taxonomy. 2-dimentional paper chromatography (2-D PC), on leaves of *Alhagi camelorum* Fisch, *Cersis silliquastrum* L., *Coronilla varia* L., *Glycirhiza glarba* L. and *Robnia peseudoacia* from Markazi Province showed all of five named species contain aglycones. *A. camelorum* and *G. glarba* had the most flavonoid variation and concentration having flavon glycoside and two subspecies of *Sophora alopecuroides* and *Medicago sativa* had not or had the least. The most flavonoid compounds similarity was between *C. silliquastrum* and *R. peseudoacia* (Table 1).

Phytochemical examination of the studied *polygonum* species showed all of *Polygonum* species contain flavon *C*- and *C*-/*O*-glycosides. *P. hyrcanicum* had the most flavonoid variation and concentration and *P. convolvolus* species with having just one flavonoid had the least. Flavonoid sulphates was found just in *P. persicaria* species (Table 1).

Chemical study of four *Reseda* species using two dimentional paper chromatography (2-DPC) and thin layer chromatography (TLC) showed all studied *Reseda* species contained flavonoid compounds in their leaves and kempferol is the most representative compound for the genus (Table 1). They may be useful taxonomic markers within the genus. Also *Reseda* flavonoids are very important for their toxicity and some different potential clinical applications such as their antiatherosclerotic, antiinflammatory, antitumor, antithrombogenic, antiosteoporotic and antiviral effects [Nijveldt et al 2001]. The presence of quercetin and absence of myrestin in *R. lutea* are taxonomic characters for separation of the species from two other species (*R. buhseana* and *R. bungei*). Among the many functions of flavonoids at the interface between plant and

environment, their activity as signals was intensively studied. Flavonoids are also beneficial for the plant itself as physiological active compounds, as stress protecting agents, as attractants or as feeding deterrents, and, in general, by their significant role in plant resistance [Treutter 2006].

Chemical studies of seven *Rumex* species using two dimentional paper chromatography (2-DPC) and thin layer chromatography (TLC) showed all of studied *Rumex* species contain flavonoid compounds with wide variation. *R. chalpensis*, *R. obtosifolius* and *R. tuberusos* species had the most flavonoid number and *R. crispus* species had the least. Identified flavonoid compounds in all of studied species with the exception *R. crispus* (lack flavonoid sulphate) are flavones *C* and *C-/O* glucoside. *R. acetosella* and *R. conglomerates* had aglycon. Rutin and luteolin found in all of studied species exceptional *R. chalpensis*, *R. obtosifolius* and *R. pulcher*. All of studied species showed wide variation in existing and concentration of myricetin, apigenin, narengenin, rhamnetin, quercetin and kaempferol. All of studied species with the exception *R. chalpensis* and *R. tuberusos* had quercetin and also kaempferol found in 3 species (*R. chalpensis*, *R. pulcher* and *R. acetosella*) (Table 1).

Phytochemical studies on five species of *Scirpus* (*S. holoschenus* L., *S. lacustris* L., *S. littoralis* Kuntze, *S. maritimus* L. and *S. multicaule*) from different parts of Markazi Province, Iran area using two-dimentional paper chromatography (2-DPC) and thin layer chromatography (TLC) showed all of studied taxa contain vitexin, luteolin, rutin and rhamnetin. There were chrysin and naringenin in all of populations with the exception of *S. lucustris* and apigenin was found in 3 species weheras others lack. Quercetin was not found in *S. maritimus* and *S. littoralis* where as three other species had (Table 1).

Our studies showed the most of collected plant species are weed and grow in poor soils and destroyed pasture. Progress continues to be made in understanding the roles of flavonoids in stress protection, as well as in defining the mechanisms that control the amount and varieties of flavonoids that are produced in plants in responses to diverse environmental cuse [Chalker-Scott 1999]. Finally, further work is needed using high performance liquid chromatography with diode array detection, atmospheric pressure chemical ionization liquid chromatography-mass spectroscopy to evaluate all flavonoid profiles in studied and other species.

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8

Phytochemicals from *Beilschmiedia anacardioides* and Their Biological Significance

Nkeng-Efouet-Alango Pépin
University of Dschang,
Cameroon

1. Introduction

Medicinal plants provide a vast array of raw materials for primary health care in Africa and other countries of the world. The World Health Organization (W.H.O) estimates that about 80% of Africans living in the continent have resort to traditional medical practitioners and the use of traditional medicine for the treatment of their diverse ailments. This practice has a considerable importance within the economic and cultural milieu of Africa.

It is estimated that less than 10% of the world's genetic resources have been studied seriously as sources of medicines. Yet from this small fraction, humanity has reaped enormous benefits.

The search for bioactive plant natural products from higher plants is gathering momentum, as they have potential to provide new lead compounds or to be of use directly. There is an increasing sense of urgency about this search due to the destruction of natural resources. With regards to these plants, it has been estimated that 25-30 million hectares of the world's rainforests are lost each year. The crucial problem already expressed by several scientists then is how to search efficiently and rapidly for bioactive components from the vast number of unstudied plants. Part of the solution is to narrow down the search-selection.

Six approaches to the selection of plant materials for study exist: the locally random, the taxonomic, the ethnobotanic, the phytochemical, the information based, and serendipity. The ethnomedical approach appears to be the method of choice for natural product chemists working in Africa and other developing countries. In this method only plants used in traditional medicine are collected.

Very little attention has been paid to *Beilshmiedia* species. Previous studies concern trees and herbs of *Beilshmiedia* species, with the aim of cultivating herbs containing the same endiandric acid derivatives as trees. Other studies led to a patent on interesting synthesis of endiandric acid derivatives.

In our own search for prospective pharmacological products from ethnobotanic data, we have been looking at some traditional medicines whose therapeutic efficiency is scientifically established towards biomedical analyses of patients on treatment in a specialized clinic. We have selected a traditional medicine based on one plant *Beilshmiedia*

anacardioides (Lauraceae), for its proven efficiency on genital infections and rheumatisms through clinical research. No phytochemical studies of *Beilshmiedia anacardioides* are however to our knowledge available in the literature. We propose that phytochemists looking for novel bioactive natural products should investigate the medicinal plants whose therapeutic efficiency has been established through clinical research on African medicine.

The genus *Beilschmiedia* comprises about 200 species widely distributed in the intertropical region (Fouilloy, 1974). *B. anacardioides* stem bark is used in the Western Province of Cameroon to cure uterine tumours (Tchouala, 2001). Some other species of the genus Beilschmiedia are used in traditional medicine in Africa for the treatment of several ailments (Tchouala, 2001; Iwu, 1993). Previous phytochemical investigations of plants of the genus *Beilschmiedia* reported the presence of bio-active lignans (Chen et al., 2006; Chen et al., 2007), flavonoids (Harbone et al., 1969), triterpenoids (Chen et al., 2006); tetracyclic endiandric acid (Bandaranayake et al., 1981; Banfield et al., 1994) and alkaloids (Clezy et al., 1966; Kitagawa et al., 1993; Chouna et al., 2011).

We have initiated a systematic phytochemical investigation of the extracts of *Beilshmiedia anacardioides* as well as the antibacterial activity of the eight new compounds isolated, towards five strains of microbes, namely *Bacillus subtilis*, *Micrococcus luteus*, *Streptococcus faecalis*, *Pseudomonas palida*, and *Escherichia coli*.

The methods used for the isolation of the compounds were mainly column chromatography and preparative TLC. The structures of all compounds were elucidated by means of modern spectroscopic techniques such as 1D-NMR (^{1}H-NMR, ^{13}C-NMR with DEPT experiments), and 2D-NMR (^{1}H-^{1}H-COSY, HMQC,HMBC,NOESY), MS , IR and X-Ray spectroscopies.

The antibacterial activities of the new compounds were examined using the dilution technique with respect to the zone of inhibition (ZI) and minimum inhibitory concentration (MIC).

We report here the results we have so far obtained and published in three renowned scientific journals (Chouna et al.,2009; 2010; 2011).

2. Study of the ethnomedical preparation

The ethnomedical preparation is a decoction.The decoction is prepared as follows: Boil about 80 g dry stem bark powder in 3 litres of water for 15 minutes. Filter when lukewarm. Drink a glass twice daily for ten days.

A treatment for fibromes could last about two to three months, depending on the patient's age.

3. Study setting

Cameroon is a bridge between Central Africa and West Africa, humid Africa and dry sahelian Africa, French speaking and English speaking Africa (French and English are official languages). The country is open to the Gulf of Guinea in his south-west border. Lake Chad is at the extreme –North border. A country of 475.442 square kilometers, Cameroon is borded in the west by Nigeria, on the east by Chad and the Central African Republic, and on the south by Congo, Gabon, and Equatorial Guinea.

The Bamoun are a Bantu people living in the west Region of Cameroon. They number more than half a million. They have a rich cultural Heritage, including famous traditional Healers. Sultan Njoya wrote a book on Bamoun traditional medicine. Important Bamoun towns are Foumban, Foumbot, Koutaba, Massangam, Magba, Malantouen. Among important villages are Mahoua, Manki 1 and Manki 2, where the plant *Beilshmiedia anacardioides* was collected.

3.1 Generalities on *Beilschmiedia* anacardioides

B. anacardioides is found in Central Africa, especially in Cameroon, Tchad and Gabon. In Cameroon, this species is found in the Adamaoua and the West Region (Eyog et al., 2006; Fouilloy, 1974). It is synonymous with *B. ngriki* and *B. Jacques-felixii* and it is commonly named *ntseum* (in Bamoun language) in the Noun subdivision of the West region of Cameroon (Eyog et al., 2006; Fouilloy, 1974; Tchouala, 2001).

3.2 Uses of *Beilschmiedia* species in traditional medicine

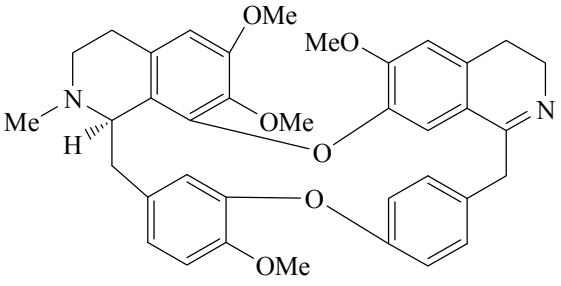
B. anacardioides stem bark is used in the Noun sub-division of the West Region of Cameroon to treat uterine tumours, rubella, rheumatisms, bacterial and fungal infections (Tchoula, 2001). Seeds are used as spices (Eyog et al., 2006). *B. lancilimba* is used in the same region to cure skin bacterial infections (Tchouala, 2001). *B. manii* is used to treat dysentery and headache. It is also used as an appetite stimulant (Iwu, 1993).

4. Phytochemistry of plant constituents of *Beilshmiedia* species

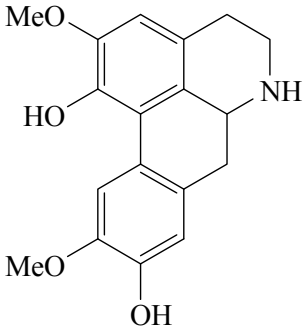
A review of the literature revealed that no phytochemial studies have been carried out on *Beilshmiedia anacardioides* prior to the initiation of our study. The various phytochemical and pharmacological studies performed and reported in the literature on the beilshmiedia genus are discussed below.

4.1 Alkaloids isolated from the *Beilschmiedia* genus

Very few alkaloids have been isolated from the *Beilschmiedia* genus.

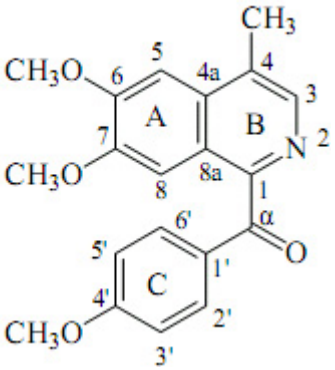
Structure and name	Source and references
	Wood of *B. madang* (Kitagawa et al., 1993)

9: Dehatrine



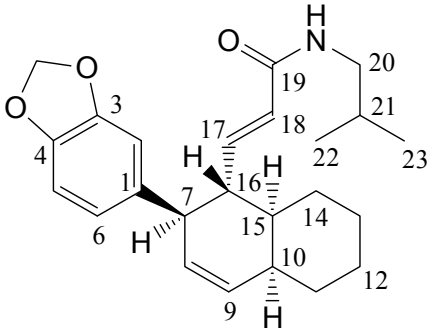
Stem bark of *B. elliptica*
(Clezy et al., 1966)

10: Laurelliptine



Leaves of *B. Brevipes*
(Pudjiastuti et al.,2010)

11: (6,7-Diméthoxy-4-methylisoquinolinyl)-(4'-methoxyphenyl)-methanone



Stem bark of *B. Obscura*
(Lenta et al.,2011)

12: Obscurine

Table 1. Structure of some alkaloids isolated from the *Beilschmiedia* genus

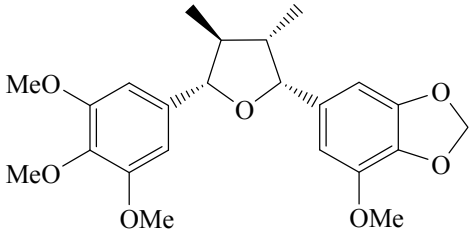
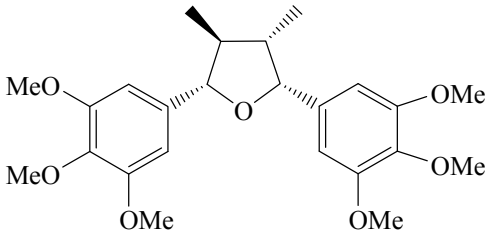
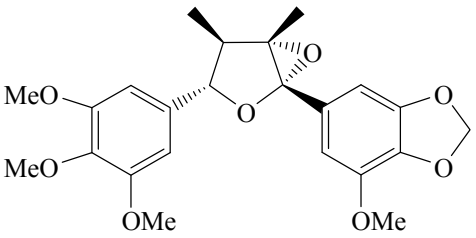
Pharmacological importance of alkaloids isolated from the Beilschmiedia genus

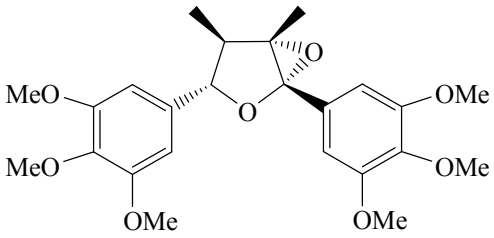
A bisbenzylisoquinoline alkaloid dehatrine (**9**) isolated from the wood of *B. madang*, exhibited potent inhibitory activity (IC_{50} value of 0.017 μM) against the proliferation of the malaria pathogen *P. falciparum* (Kitagawa et al., 1993). Paulo and coworkers (1992) demonstrated the antimicrobial properties of laurelliptine (**10**).

4.2 Phenolic and phenolic derived compounds

4.2.1 Lignans and neolignans

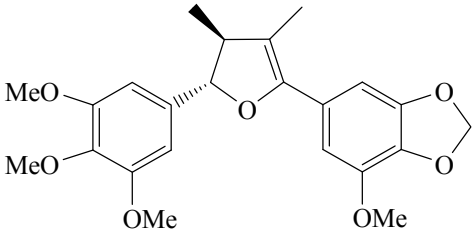
Lignans and neolignans and flavonoids are the main phenolic compounds encountered in the *Beilschmiedia* genus.

Structure and name	Source and references
 **13**: Beilschmin A	Stem of *B. tsangii* (Chen et al., 2006)
 **14**: Beilschmin B	Stem of *B. tsangii* (Chen et al., 2006)
 **15**: 4α,5α,-Epoxybeilschmin A	Leaves of *B. tsangii* (Chen et al., 2007)



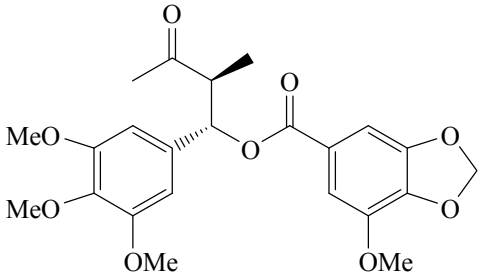
Stem of *B. tsangii* (Chen et al., 2006)

16: 4α,5α,-Epoxybeilschmin B



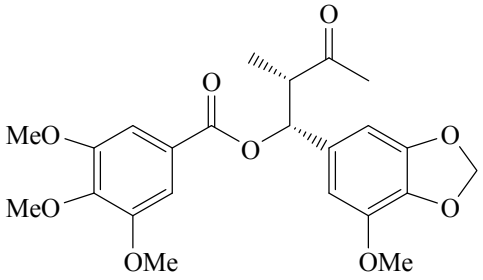
Stem of *B. tsangii* (Chen et al., 2006)

17: Beilschmin C



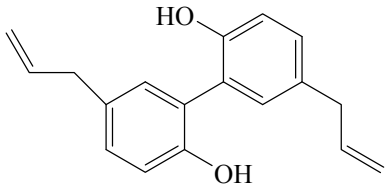
Stem of *B. tsangii* (Chen et al., 2006)

18: Tsangin A



Stem of *B. tsangii* (Chen et al., 2006)

19: Tsangin B



Leaf of *B. volckii* (Banfield et al., 1994)

20: Magnolol

Table 2. Structure of some lignans and neolignans isolated from *Beilschmiedia* genus

Pharmacological importance of lignans and neolignans isolated from the Beilschmiedia genus

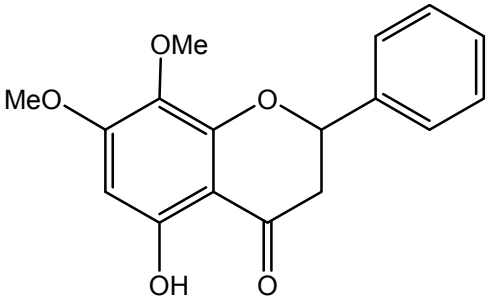
Tetrahydrofuran-type lignans beilschmin A (**13**) and B (**14**), dihydrofuran-type lignan beilschmin C (**17**) together with tsangin A (**18**) and B (**19**) were found cytotoxic (IC_{50} value below 4 μg/mL) in P-388 and/or HT-29 cell lines *in vitro* (Chen et al., 2006). In addition, beilschmin A (**13**) and B (**14)** exhibited potent antitubercular activity (MIC values of 2.5 and 7.5 μg/mL, respectively) against *Mycobacterium tuberculosis* 90-221387 in *vitro* (Chen et al., 2007). A neolignan, magnolol (**20**) displayed wide biological properties, mainly cytotoxic (Li et al., 2007), antidepressant (Li et al., 2007), antimicrobial (Park et al., 2004) and anti-inflammatory (Lee et al., 2005).

4.2.2 Some flavonoids isolated from *Beilschmiedia* genus

Pharmacological importance of flavonoids isolated from the Beilschmiedia genus

Lenta and coworkers (2009), evaluated the antibacterial activities of the extract and flavonoids isolated from the stem of *B. zenkeri*, in vitro against three strains of microbes, *pseudomonas agarici, Bacillus subtilis,* and *streptococus minor*. Their activities were moderate compare to reference drugs ampicillin and gentamicin. (2S,4R)-5,6,7-trimethoxyflavan-4-ol (**22a**) exhibited the best potency against *S. minor* (IC_{50} of 197.5 µM) (Lenta et al., 2009).

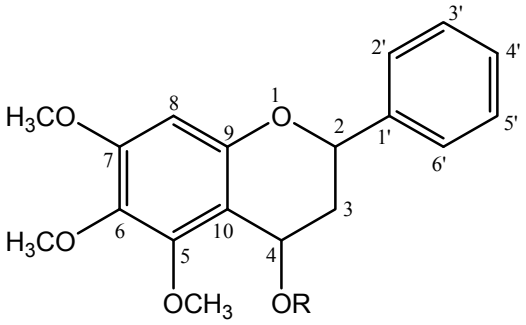
Structure and name **Source and references**



Stem of *B. zenkeri* (Lenta et al., 2009)

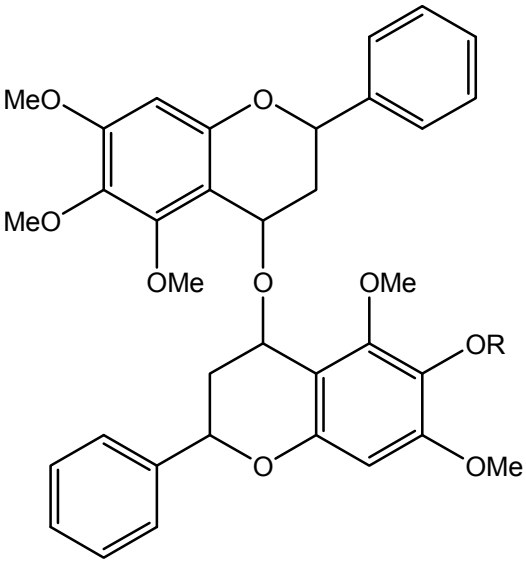
21: 5-Hydroxy-7,8-dimethoxyflavanone

Stem of *B. zenkeri* (Lenta et al., 2009)



22a: R = H: (2S,4R)-5,6,7-trimethoxyflavan-4-ol
22b: R = CH3: (2S,4R)-4,5,6,7-trimethoxyflavan

Stem of *B. zenkeri* (Lenta et al., 2009)



23a: R = CH3: Beilschmieflavonoid A
23b: R = H: Beilschmieflavonoid B

Table 3. Structure of flavonoids isolated from the *Beilschmiedia* genus

4.2.3 Other phenolic and phenolic derived compounds from the *Beilschmiedia* genus and their pharmacological importance

Vanillin (**21a**) and 4-hydroxybenzaldehyde (**24b**) were isolated from *Beilschmiedia tsangii* (Chen et al., 2006). Both compounds were reported to exhibit analgesic, anti-inflammatory and antifungal activities (Lee et al., 2005; Lee et al., 2006; Fitzgerald et al., 2005).

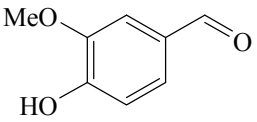
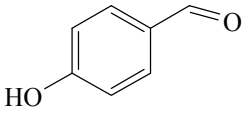
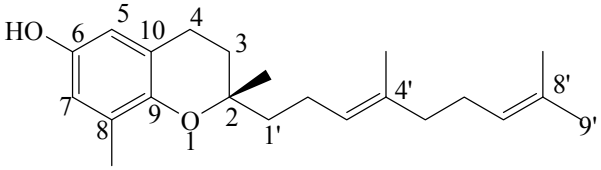
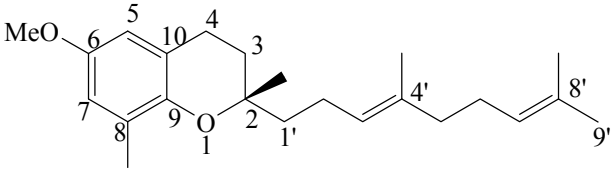
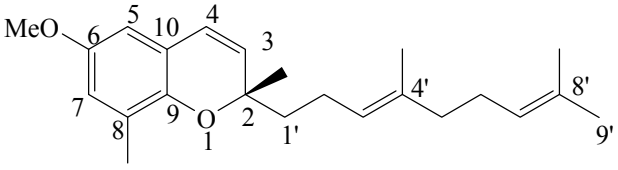
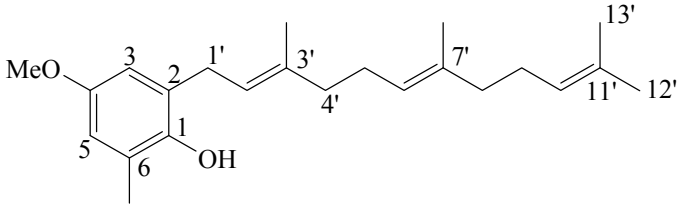
Structure and name	Source and references
 **24a**: Vanillin	Stem of *B. tsangii* (Chen et al., 2006)
 **24b**: 4-hydroxybenzaldehyde	Stem of *B. tsangii* (Chen et al., 2006)
 **25a**: Oligandrol	Bark of *B. oligandra* (Banfield et al., 1994)
 **25b**: Oligandrol methyl ether	Root of *B. erytrhophloia* (Yang et al., 2008)
 **25c**: 3,4-Dehydrooligandrol methyl ether	Root of *B. erytrhophloia* (Yang et al., 2008)
 **26**: Farnesylol	Root of *B. erytrhophloia* (Yang et al., 2008)

Table 4. Structure of other phenolic and phenolic derived compounds isolated from the *Beilschmiedia* genus

4.3 Endiandric acids

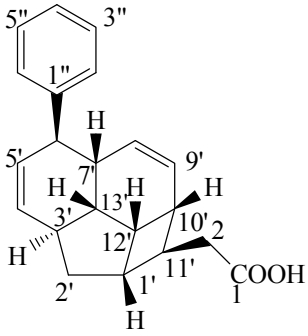
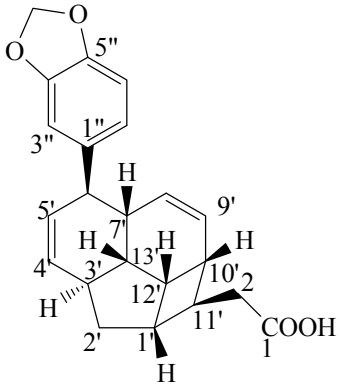
Endiandric acids are a rare class of secondary tetracyclic metabolites generally encountered in *Beilschmiedia* and *Endiandra* species of the Lauraceae family. Endiandric acids are products of electrocyclic cyclization of naturally occurring polyketides (Bandanarayake et al., 1980).

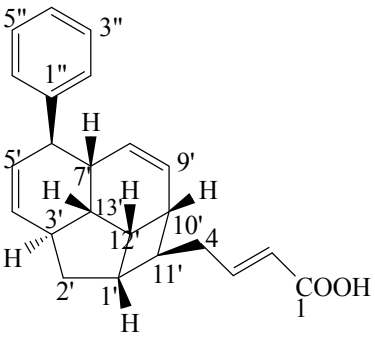
4.3.1 Some endiandric acids previously isolated

Pharmacological importance of the endiandric acids

Very few pharmacological studies have been done in this class of metabolites. Endiandric acid H (**41**) is used for the manufacture of medication, in particular for the treatment of asthmatic disorders or concomitant inflammatory symptoms of asthma (Eder et al., 2004).

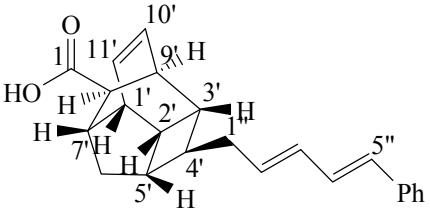
Erytrophloin C (**34**) exhibited antitubercular activity (MIC value of 50 μg/mL) against *Mycobacterium tuberculosis* H37Rv in *vitro* (Yang et al., 2009).

Structure and name	Source and references
 **27**: Endiandric acid A	Leaves of *Endiandra entrorsa* and *Endiandra oligandra* (Bandaranayake et al., 1981) Bark of *B. oligandra* (Banfield et al., 1994)
 **28**: Methylenedioxyendiandric acid A	Leaves of *Endiandra entrorsa* (Banfield et al., 1994) and Stem bark of *B. manii* (Mpetga, 2005)



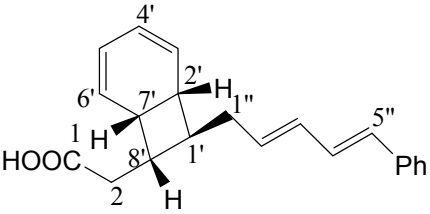
Leaves of *Endiandra entrorsa* (Bandaranayake et al., 1982)
Barks and leaves of *Endiandra jonesii* (Banfield et al., 1994)

29: Endiandric acid B



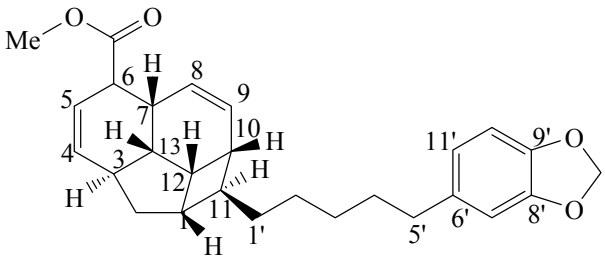
Leaves of *Endiandra entrorsa* (Bandaranayake et al., 1982)
Bark and leaves of *Endiandra jonesii* (Banfield et al., 1994)

30: Endiandric acid C



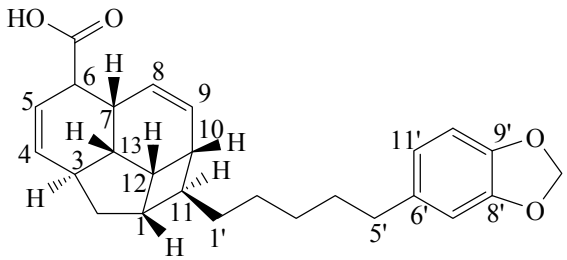
Leaves of *Endiandra entrorsa* (Banfield et al., 1983)

31: Endiandric acid D



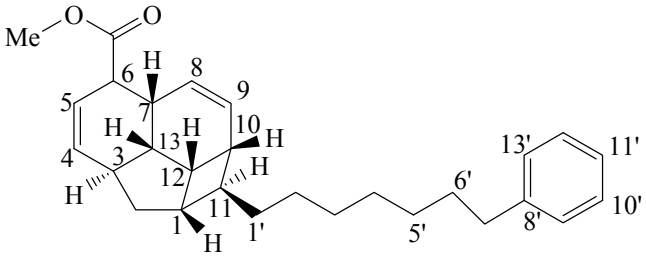
Root of *B. erytrhophloia* (Yang et al., 2009)

32: Erytrophloin A



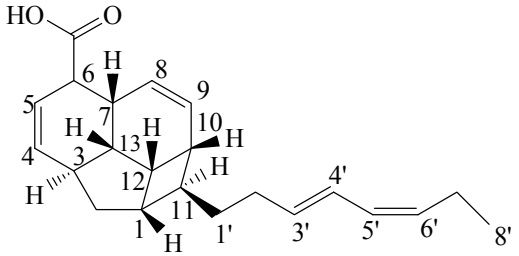
Root of *B. erytrhophloia* (Yang et al., 2009)

33: Erytrophloin F



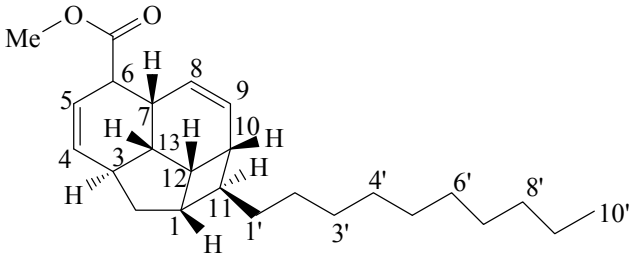
Root of *B. erytrhophloia* (Yang et al., 2009)

34: Erytrophloin C



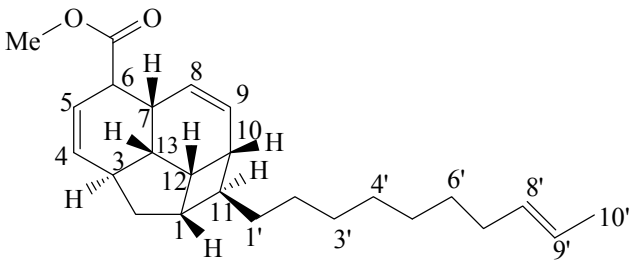
Root of *B. erytrhophloia* (Yang et al., 2009)

35: Erytrophloin E



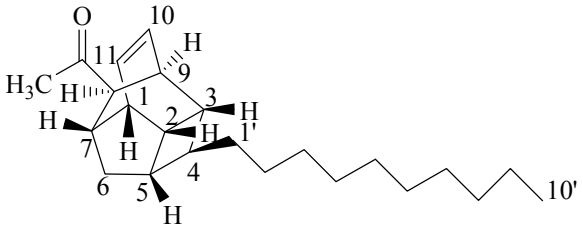
Root of *B. erytrhophloia* (Yang et al., 2009)

36: Erytrophloin B



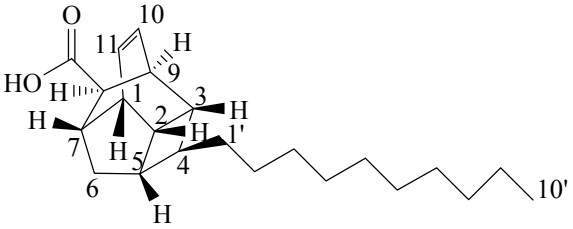
Root of *B. erytrhophloia* (Yang et al., 2009)

37: Erytrophloin D



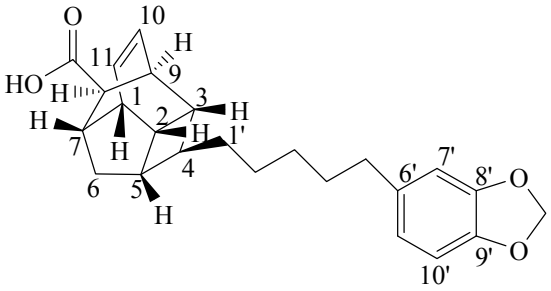
Root of *B. erytrhophloia* (Yang et al., 2009)

38: Beilcyclone



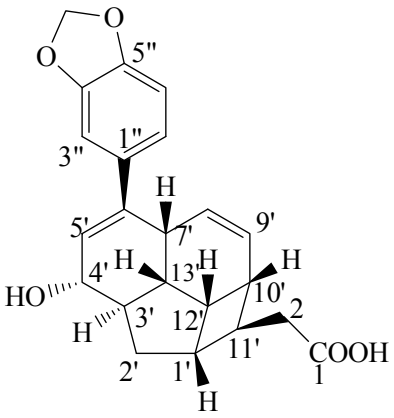
Root of *B. erytrhophloia* (Yang et al., 2008)

39: Endiandric acid J



Root of *B. erytrhophloia* (Yang et al., 2008)

40: Endiandric acid I



Stem of B. fulva (Eder et al., 2004)

41: Endiandric acid H

Table 5. Structure of some endiandric acids previously isolated

5. Results of our own studies

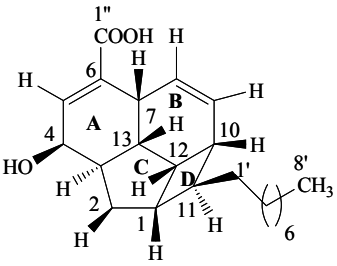
We have initiated a systematic phytochemical investigation of the extracts of *Beilshmiedia anacardioides* and have so far obtained the following results which have led to three publications in renowned scientific journals (Chouna et al.,2009; 2010; 2011).

Air-dried and ground stem bark of *B. anacardioides* was extracted successively at room temperature with MeOH. The methanol extract was re-extracted in turn with CH_2Cl_2 and EtOAc. These extracts were concentrated to dryness under reduced pressure.

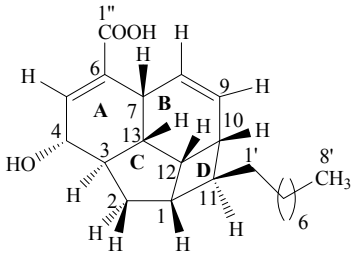
The CH_2Cl_2 extract was submitted to repeated column chromatography on silica gel, yielding beilschmiedic acids A (**1**), B (**2**) and C (**3**) and the known β-sitosterol (Chouna et al.,2009) .

Further Successive purifications by column chromatography over silica gel and preparative TLC afforded three new endiandric acid derivatives: beilschmiedic acids D(**4**) and E(**5**), and Beilshmiedin (**8**) (Chouna et al.,2010), together with the known compounds bisabolene (Mossa et al 1992; Barrero et al. 1990), and tricosanoic acid (Erdemoglu et al., 2008).

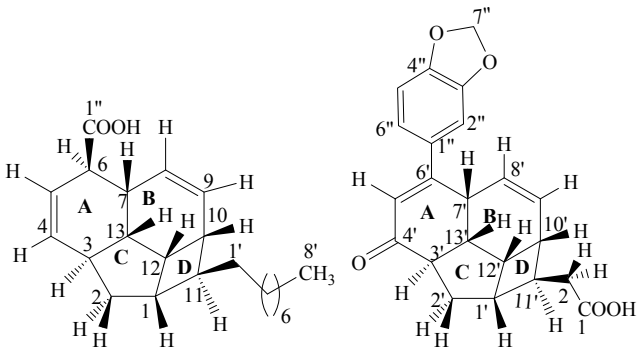
The ethyl acetate soluble part of the MeOH extract of the stem bark of *B. anacardioides* was fractionated by column chromatography over silica gel. Successive purifications by column chromatography and preparative TLC afforded two new endiandric acid derivatives: beilschmiedic acids F(**6**) and G(**7**) Chouna et al.,2011, along with the known constituents beilschmiedic acid A(**1**), beilschmiedic acid C(**3**) [6] and sitosterol-3-*O*-*β*-*D*-glucopyranoside (Chouna et al.,2011).



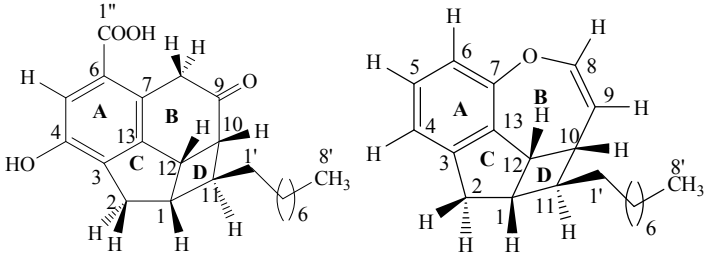
Beilshmiedic acid A (**1)** Beilshmiedic acid B (**2)**



Beilshmiedic acid C (**3)** Beilshmiedic acid D (**4)**



Beilshmiedic acid E (**5)** Beilshmiedic acid F (**6)**



Beilshmiedic acid G (**7)** Beilshmiedin (**8**)

Scheme 1. endiandric acid derivatives from *Beilshmiedia anacardioides.*

6. Biological activity and the significance of some compounds

Our preliminary antibacterial studies on the new endiandric acid derivatives have yielded chemical entities that have been shown to possess significant activities (Chouna et al., 2009) .

Antibacterial assay on some compounds isolated from *B.anacardioides*

Compounds **1-8** were tested in vitro for their antibacterial activity against *Bacillus subtilis*, *Streptococcus ferus*, *Streptococcus minor*, *Micrococcus luteus*, *Escherichia coli*, and *Pseudomonas agarici*, using the dilution technique.

The ZI (Table 1) and MIC (Table 2) obtained for these compounds indicated that they possessed strong to weak antibacterial activity against *gram* positive bacteria.

Beilshmiedic acid C (**3)** demonstrated the best potency against *B. subtilis* and *M. luteus*, compared to the reference drug ampicillin. The MIC values (Table 2) of Beilshmiedic acids B(**2)**, C (**3)** and G(**7),** against *B. subtilis* and Beilshmiedic acid C (**3)** against *M. luteus* were found to be greater than that of standard drug ampicillin, indicating that this series of compounds might be possible candidates as antibacterial drugs.

None of the tested compounds was active against *Gram* negative *P. palida* and *E. Coli*. Therefore, they might be well tolerated as antibiotics even for long term treatments.

Compound tested	*B. subtilis*	*M. luteus*	*S. faecalis*	*S. minor*	*S. ferus*	*P. palida*	*E. coli*
1	15	12	14	n.t.	n.t.	-	-
2	16	15	15	n.t.	n.t.	-	-
3	13	30	18	n.t.	n.t.	-	-
4	10	n.t.	n.t.	10	-	-	-
5	12	n.t.	n.t.	-	12	-	-
6	10	n.t.	n.t.	-	-	-	-
7	20	15	16	-	-	-	-
8	10	n.t.	n.t.	-	-	-	-
Ampicillin	29	26	25	22	23	-	-

(-) inactive, n.t. (not tested)

Table 6. Antibacterial activity (Zone of inhibition of compounds in mm) of compounds **1-8** (500μg/mL) against *B. Subtilis, M. luteus*, *S. faecalis, S. minor, S. ferus*, *P. palida* and *E. Coli.*

Compound tested	*B. subtilis*	*M. luteus*	*S. faecalis*	*S. minor*	*S. ferus*
1	181.60	173.60	363.30	n.t.	n.t.
2	11.30	347.20	45.30	n.t.	n.t.
3	5.60	< 0.70	22.70	n.t.	n.t.
4	381.00	n.t.	n.t.	190.50	-
5	381.00	n.t.	n.t.	190.50	-
6	343.40	n.t.	n.t.	-	-
7	87.78	10.95	87.78	-	-
8	422.20	n.t.	n.t.	-	-
Ampicillin	89.5	1.95	3.9	1.05	5.25

(-) inactive, n.t. (not tested)

Table 7. Antibacterial activity (MIC in μM) of compounds **1-8** against *B. Subtilis*, *M. luteus*, *S. faecalis*, *S. minor*, *S. ferus*, *P. palida* and *E. coli*.

Beilshmiedic acid C (**3)** was more active than Beilshmiedic acid D (**4)** . The enhanced activity may be due to the additional hydroxyl group at C-4 position in Beilshmiedic acid C (**3)** . Beilshmiedic acid B(**2)** which possesses one hydroxyl group more than Beilshmiedic acid A (**1)** and Beilshmiedic acid C (**3)** was less active. Beilshmiedic acid A (**1)** was more active than Beilshmiedic acid C (**3)** . They are epimers at C-4 position; the modification of the configuration at this position influences significantly the activity.

Based on the skeletal features, it is difficult at this stage to define the contribution of the different functional groups with respect to the activity. The mechanism of action of this class of metabolites on these strains is not yet known. Further investigations will help to establish the mode of action of this particular skeleton. These interesting results highlight the potency of this rare class of metabolites that might be investigated for the search of new antibacterial drugs.

7. Conclusion

In our hypothesis we proposed that phytochemists looking for novel bioactive natural products should investigate the medicinal plants whose therapeutic efficiency has been established through clinical research on African medicine.

We suggested that Natural products from *Beilshmiedia anacardioides* may play a role in treating genital infections due to *B-subtilis* and *M. luteus*, and rheumatisms due *Streptococcus ferus* and *S. minor*. The biological activities of some of the constituents isolated in our studies, Beilshmiedic acid C presented above, more than lend support to this suggestion.

It is certain that as more and more data become available from the phytochemical and biological analysis of the constituents of therapeutic efficient medicinal plants selected after

clinical research, the role of these plants in the treatment of diseases will become more defined. Thus African Traditional medicine will gain universal status.

8. Acknowledgments

Financial support for this survey from the Alango Foundation (Centre de Phytomédecine Africaine) at Dschang / Cameroon is gratefully acknowledged.

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9

Phenolic Constituents and Antioxidant Properties of some Thai Plants

Pitchaon Maisuthisakul
University of the Thai Chamber of Commerce, Thailand

1. Introduction

Thai plants have been used as medicines for many centuries because they contain active phytochemicals including phenolic compounds. These components function as antibiotics, help to make cell walls impermeable to gas and water, act as structural materials to give plants stability and provide protection against ultraviolet (UV) light. Hence, plants in the tropical zone including Thailand contain a high concentration of phenolic compounds formed as secondary metabolites in plants (Shahidi & Naczk, 2003).

Several parts of edible plants from tropical and subtropical climates are known to contain many phenolic compounds which are receiving increasing interest from consumers for several reasons (Leong & Shui, 2002). Epidemiological studies have suggested that relationships exist between the consumption of phenolic-rich foods or beverages and the prevention of diseases such as cancer, stroke, coronary heart desease and others. This association has been partially explained by the fact that phenolic compounds retard oxidative modification of low density lipoproteins (LDL), which is implicated in the initiation of arteriosclerosis. More recently, alternative mechanisms have been proposed for the role of antioxidants in reducing the incidence of cardiovascular disease, besides that of the simple protection of LDL from reactive oxygen species (ROS)-induced damage. Several phenolic antioxidants significantly affect cellular responses to different stimuli, including cytokines and growth factors. Although many papers have reported studies on the composition and antioxidant activity of phenolic compounds in tropical edible plants (Auddy et al., 2003; Nūnez sellés et al., 2002; Habsah et al., 2000), information about the phenolics and antioxidant potential of Thai edible plant species is limited compared to the broad biodiversity of edible plants grown. Moreover, many studies have been reported in the Thai language, and are not available to English speaking scientists.

The aim of this chapter is to provide a critical review of the composition and antioxidant properties of phenolic compounds of some Thai plants. In addition, factors affecting extraction of these components from Thai plants are reported.

2. Phenolic compounds of some Thai plants

Plant materials contain many phytochemicals including compounds with antioxidant activity, which are mostly phenolic in structure(Johnson, 2001). Compounds with

antioxidant activity are mainly phenolic acids, flavonoids and polyphenols (Dillard & German, 2000). Phenolic acids such as caffeic acid and gallic acid are widely distributed in the plant kingdom. The most widespread and diverse phenolics are the flavonoids which have the same C15 (C6-C3-C6) skeleton and retard oxidationof a variety of easily oxidizable compounds (Zheng & Wang, 2001). Flavonoids include catechins, proanthocyanins, anthocyanidins, flavones, flavonols and their glycosides (Ho, 1992). Flavonoids are ubiquitous in plants, since almost all plant tissues are able to synthesize flavonoids. Among the most widely distributed are the flavonols quercetin and rutin.

Investigations have increased considerably in recent years in order to find natural plant antioxidants to replace synthetic compounds whose use is being restricted due to possible side effects such as carcinogenecity (Zeng & Wang, 2001). Many papers have reported that phenolic plant constituents provide protection against oxidation (Amarowicz et al., 2003; Pokorny, 2001). Phenolic substances inhibit propagation of the oxidation chain reactions due to their resonance stabilized free-radical forms (Lindsay, 1996).

Phenolic compounds possess one or more aromatic rings bearing two or more hydroxyl groups (Ho, 1992). They are closely associated with the sensory and nutritional quality of fresh and processed foods. In general, the leaves, flowers, fruits and other living tissues of the plant contain glycosides, woody tissues contain aglycones, and seeds may contain either (Huang & Ferraro, 1992).

Agricultural based manufacturers in Thailand produce and export many fruit and vegetable products. These Thai plants are widely distributed throughout the tropics particularly in Southeast Asia. Many researchers have shown that several parts of tropical and subtropical plants contain large amounts of natural phenolic phytochemicals, such as flavonoids (Leong & Shui, 2002; Kähkonen et al., 1999; Demo et al., 1998). Hence, there is a potential for Thai plants to be used as sources of phenolic antioxidants and commercial extracts could be prepared from numerous available raw plant materials. Cost, simplicity and safety should be considered in the development of an acceptable extraction procedure (Pokorny & Korczak, 2001).

Due to the diversity and complexity of natural mixtures of phenolic compounds in plant extracts, it is rather difficult to characterize every phenolic compound. Each plant generally contains different mixtures of phenolic compounds. The Folin-Ciocalteu method is a rapid, widely used assay to determine the total concentration of phenolic compounds. It is known that different phenolic compounds vary in their responses in the Folin-Ciocalteu method. Many researchers have reported the total phenolic content of Thai plants as shown in Table 1.

The data clearly indicate that some of these plants are rich in natural phenolic compounds. Many plants with a high phenolic content have an astringent taste (for leaves) or strong colors (for flowers and fruits) due to flavonoid components. Proanthocyanidins contribute astringency to plants and other flavonoids contribute red or violet colors (anthocyanins) or yellow colors (flavonols). Higher total phenolic and flavonoid contents have been found in seeds compared to other tissues. Typically, leaf photosynthesis products including essential nutrients such as sucrose are translocated from leaves to fruits and seeds which are the food storage organs of the plants (Salisbury & Ross, 1992). This leads to a concentration of phenolic compounds in seeds. Thai plant samples (Table 1) may be classified into two groups with high and low contents of polyphenolic phytochemicals, Samples having

Scientific name	Common name	Plant part	Moisture Content (%)	Total phenolics (mg GAE/g db plant)¥	Total flavonoids (mg RE/g db plant)	Total carotenes (mg%)	Total xanthophyll (mg%)	Tannin (mg% of tannic acid equivalent)
	Herb and vegetable							
Acacia pennata	Acacia leaf	Young leaves	-	121.00 [a]	-	1.27	1.59	11.1
Acanthopanax trifoliatum		Leaves	-	275.00[a]	-	2.54	3.17	57.30
Allium ascalonicum Linn.¥	Onion	Flower	94.70	55.70	20.20	-	-	-
Artemisia dubia Wall. ex DC. (Syn. *A. vulgaris* L. var. *indica* Maxim.)		Stem and leaves	-	14.24	-	-	-	-
Aspidistra sutepensis K. Larsen		Flower	-	5.06	-	-	-	-
Azadirachta indeca A. Juss Var. siamensis valeton¥		Flower	77.20	40.30	29.80	-	-	-
Basella alba Linn.¥	Ceylon spinash	Leaves	93.5	15.50	6.2	-	-	-
Bidens bipinnata , L.		Stem and leaves	-	34.18	-	-	-	-
Bidens pilosa Linn.		Stem and leaves	-	24.62	-	-	-	-
Buddieia asiatica Lour	Rachawadi pa	Stem and leaves	-	19.17	-	-	-	-
Cassia siamea Britt.¥,	Thai copper pod	Flower	74.8	51.50	24.8	-	-	-
		Leaves	-	384.00 [a]	-	1.92	1.59	110.00
Careya sphaerica Roxb.¥	Tummy wood	Young leaves and Leaves	75.3	54.50	20.5	-	-	-
Centella asiatica Linn.¥,	Pennywort	Leaves	86.6	12.40	10.6	12.80	10.60	24.30
Cratoxylum formosum Dyer.¥		Young leaves and Leaves	85.5	63.40	25.5	-	-	-
Coccinia grandis	Ivy gourd	Leaves	-	74.70 [a]	-	1.94	2.65	17.70
Coleus amboinicus	Country borage	Leaves	-	54.80	-	2.54	4.24	24.30
Commelina diffusa Burm.f.		Stem and leaves	-	19.73	-	-	-	-
Conyza sumatrensis (Retz.) Walker		Stem and leaves	-	15.66	-	-	-	-
Coriandrum sativum	Coriander	Leaves	-	33.0 [a]	-	2.52	1.05	24.30
Cucurbita moschata	Pumpkin	Leaves	-	87.8 [a]	-	1.92	1.59	4.48
Cuscuta australis R. Br.		Stem	-	33.21	-	-	-	-
Diplazium esculentum (Retz.) Sw.		Stem and leaves	-	19.48	-	-	-	-
Dolichandrone serrulata (DC.) Seem.		Flower	-	13.25	-	-	-	-
Dregea volubilis		Leaves	-	100.00[a]	-	6.14	1.07	17.70
Embelia ribes Burm.f.		Leaves	-	57.89	-	-	-	-
Embelia sessiliflora Kurtz		Leaves	-	65.08	-	-	-	-
Erythrina crista Galli.¥	Coral tree	Leaves	81.7	67.50	20.2	-	-	-
Gymnema inodorum		Leaves	-	188.00[a]	-	1.31	1.07	11.1
Hydrocharis dubia (Bl.) Back.*	Frogs bit	Bud	95.0	20.40	8.9	-	-	-

Scientific name	Common name	Plant part	Moisture Content (%)	Total phenolics (mg GAE/g db plant)¥	Total flavonoids (mg RE/g db plant)	Total carotenes (mg%)	Total xanthophyll (mg%)	Tannin (mg% of tannic acid equivalent)
Lasia spinosa (Linn.) Thw.¥		Leaves	94.8	6.40	4.4	-	-	-
Leucaena glauca Benth¥	Lead tree	Young leaves and Leaves	79.9	51.20	22.3	-	-	-
Limnocharis flava Buch.¥		Leaves	94.7	5.40	3.7	-	-	-
Macropanax dispermus		Leaves	-	651.00[a]	-	3.89	1.06	37.4
Marsdenia glabra		Leaves	-	51.50[a]	-	8.92	7.42	4.47
Melicope pteleifolia (Champ.ex Benth.)		Hartley Leaves	-	40.31	-	-	-	-
Mentha arvensis	Japanese mint	Leaves	-	70.0[a]	-	4.48	26.5	21.0
Mentha cordifolia	Kitchen mint	Leaves	-	280.00[a]	-	2.58	4.24	73.7
Micromelum minutum Wight & Arn		Stem and leaves	-	61.15	-	-	-	-
Momordica charantia Linn.*,	Balsum pear	Bud and Leaves	87.0	50.90	21.6	1.31	0.54	4.48
-*Musa spiantum* Linn.¥	Banana	Flower	92.8	45.30	20.3	-	-	-
Neptunia oleracea	Water cress	Leaves	-	104.00[a]	-	3.18	1.06	21.00
Ocimum americanum	Hairy basil	Leaves	-	43.6[a]	-	5.12	9.52	11.10
Ocimum basilicum Linn.¥,	Sweet basil	Leaves	89.8	50.50	15.3	10.80	13.30	30.90
Ocimum basilicum cultivar Purple Petra€		Leaves	-	3.40 (by fresh weight)	-	-	-	-
Ocimum sanctum Linn.¥,	Holy basil	Young leaves and Leaves	87.6	41.90	12.6	5.13	3.18	40.80
Oenanthe stolonifera	Chenese celery	Leaves	-	329.00[a]	-	3.83	14.8	34.2
Orthosiphon grandiflorus	Cat's whisker	Leaves	-	145.00[a]	-	3.20	25.40	30.90
Piper retrofractum	Long pepper	Flower	-	57.5[a]	-	1.28	5.31	7.78
Polycia fruticosa		Leaves	-	46.30[a]	-	2.52	2.13	24.30
Sauropus androgynus Linn.¥		Young leaves and Leaves	89.9	11.50	10.4	-	-	-
Schima wallichii (DC.) Korth.		Leaves	-	206.10	-	-	-	-
Sechium edule	Chayote	Leaves	-	66.1[a]	-	3.83	2.13	4.48
Sesbania grandiflora Desv.¥	Cork wood	Flower	91.1	58.60	13.1	-	-	-
Spondias pinnata Kurz.¥	Hog plum	Young leaves and Leaves	76.4	42.60	14.8	-	-	-
Suaeda maritima		Red mature Leaves	-	38.60	-	-	-	-
		Green young Leaves	-	66.90	-	-	-	-
		Green flower	-	59.30	-	-	-	-
Syzygium gratum (Wight) S.N.Mitra var. gratum¥		Young leaves and Leaves	79.6	57.30	23.6	-	-	-
Tamarindus indica	Tamarind	Young leaves	-	121.00[a]	-	0.64	1.05	77.00

Scientific name	Common name	Plant part	Moisture Content (%)	Total phenolics (mg GAE/g db plant)¥	Total flavonoids (mg RE/g db plant)	Total carotenes (mg%)	Total xanthophyll (mg%)	Tannin (mg% of tannic acid equivalent)
Telosma minor	Tonkin jasmine	Flowers	-	98.40 [a]	-	1.29	4.24	17.70
Vaccinium sprengelii (G.Don) Sleum.		Leaves	-	95.42	-	-	-	-
	Berries and fruits							
Amomum krervanh	Siam cardamon	Fruit	-	46.30 [a]	-	1.29	1.07	7.77
Capsicum frutescus Linn.¥	Chilli pepper	Fruit	85.4	40.30	13.3	-	-	-
Eugenia siamensis Craib.¥	Jambolan plum	Fruit	85.1	82.40	44.3	-	-	-
Euginia malaccenses Linn.¥	Malay apple	Fruit	93.7	69.20	28.7	-	-	-
Momordica charantia Linn.¥	Balsam pear	Fruit	75.9	50.90	21.6	-	-	-
Phyllanthus emblica¥	Indian gooseberry	Fruit	82.6	69.10	23.4	-	-	-
Spondias pinnata Kurz.¥	Hog plum	Fruit	77.3	47.20	12.6	-	-	-
	Berry and fruit seeds							
Antidesma velutinum Tulas*		Seed	38.4	123.30	50.3	-	-	-
Cleistocalyx operculatus var. paniala (Roxb.)*		Seed	55.1	173.60	44.2	-	-	-
Eugenia siamensis Craib.*	Jambolan Plum	Seed	50.3	180.50	50.4	-	-	-
Leucaena glauca Benth. *	Leadtree	Seed	76.5	20.40	5.3	-	-	-
Nephelium lappaceum Linn.¥	Rambutan	Seed	36.3	43.50	13.3	-	-	-
Parkia speciosa Hassk.¥		Seed	70.7	51.90	20.3	-	-	-
Piper nigrum Linn.¥	Pepper	Seed	72.5	53.10	22.8	-	-	-
Tamarindus indica Linn¥	Tamarind	Seed	49.5	40.70	23.2	-	-	-
	Chewing plants							
Acacia catechu (L.F) Wild.*	Black catechu	Bark	16.3	177.70	41.8	-	-	-
Areca catechu Linn.*	Betel nut	Whole fruit	90.2	52.50	12.6	-	-	-
		Kernel	91.2	137.30	42.8	-	-	-
Cassia fistula Linn.*	Golden shower	Stem core	11.4	103.60	25.4	-	-	-
Piper betel Linn*	Betel leaf	Leaf	82.6	57.50	14.9	-	-	-

Source: Adapted from ¥Maisuthisakul et al. (2008); *Maisuthisakul et al. (2007a); ᶜLee & Scagel (2009); ✪ Chuenarom et al. (2010); ✂Phomkaivon & Areekul (2009); ⌖Chanwitheesuk, Teerawutgulrag & Rakariyatham (2005)

- means not reported or not determined.

[a] The data were calculated as mg% of pyrocatechol equivalent.

Table 1. Ranges of some phenolic constituents in some Thai plants

relatively high concentrations of phenolic phytochemicals (more than 100 mg GAE/g dry weight of material) included seeds of *Antidesma velutinum* Tulas, *Cleistocalyx operculatus* var. paniala (Roxb.), *Eugenia siamensis* Craib., bark of *Acacia catechu* (L.F) Wild., kernel of *Areca catechu* Linn. and stem of *Cassia fistula* Linn. Chanwitheesuk et al. (2005) reported the total phenolic content of Thai plants using Folin Denis reagent and pyrocatechol as reference, with data calculated as mg% of pyrocatechol equivalents. For comparison, the total phenolic content of Leucaena was reported as 405 mg% (Chanwitheesuk et al., 2005) and 51.20 mg GAE/g db plant (Maisuthisakul et al., 2008). The data showed that the different reference compound and the units used required a conversion factor of around eight to convert the values (Table 1).

Quantitative determination of individual flavonoid glycosides is difficult because most standards are not commercially available. Hence, the total flavonoid content determined using a colorimetric method (Bonvehí et al., 2001) is commonly used for flavonoid evaluation. Generally, the calculation of the total flavonoid content is quoted in units of mg rutin equivalent of flavonoid compounds in one gram of plant extract based on the dry weight of the original plant sample. It is well known that flavonoids possess antioxidant properties both in vitro and in vivo. The flavonoids contain a number of phenolic hydroxyl groups attached to aromatic ring structures, which confer the antioxidant activity. Flavonoids were found in leaves, seeds and fruits and were good antioxidants (Bonvehí et al., 2001). The data clearly indicate that some plants in Thailand are rich in natural antioxidant flavonoids. Some data in Table 1 were used to find a relationship between total phenolic and flavonoid content. The flavonoid content correlates moderately with the phenolic content as shown in Fig. 1.

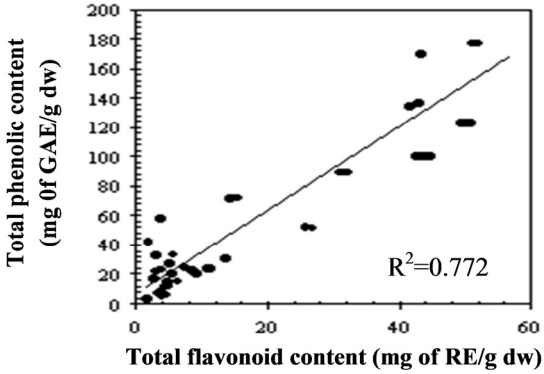


Fig. 1. Relationship between total phenolic and total flavonoid content in Thai plant extracts.

Chanwitheesuk et al. (2005) reported the total carotene, total xanthophyll and tannin contents of edible plants grown in Thailand as shown in Table 1. Total carotenes and total xanthophylls were determined according to Helrich (1990). Tannin contents were determined colorimetrically using the Folin-Dennis reagent according to Helrich (1990).

Identification of the phenolic components in Thai plants has not commonly been reported because it is expensive and standards required for identification are often not available. Hence, this chapter will report the phenolic profile of only four of the Thai plants

investigated; Piper betel Linn, *Careya sphaerica*, *Cratoxylum formosum* Dyer., and *Leucaena leucocephala* de Wit.

Piper betel Linn.; which is commonly known as Betel leaf, is chewed with Betel nut and lime by some people in Asia. Its Thai name is different in different areas, for instance, Plu-Cheen, Se-ke, Bul-plao-yuan, she-ke (South), Pu (North-West). The leaves are chewed alone or with other plant materials including the areca nut (Areca catechu Linn.) and lime. Many researchers have focused on the red lime betel quid in the past few years. A little information about the Betel leaf was found. The Betel leaf itself has a spicy taste and yields an essential oil widely used as a medicine. In Thailand, it is used to treat bruises, heal urticaria, cure ringworm and joint pain as well as relieving toothache. It is also used in cough and mucus remedies and infusions to cure indigestion, as a topical cure for constipation, as a decongestant and as an aid to lactation. The characteristics and chemical composition of 100 grams of Betel leaves are 44 kcal for energy, 85-90 g of water, 3-3.5 g of protein, 2.3 g of fiber, 0.63-0.89 mg of nicotinic acid, 0.005-0.01 g of vitamin C, 1.9-2.9 mg of Vitamin A, 10-70 µg of thiamine, 1.9-30 µg of riboflavin, 0.1-1.3 g of tannin, 0.05-0.6 g of phosphorus, 1.1-4.6 g of potassium, 0.2-0.5 g of calcium, 0.005-0.007 g of iron, 3.4 µg of iodine (Guha, 2006).

Other biological activities described for the essential oil include antifungal, antiseptic and anthelmintic effects (Evans et al., 1984). It was reported that Betel leaf was rich in carotenes (80 IU/g fresh wt.) and phenolics. Data on the phenolic compounds of this plant have been reported for chavicol (Amonkar, et al., 1986), chavibetol, chavibetol acetate (Rimando, et al., 1986) and eugenol (Nagabhushan, et al., 1989). The major bioactive phenolic compoundsin Thai Betel leaf extracted with ethyl acetate were found to be relatively low in polarity. They are chavicol and two-unknown compounds which show higher polarity than chavicol (Maisuthisakul, 2008). The chemical structures of phenolic compounds found in betel leaf are shown in Fig. 2.

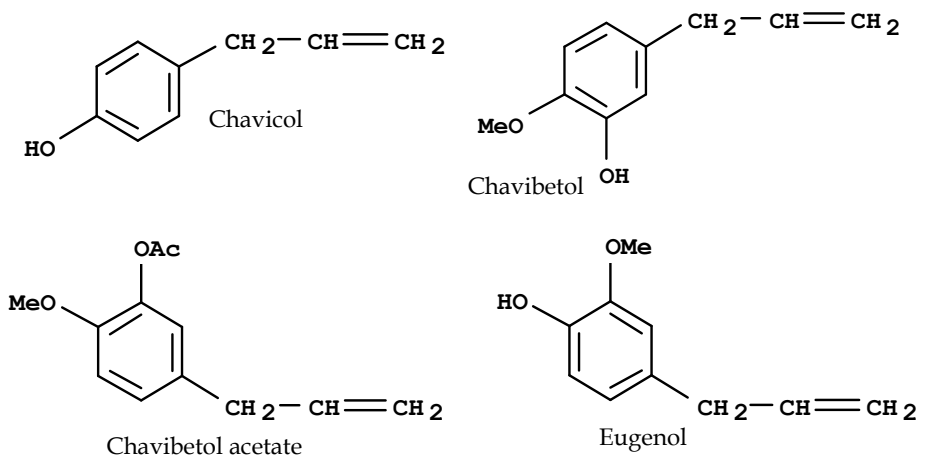


Fig. 2. Some phenolic compounds found in *Piper betel* Linn.

Careya sphaerica Roxb.; This plant is also known as *Careya arborea* Roxb. This plant is normally consumed fresh and mostly found in the North – East of Thailand. Its name is different in different areas, for instance, Kradon, Phak–Kradon, Kradonbok, Kradonkhon (North – East), Khui (Khanchanaburee), Phuk-Pui (North), Puikradon (South), Pui - khao (Chiangmai). Thai people traditionally eat shoots, young leaves and young flowers of this plant. It tastes a little astringent due to the phenolic compounds present. The harvesting season of Kradonbok is during March to May of each year. Kradonbok trees are planted commercially in Sakolnakhon, Kalasin, Yasothorn, Mahasarakham, and Bureerum which are provinces in the North – East of Thailand. Kradonbok has some health benefits such as the use of Kradonbok leaf for healing a wound and flowers for remedying a cough. The characteristics and chemical composition of 100 grams of Kradonbok leaves are 83 Kcal for energy, 1.9 g of fiber, 13 mg of calcium, 18 mg of phosphorus, 17 mg of iron, 3958 IU of riboflavin, 1.8 mg of niacin and 126 mg of vitamin C (Nutrition division, 1992).

There have been few reports about the phenolic compounds of *Careya sphaerica* Roxb. during the last 30 years. Gupta et al. (1975) reported that the phenolic consituents present in the leaf extracts when extracted with petroleum ether at room temperature were lupeol, hexacosanol ($C_{26}H_{54}O$), α-spinosterol ($C_{29}H_{48}O$), teraxerol ($C_{30}H_{50}O$), β-sitosterol ($C_{29}H_{50}O$), quercetin ($C_{15}H_{10}O_7$), taraceryl acetate ($C_{32}H_{52}O_2$) and ellagic acid ($C_{14}H_6O_8$). Careaborin, β-amyrin, careyagenolide, maslinic acid and α-hydroxyursolic acid were also found in the leaves (Das & Mahato, 1982 Das & Mahato, 1982; Talapatra et al., 1981). The chemical structures of some componentsextracted from *Careya sphaerica* Roxb.leaf are shown in Fig. 3.

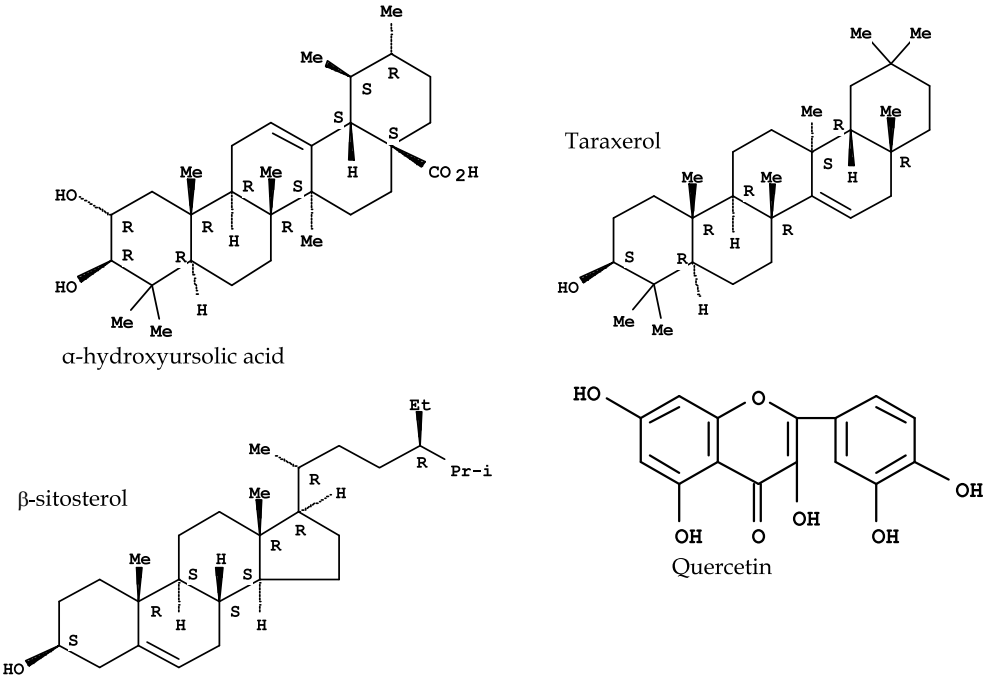
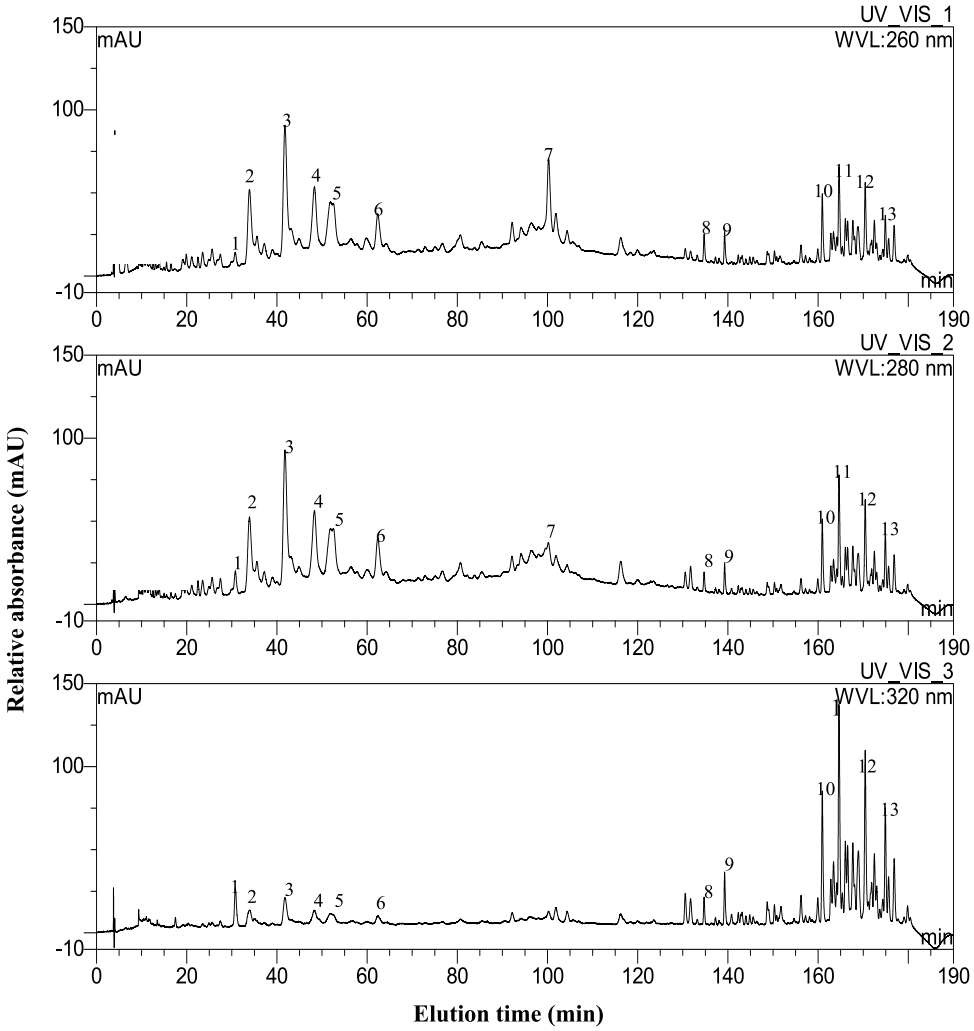


Fig. 3. Some phytochemicals extracted from *Careya sphaerica* Roxb.

The HPLC chromatogram of the Kradonbok extract is shown in Fig. 4. The ethanol extract was dissolved in methanol and passed through a Sep-Pak C18 cartridge (Waters, Milford, MA.). The C-18 cartridge was first conditioned by suction with 1 column volume of methanol followed by 2 column volumes of a 3% HCl solution (v/v) in HPLC grade water. The cartridge was not allowed to dry out during conditioning. The aqueous extract was then transferred to the cartridge. The cartridge bed was then rinsed with HCl (3%, 5 mL) and air-dried under vacuum for ~10 min. Phytochemicals were eluted with HPLC grade methanol. Samples were filtered through a 0.20 mm Millipore filter (type HA) into a 2 mL autosampler



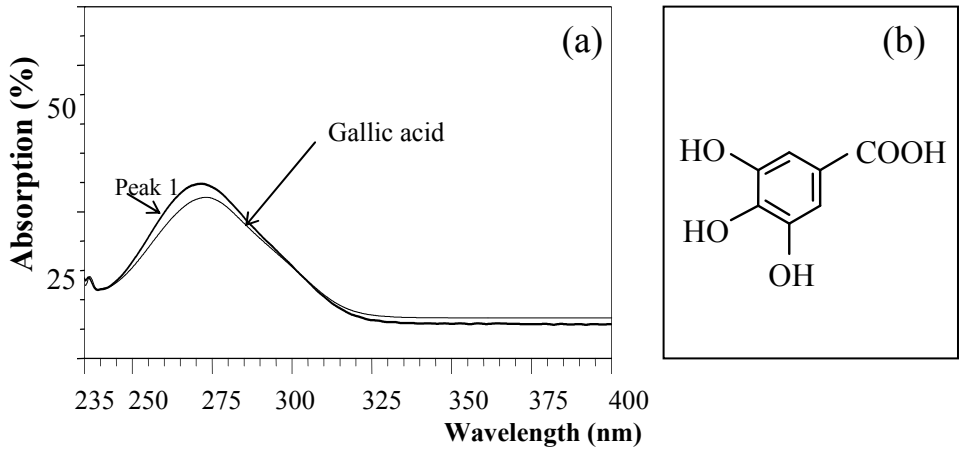
Source: Maisuthisakul (2007)

Fig. 4. HPLC chromatogram for the extract from *Careya sphaerica* Roxb. (Kradonbok) detected at 260, 280 and 320 nm.

vial for subsequent analysis by HPLC. The solution (10 mL) was injected into the HPLC and analyzed according to the following conditions: column, Synergi Hydro RP column (150 × 4.6 mm id., 4mm, Phenomenex), fitted with a Allsphere ODS-2 guard column (10 × 4.6 mm id., Alltech). The HPLC system was equipped with diode array detector (Dionex PDA 100 photodiode array, USA) controlled by Chromeleon software version 6.60 Build 1428 (Dionex Corporation, Sunnyvale, USA). Chromatograms were recorded at 260, 280 and 320 nm.

Thirteen main peaks were detected in Thai Tummy wood leaf extracted with ethanol and the components corresponding to peaks 1, 2, 3, 4, 5, and 6 eluted at 11.06, 33.91, 41.79, 48.35, 51.91 and 62.36 min in the more hydrophilic region (short retention time). The other main components eluted at 100.19 min (peak 7), 134.71 min (peak 8), 139.27 min (peak 9), 160.93 min (peak 10), 164.63 min (peak 11), 170.44 min (peak 12) and 174.90 min (peak 13) in the more hydrophobic region (long retention time).

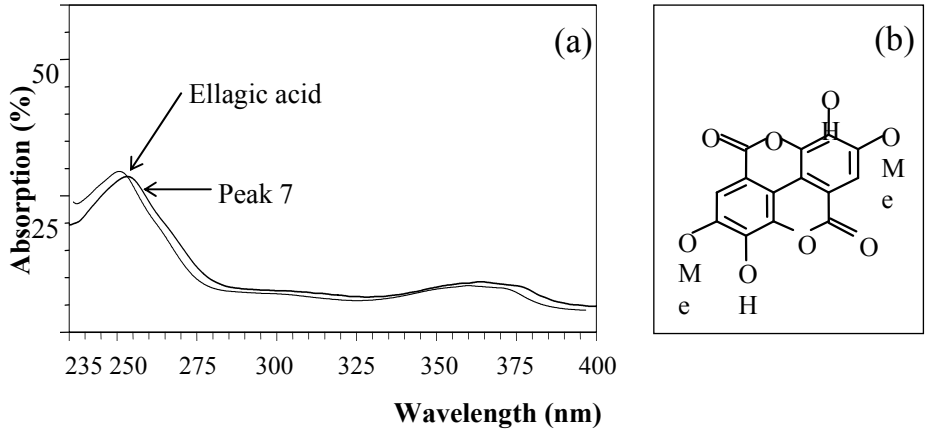
The identity and purity of peak 1 in the HPLC chromatogram of the extract of *Careya sphaerica* Roxb. was determined by comparison of the retention time and UV spectrum (Fig. 5) with that of pure gallic acid. The identification of peak 1 as gallic acid was confirmed by co-injection of gallic acid with the plant extract. HPLC-ESI-MS confirmed the identification of this compound (MS [M + H]+ at m/z 171, MS [M + Na]+ at m/z 193 and MS [M+4Na]+ at m/z 262), which is consistent with the molecular weight of 170.12 for gallic acid. Compound 7 was identified by comparison of the retention time and UV spectrum (Fig. 6) with that of pure ellagic acid. The identification of peak 7 as ellagic acid was confirmed by co-injection of ellagic acid with the plant extract. HPLC-ESI-MS confirmed the identification of this compound (MS [M + H]+ at m/z 303, MS [M + Na]+ at m/z 325), confirming the molecular weight of 302.19 for ellagic acid. Talapatra et al. (1981) reported that ellagic acid was isolated from the leaves of *Careya arborea*, which is the synonym of *Careya sphaerica* Roxb.



Source: Maisuthisakul (2007)

Fig. 5. UV spectrum of (a) peaks 1 of *Careya sphaerica* Roxb. (Kradonbok) extract, gallic acid and (b) structure of gallic acid.

The known antioxidant components 1 (gallic acid) and 7 (ellagic acid) were present at 0.93 % and 2.37 % of the extract. These concentrations were calculated according to the peak areas from the HPLC chromatogram. The thirteen phenolic compounds represented about 56.16% of the total phenolic compounds of Kradonbok extract.



Source: Maisuthisakul (2007)

Fig. 6. UV spectrum of (a) peak 7 of *Careya sphaerica* Roxb. (Kradonbok) extract, ellagic acid and (b) structure of ellagic acid.

Cratoxylum formosum **Dyer.**; The name of this plant differs in different local areas, for instance, Teawkon (Central), Teawdang (North) and Tao (South). Thai people traditionally eat shoots and young leaves of this plant. It tastes sour and a little astringent due to the phenolic phytochemicals present. The harvesting season of *Cratoxylum formosum* Dyer. is during March to May of each year. Trees of this plant are planted commercially in Sakolnakhon, Kalasin, Yasothorn, Mahasarakham, Bureerum, which are the provinces in North – East Thailand. The chemical composition per 100 grams of Teaw leaves which provides 58 Kcal for energy includes 1.5 g of fiber, 67 mg of calcium, 19 mg of phosphorus, 205 mg of iron, 4500 µg of β-carotene, 750 µg vitamin A as retinol, 10.04 mg of thiamin, 0.67 mg of riboflavin, 3.1 mg of niasin and 58 mg of vitamin C (Nutrition division, 1992).

Relatively little work has been done on the phytochemicals in *Cratoxylum* sp. The only studies concerning the phytochemistry of *Cratoxylum* sp. was published by Kitanov & Assenov (1988), and Kumar et al. (2004) who reported that the phenolic compounds in *Cratoxylum pruniflorum* Kurz were quercetin ($C_{15}H_{10}O_{12}$), hyperoside ($C_{21}H_{20}O_{12}$), 1,3,6,7-tetrahydroxyxanthone, mangiferin ($C_{19}H_{18}O_{11}$) and isomangiferin ($C_{19}H_{18}O_{12}$), (Kitanov & Assenov, 1988). The phenolic constituents in *Cratoxylum neriifolium* Kurz were biflavonol GB-2, pentahydroxyflavanone chromone and stigmasterol (Kumar et al., 2004). The chemical structures of some phytochemicals found in *Cratoxylum* sp. leaf are shown in Fig. 7.

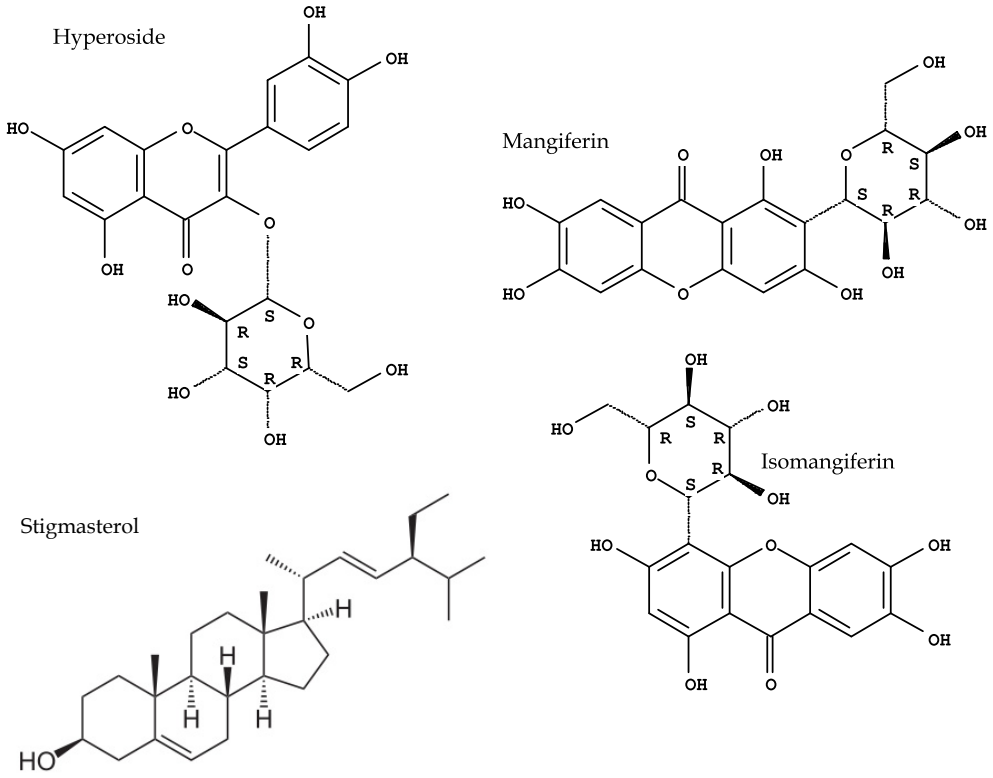
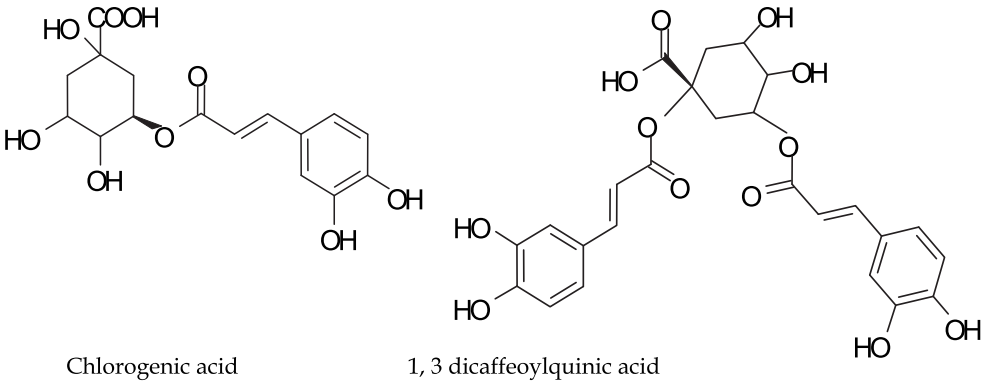


Fig. 7. Some phytochemicals found in *Cratoxylum* sp.

With regards to Thai plants, three active phenolic ingredients were chlorogenic acid, dicaffeoylquinic acid and ferulic acid hexose derivative (Maisuthisakul et al., 2007b). Chlorogenic acid was present at 60 % of the extract. Two minor components (dicaffeoylquinic acid and ferulic acid hexose derivative) were present at 7 % and 2 %, and other components that were present at lower concentrations were also detected. Some chemical structures of phenolic compounds found in Thai *Cratoxylum formosum* leaf are shown in Fig. 8.

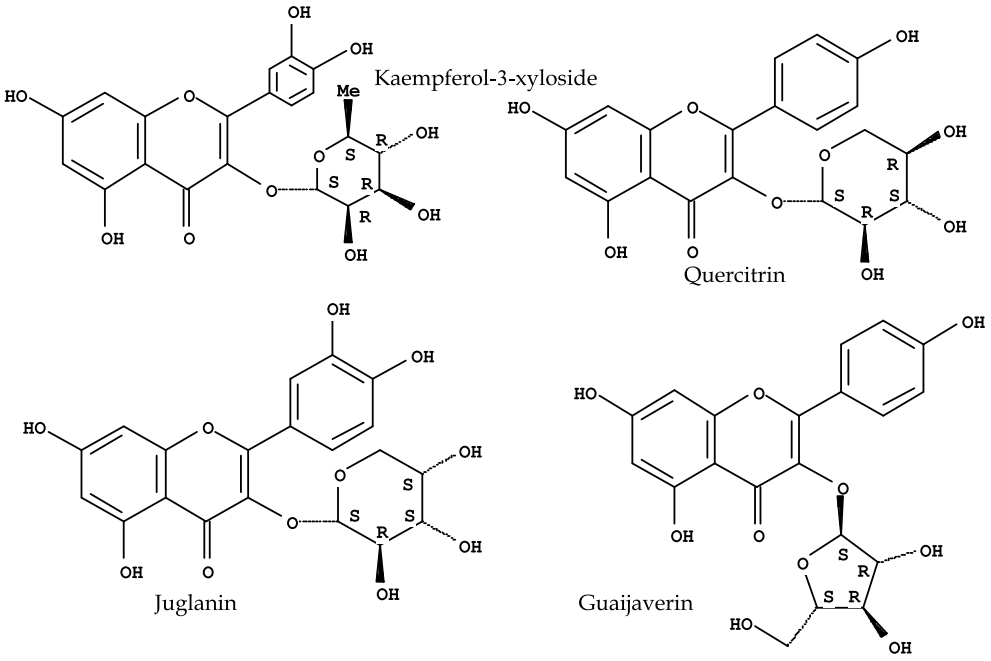
Leucaena glauca **Benth**; the plant is found throughout Thailand in the settled areas at low and medium altitudes. It occurs widely and is abundant. Its name is different in different areas, for instance, Kratin-Thai (Central), Satorban (South), Katong and Kratin. Thai people traditionally eat young leaves and the young pod of this plant. The young leaf is found in all seasons, however, it is most abundant during March to May of each year. Kratin trees are planted commercially in Roi-ed, Amnat Charoen, Pichit, Nakhonsawan, Songkla, Krabi, Pattani and Trang. Kratin leaves contain leucine which can absorb selenium. The charcateristics and chemical composition of 100 grams of Kratin leaves are 62 Kcal for energy, 8.4 g of protein, 3.8 g of crude fiber, 137 mg of calcium, 11 mg of phosphorus, 9.2 g of iron, 7883 IU of total vitamin A, 0.33 mg of thiamin, 0.09 mg riboflavin, 1.7 mg of niacin and 8 mg of vitamin C (Nutrition division, 1992).



Chlorogenic acid 1, 3 dicaffeoylquinic acid

Fig. 8. Some phenolic compounds found in *Cratoxylum formosum* in Thailand.

Relatively little information on the phenolic constituents of *Leucaena glauca* Benth has been published during the last 30 years. Chen (1979) found foeniculin ($C_{14}H_{18}O$) and kaempferol-3-xyloside ($C_{20}H_{18}O_{10}$) in the leaves. Guaijaverin ($C_{20}H_{18}O_{11}$), juglania ($C_{20}H_{18}O_{10}$), kaempferol-3-O-β-xyloside and quercitrin ($C_{21}H_{20}O_{11}$) were also found in the leaves (Morita et al., 1977). The chemical structures of phenolic compounds found in Thai *Leucaena glauca* Benth leaf are shown in Fig. 9.



Kaempferol-3-xyloside

Quercitrin

Juglanin

Guaijaverin

Fig. 9. Some phenolic compounds found in *Leucaena glauca* Benth

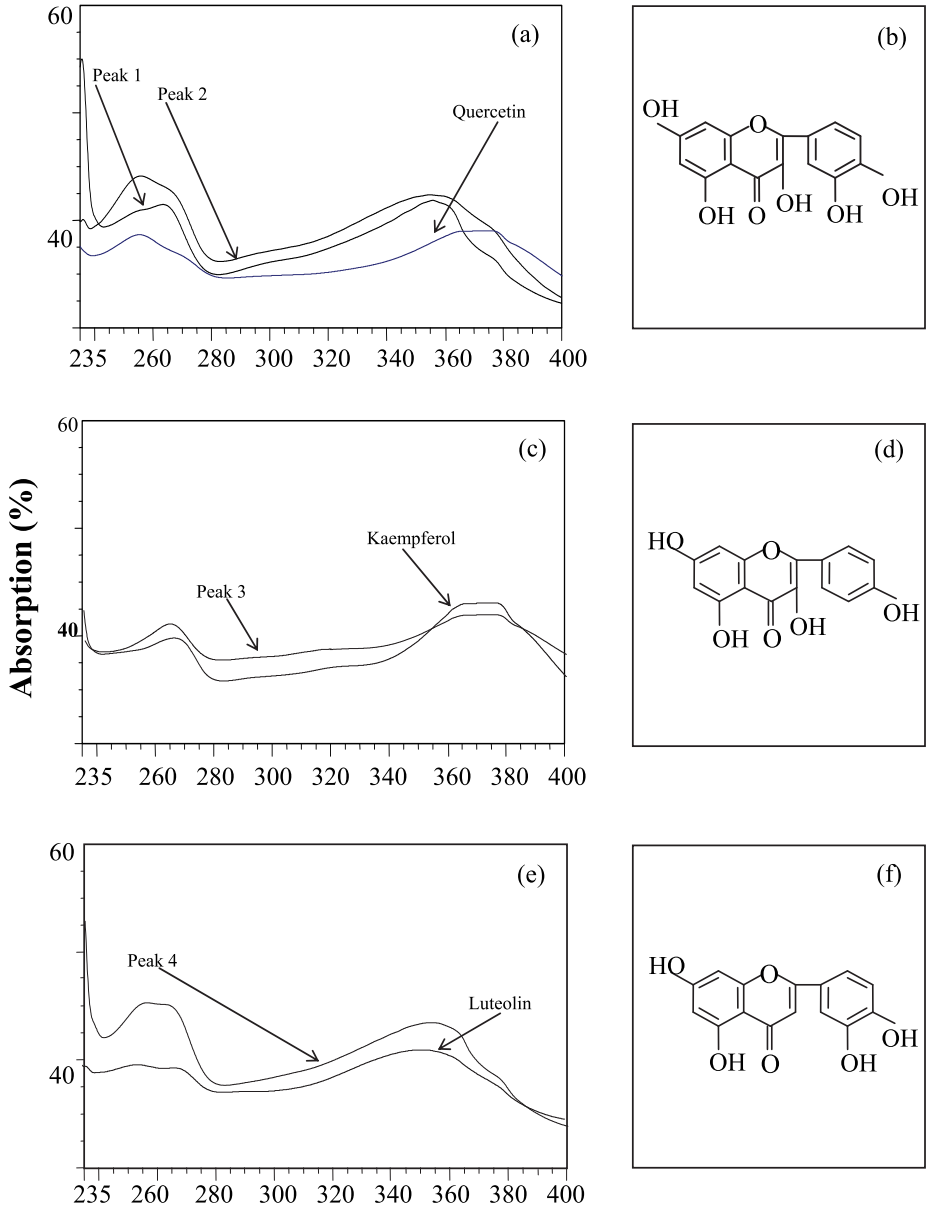
The phenolic components of Leucaena glauca Benth in the HPLC chromatogram appeared as 4 peaks which were present at 15.28%, 6.50%, 9.71% and 36.91%, respectively, and other components that were present at lower concentrations were also observed. These concentrations were calculated from each area by dividing by the total peak area from the HPLC chromatogram. The four phenolic compounds were about 68.4% of the total phenolic content of Leucaena glauca Benth extract. The compounds corresponding to peaks 1, 2, 3, and 4 had a similar UV spectrum to that of flavonoids such as quercetin, and kaempferol which are flavonols and luteolin which is a flavone (Fig. 10). The identification of each peak was confirmed by using comparisons of their UV spectra and LC-MS in both positive and negative mode in order to obtain more information on the structural features of the conjugated forms of the phenolic compounds.

3. Antioxidant properties of some Thai plants

In general, the antioxidant activity of plant extracts is associated with specific compounds or classes of compounds, such as flavones, flavonols and proanthocyanidins in plant materials native to the Mediterranean area (Skerget et al. , 2005), carotenoids (Stahl & Sies, 2003) and melatonin (Chen et al., 2003). Most of the antioxidant substances in plants are phenolic compounds. Phenolic compounds serve as oxidation terminators by scavenging radicals to form resonance stabilized radicals (Rice-Evans et al., 1997).

Although the antioxidant capacities are influenced by many factors, which cannot be fully described with a single method, the DPPH radical scavenging activity is the most commonly used method for assessment of antioxidant properties of natural products. The DPPH• assay overcomes the limitations of monitoring the activity of the numerous samples over a specified period of time. It is reproducible and strongly correlated with phenolic compounds (Maisuthisakul et al., 2007; Katalinic et al., 2006; Miliauskas et al., 2004; Matsuda et al., 2001). In addition the radical scavenging method had many benefits compared to the lipid oxidation method (Nuutila et al., 2002). Gallic acid, which has been used as a standard, has been reported to be the most abundant phenolic compound in plants (Witzell et al., 2003., Nuutila et al., 2002). The EC_{50} is defined as the amount of antioxidant required to cause a 50% reduction in the absorbance of DPPH. These values were changed to antiradical activity defined as $1/EC_{50}$, since this parameter increases with antioxidant activity. The antiradical activity of α-tocopherol was 0.67 (Maisuthisakul et al., 2008). Many researchers have reported the total phenolic content of Thai plants as shown in Table 2. The plants which had strong antiradical activity (Table 2) had high total phenolic and flavonoid contents (Table 1).

In Thailand, there are many plants which show antiradical activity higher than α-tocopherol such as *Careya sphaerica* Roxb., *Cratoxylum formosum* Dyer., *Erythrina crista* Galli., *Leucaena glauca* Benth, *Momordica charantia* Linn., *Ocimum basilicum* Linn., *Ocimum sanctum* Linn., *Syzygium gratum* (Wight) S.N.Mitra var. gratum, *Allium ascalonicum* Linn., *Azadirachta indeca* A. Juss Var. siamensis valeton, *Cassia siamea* Britt., *Musa spiantum* Linn., *Sesbania grandiflora* Desv. The antiradical activity of Thai fruits and fruit seeds were higher than that of α-tocopherol except for seeds of *Leucaena glauca* Benth (Table 2).



Source: Maisuthisakul (2007)

Fig. 10. UV spectrum of (a) peaks 1, 2 of *Leucaena glauca* Benth (Kratin) extract, quercetin, (b) structure of quercetin, (c) UV spectrum of peaks 3 of Kratin extract, kaempferol, (d) structure of kaempferol, (e) UV spectrum of peaks 4 of Kratin extract, luteolin and (f) structure of luteolin.

Scientific name	Local name	Plant part	Antiradical activity (1/ EC_{50})
Herb and vegetable			
Allium ascalonicum Linn.¥	Hom	Flower	2.6
Artemisia dubia Wall. ex DC. (Syn. *A. vulgaris* L. var. *indica* Maxim.)⌘	Hia	tem and leaves	0.19
Aspidistra sutepensis K. Larsen ⌘	Nang-laeo	Flower	0.06
Azadirachta indeca A. Juss Var. siamensis valeton¥	Sa-dao	Flower	1.2
Basella alba Linn.¥	Plang	Leaf	0.7
Bidens bipinnata , L.⌘	Ya-Puen-Laem Nok-Sai	Stem and leaves	1.04
Bidens pilosa Linn. ⌘	Peen-nok-sai	Stem and leaves	1.22
Buddieia asiatica Lour ⌘	Ra-cha-wa-di-pa	Stem and leaves	0.26
Careya sphaerica Roxb.¥	Kra-don	Young leaf and leaf	2.3
Cassia siamea Britt. ¥	Kee-lek	Flower	2.4
Centella asiatica Linn.¥	Bua-bok	Leaf	0.7
Cratoxylum formosum Dyer.¥	Tew	Young leaf and leaf	4.4
Commelina diffusa Burm.f. ⌘	Plap	Stem and leaves	0.64
Conyza sumatrensis (Retz.) Walker ⌘	Ya-khamai	Stem and leaves	0.46
Cuscuta australis R. Br. ⌘	Khruea-kham	Stem	1.08
Diplazium esculentum (Retz.) Sw. ⌘	Kut-khao	Stem and leaves	0.63
Dolichandrone serrulata (DC.) Seem. ⌘	Khae-pa	Flower	0.06
Embelia ribes Burm.f. ⌘	Som-jee	Leaves	2.86
Embelia sessiliflora Kurtz ⌘	Som-kui	Leaves	5.0
Erythrina crista Galli.¥	Tong-lang	Leaf	3.1
Hydrocharis dubia (Bl.) Back.*	Tub-tao	Bud	0.82
Lasia spinosa (Linn.) Thw.¥	Nham	Leaf	0.1
Leucaena glauca Benth¥	Kra-tin	Young leaf and leaf	1.5
Limnocharis flava Buch.¥	Pai	Leaf	0.1
Melicope pteleifolia (Champ.ex Benth.)⌘	Sa- Riam -Dong	Hartley Leaves	0.18
Micromelum minutum Wight & Arn ⌘	Sa-mui	Stem and leaves	0.83
Momordica charantia Linn.*	Ma-ra-khee-nok	Bud and leaf	1.70
Musa spiantum Linn.¥	Hua-plee	Flower	1.8
Ocimum basilicum Linn.¥	Ho-ra-pa	Young leaf and leaf	1.80
Ocimum sanctum Linn.¥	Ka-prow	Young leaf and leaf	1.8
Sauropus androgynus Linn. ¥	Whan-ban	Young leaf and leaf	0.7
Schima wallichii (DC.) Korth. ⌘	Talo	Leaves	12.5
Sesbania grandiflora Desv.¥	Kae	Flower	1.7
Spondias pinnata Kurz.¥	Ma-kok	Young leaf and leaf	0.7
Stachytarpheta jamaicensis (L.) Vahl (*S.indica* Vahl)✡	Pun-ngu-keaw	Leaf	0.016
		Stem	0.018
		Root	0.018
		Inflorescence	0.020
Syzygium gratum (Wight) S.N.Mitra var. gratum¥	Mek	Young leaf and leaf	1.8
Tiliacora triandra (Colebr.) Diels ⌘	Ya-nang	Leaves	0.15
Vaccinium sprengelii (G.Don) Sleum. ⌘	Som-pi	Leaves	1.89
Berries and fruits			
Capsicum frutescus Linn.¥	Prik	Fruit	1.8

Scientific name	Local name	Plant part	Antiradical activity (1/ EC_{50})
Eugenia siamensis Craib.¥	Chom-pu-nam	Fruit	5.0
Euginia malaccenses Linn.¥	Chom-pu-ma-meaw	Fruit	2.2
Momordica charantia Linn.¥	Mara-khee-nok	Fruit	1.7
Phyllanthus emblica¥	Ma-kham-pom	Fruit	2.0
Spondias pinnata Kurz.¥	Ma-kok	Fruit	1.6
	Seeds		
Antidesma velutinum Tulas*	Ma-mao	Seed	14.28
Cleistocalyx operculatus var. paniala (Roxb.)*	Ma-kieng	Seed	11.11
Eugenia siamensis Craib.*	Chom-pu-nam	Seed	6.67
Leucaena glauca Benth. *	Kra-tin	Seed	0.14
Nephelium lappaceum Linn.¥	Ngo	Seed	2.2
Parkia speciosa Hassk.¥	Sa-tor	Seed	1.5
Piper nigrum Linn.¥	Prik-Thai-dum	Seed	3.0
Tamarindus indica Linn¥	Ma-kham	Seed	2.0
	Chewing plants		
Acacia catechu (L.F) Wild.*	See-sead	Bark	20.0
Areca catechu Linn.*	Mhak	Whole fruit	2.13
		Kernel	5.56
Cassia fistula Linn.*	Kaen-khun	Stem core	6.25
Piper betel Linn*	Bai-plu	Leaf	3.13

Source: Adapted from ¥Maisuthisakul et al. (2008); *Maisuthisakul et al., 2007a; €Lee & Scagel, 2009; ✪ Kerdchoechuen & Laohakunjit, 2010; ✡Ongard & Dara, 2010; ⌘Phomkaivon & Areekul (2009)
- means not reported or not determined.

Table 2. Ranges of antiradical activity in some Thai plants

4. Factors affecting extraction of plant phenolics

Natural antioxidants are available from raw materials of variable composition. Both the content of active substances and the content of various other compounds may vary. The quality of natural extracts and their antioxidative activity depends not only on the quality of the original plant, date and storage, but also the extraction conditions which affect the plant phenolic compounds extracted (Moure et al., 2001).

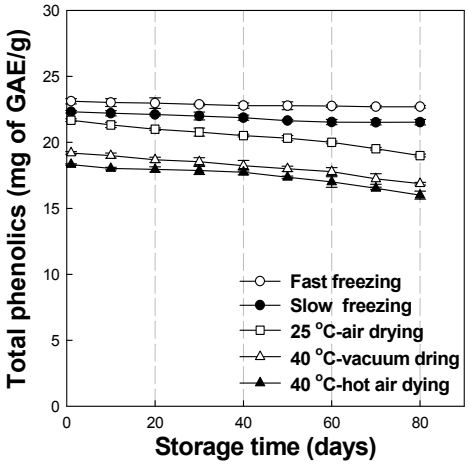
4.1 Sample preparation for storage

Sample preparation is required to keep samples for a certain period of time before analysis since even the total phenolic content was shown to decrease during storage compared with the fresh leaves. In addition, the availability of raw materials is usually limited to a harvesting season. The preliminary processing of plants is necessary for storage.

Temperature is the major factor influencing changes in antioxidant activity during storage (Moure et al., 2001). From numerous publications, the preservative processes before storage vary such as drying citrus peel and seed in an oven at 40 ^{o}C and keeping at room temperature (Bocco et al., 1998) or drying Indian Laburnum by air at 25^{o}C and keeping at room temperature (Siddhuraju et al., 2002) or keeping berries in a still air freezer at -25 to -30^{o}C (Amakura et al., 2000).

Sample preparation methods gave significant effects on total phenolic content and antioxidant activity but had no marked effects on yield of the extract of *Careya sphaerica* or Kradonbok (Maisuthisakul & Pongsawatmanit, 2005). Freezing, especially fast freezing, for keeping the leaves until extraction gave a higher total phenolic content compared with those obtained from other drying methods and slow freezing (Fig. 11). The drying methods studied were (1) hot air drying by tray dryer at 40°C for 18 h with air velocity about 0.5 m/s; (2) vacuum drying by vacuum dryer at 40°C, 100 mmHg (EYELA, model VOS-300SD, Japan) for 10 h; (3) air drying at room temperature 25°C for 12 h with air velocity about 3.2 m/s. The rate of freezing affected the total phenolic content obtained because larger ice crystals grew during slower freezing which would damage plant cells and cause a loss of antioxidant activity. Enzymes from plant cells such as lipoxygenase can oxidize polyphenols (Akoh & Min, 1997). The total phenolic content obtained from air drying was higher than those obtained from vacuum drying and hot air drying because some phenolic components may be degraded by higher temperature (Moure et al., 2001). This effect also found in fresh Mulberry leaves, where the amount of flavonoids was higher in air-dried samples than that in oven-dried samples, probably due to decomposition after storage (Zhishen et al., 1999).

Normally, temperature affects the compounds' stability due to chemical and enzymatic degradation. These mechanisms were reported as mainly responsible for a reduction in phenolic content (Larrauri et al., 1997). Maisuthisakul & Pongsawatmanit also reported that the reduction in antioxidant activity was higher than that expected from the reduction in phenolic contents, probably due to the synergistic effect of natural phenolics (Moure et al., 2001). In addition, phenolics can react with other plant components, and prolonged exposure at moderate temperatures can also cause phenolic degradation. Therefore, sample preparation conditions including temperature and time before storage should be controlled.



Source: Adapted from Maisuthisakul & Pongsawatmanit (2005)

Fig. 11. Total phenolic content of the dried extracts from *Careya sphaerica* leaves obtained by various sample preparation methods.

4.2 Extraction conditions

Sovent effect; solvent extraction is more frequently used for isolation of antioxidants and both extraction yield and antioxidant activity of extracts are strongly dependent on the solvent, due to the different antioxidant potential of compounds with different polarity (Marinova & Yanishlieva, 1997). Apolar solvents are among the most common solvents for removing polyphenols from water. Ethyl acetate and diethyl ether have been used for extraction of low molecular weight phenols from oak wood (Fernández de Simón et al., 1996). Ethanol and water are the most widely employed solvents for reasons of lack of toxicity and abundance, respectively.

With regards to Thai plants, Maisuthisakul (2007) reported that the solvent used had significant effects on antioxidant activity, total phenolic content, yield and partition coefficient of Thai betel leaf extract ($p < 0.05$). The antioxidant activity assessed by DPPH and ABTS radicals was stronger with less polar solvents (Table 3). The results showed that the DPPH activity of the extract obtained with ethyl acetate was significantly higher than that obtained with the other solvents. Total phenolic content confirmed this finding. The extract with a solvent which has higher polarity was found to contain rather small amounts of phenolic compounds. The EC50 and TEAC value of α- tocopherol was also measured and gave values of 14.95±0.23 μg.mL-1 and 2.30±0.03 mmol of Trolox/g sample, respectively. Betel leaf extracted with ethyl acetate (Table 3) had a weak antioxidant activity compared to α- tocopherol. The antioxidant activity and total phenolic contents were significantly different from various solvent extractions. The antioxidant activity values were consistent with those of Chen et al. (2001).

The effectiveness of phenolic antioxidants is often dependent on their polarity. Decker (1998) used the term "antioxidant paradox" to describe how polar antioxidants are most effective in bulk lipids while nonpolar antioxidants are most effective in dispersed lipids. The polarity of the Betel leaf extract was assessed by determination of the oil-water partition coefficient by HPLC. The oil-water partition coefficient was calculated by summing the areas of the three phenolic peaks in the HPLC chromatogram. The oil-water partition coefficient of the Betel leaf extract from ethyl acetate was significantly different from those extracted with other solvents as shown in Table 4.

Solvent used	DPPH activity (EC_{50}, μg.mL^{-1})	TEAC (mmol Trolox/g sample)	Total phenolic content (mg GAE/g sample)
Methanol	36.65 ± 2.60^{a}	2.01± 0.06^{c}	49.89 ± 0.21^{d}
Ethanol	33.85 ± 2.81^{ab}	2.12± 0.03^{bc}	50.38 ± 0.08^{c}
Acetone	30.09 ± 1.21^{b}	2.21± 0.04^{ab}	53.28 ± 0.19^{b}
Ethyl acetate	17.04 ± 0.51^{c}	2.34± 0.06^{a}	55.35 ± 0.14^{a}

Note: ¥ Data followed by different letters within each column are significantly different according to Duncan's multiple range tests at $P < 0.05$. Data were represented as means from three replicate measurements.
Source: Maisuthisakul (2007)

Table 3. Antioxidant activity and total phenolic content of Betel leaf extracted by different solvents¥

Solvent used	Partition coefficient of solvent#	Partition coefficient of extract
Methanol	-0.77	2.02± 0.01[a]
Ethanol	-0.32	2.09± 0.02[ab]
Acetone	-0.24	2.15± 0.01[b]
Ethyl acetate	0.66	2.31± 0.03[c]

Note: ¥ Data followed by different letters within each column are significantly different according to Duncan's multiple range test at $P < 0.05$. Data were represented as means from three replicate measurements.
Data obtained from literature review.
Source: Maisuthisakul (2007)

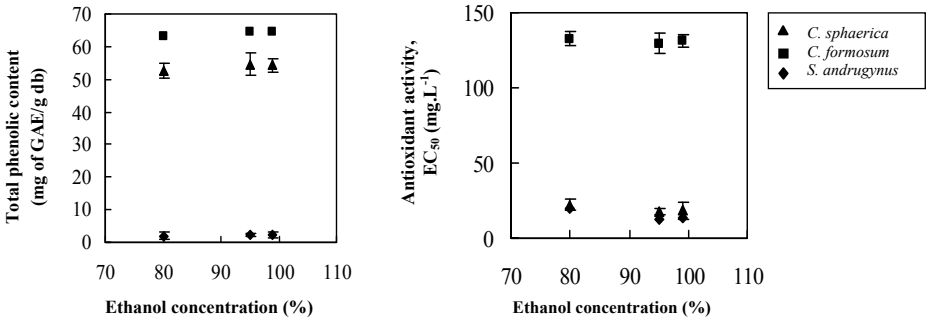
Table 4. Partition coefficient of solvent used and Betel leaf extract extracted by different solvents¥

The phenolic compounds in Betel leaf from the literature include low polarity compounds such as chavicol (log P = 2.50±0.21), chavibetol (log P = 2.30±0.23), chavibetol acetate (log P = 2.39±0.24), and eugenol (log P = 2.20±0.23). Normally, a frequently used descriptor for the estimation of the lipophilicity of phenolic compounds is the partition coefficient. The partition coefficients of Betel leaf extracts were those of low polarity compounds since the value is higher than 0 (Munishwar, et al., 1997) (Table 4). Less polar solvents showed higher extraction efficacy due to the low polarity of the phenolic compounds of Betel leaf extract. Compounds, which have a polarity similar to the solvent, are able to dissolve more than compounds with different polarity. It can be noted here that Betel leaf extract is rich in less polar phenolic compounds. The solvent used for extraction also affected the total phenolic content in the extracts.

The ratios of solvents used in mixed solvents affected the phenolic content extracted from some Thai plants. *Careya sphaerica* Roxb. (Kradonbok), *Cratoxylum formosum* Dyer. (Teaw) and *Sauropus andrugynus* Merr. (Phak whan ban) leaves were used to study the effect of ethanol concentration used in the extraction. The total phenolic content of plant extracts readily increased with increasing concentration of ethanol from 80% to 95%, but there was no significant difference between the extracts using ethanol concentrations of 95% and 99% for each plant. The effect of ethanol concentration on antioxidant activity and total phenolic content was similar (Fig. 12).

The inflorescence, leaves, stem and root of *Stachytarpheta indica* Vahl were extracted with three methanol and water solvent mixtures, namely water, 75% methanol and 50% methanol. The results showed that the leaf extract from 75% methanol had the highest antioxidant activity in both fresh and dry samples (Ongard & Dara, 2010).

Extraction temperature effect; the temperature of extraction affects the compounds' stability due to the decomposition of phenolic compounds. The effect of temperature has been studied in the extraction of anthocyanins. They were shown to be degraded since the visible spectrum showed both a reduction in the peak at 400-500 nm and reduction in the red color. The temperature during extraction can affect extractable compounds to different extents; boiling and resting increases the total phenol content extracted from *Quercus suber* cork (Conde et al., 1998). Milder extraction temperatures are desirable in those cases where some compounds can be degraded, e.g. carnosic acid (Ibañez et al., 1999).



Source: Adapted from Maisuthisakul (2007)

Fig. 12. Total phenolic contents and antioxidant activity of the extracts from *Careya sphaerica* Roxb., *Cratoxylum formosum* Dyer. and *Sauropus andrugynus* Merr. leaves obtained with various ethanol concentrations.

Other factors; such as extraction time, pH, the particle size of materials, and extraction methods were reported to affect the antioxidant activity and concentrations of phenolic compounds extracted. Sheabar & Neeman (1988) reported the maximum solubility of phenolic compounds from olive rape at pH 4 in the organic phase. The yield of extracted phenolics was correlated with plant cell wall breakdown. Particle size reduction significantly increased the antioxidant activity as a result of both increased extractability and enhanced enzymatic degradation of polysaccharides (Weinberg et al., 1999). Various process conditions (refluxing, shaking and ultrasonic extraction) also affected the concentrations of antioxidants in extracts from balm leaves (Herodež et al., 2003).

5. Conclusion

Many plants in Thailand show potential as a source of extracts rich in phenolic constituents and natural antioxidants. Phenolic compounds are the major antioxidants in plants. Moreover, practical aspects relevant to the use of this class of compounds need to be considered including extraction efficiency, availability of sufficient raw material, and toxicity or safety considerations. To utilize these significant sources of natural antioxidants, further characterization of the phenolic composition is needed.

6. Acknowledgement

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10

Lignans: Chemical and Biological Properties

Wilson R. Cunha[1], Márcio Luis Andrade e Silva[1], Rodrigo Cassio Sola Veneziani[1], Sérgio Ricardo Ambrósio[1] and Jairo Kenupp Bastos[2]
[1]*Universidade de Franca,*
[2]*Universidade de São Paulo,*
Brazil

1. Introduction

The plant kingdom has formed the basis of folk medicine for thousands of years and nowadays continues to provide an important source to discover new biologically active compounds (Fabricant & Farnsworth, 2001; Gurib-Fakim, 2006; Newman, 2008). The research, development and use of natural products as therapeutic agents, especially those derived from higher plants, have been increasing in recent years (Gurib-Fakim, 2006). Several lead metabolites such as vincristine, vinblastine, taxol and morphine have been isolated from plants, and many of them have been modified to yield better analogues for activity, low toxicity or better solubility. However, despite the success of this drug discovery strategy, only a small percentage of plants have been phytochemically investigated and studied for their medicinal potential (Ambrosio et al., 2006; Hostettmann et al., 1997; Soejarto, 1996).

The first step in the search of new plant-based drugs or lead compounds is the isolation of the secondary metabolites. In the past, the natural products researchers were more concerned with establishing the structures and stereochemistry of such compounds but, in recent years, a great number of studies have concentrated efforts on their biological activities (Ambrosio et al., 2006). This multidisciplinary approach was reinforced by the substantial progress observed in the development of novel bioassay methods. As a consequence, a great number of compounds isolated from plants in the past have been "rediscovered" (Ambrosio et al., 2008; Ambrosio et al., 2006; Houghton, 2000; Porto et al., 2009a; Porto et al., 2009b; Tirapelli et al., 2008).

Several classes of secondary metabolites are synthesized by plants and, among those, lignans are recognized as a class of natural products with a wide spectrum of important biological activities. **Table 1** summarizes the main biological properties described in the literature for lignans.

The term "Lignan" was first introduced by Haworth (1948) to describe a group of dimeric phenylpropanoids where two C_6-C_3 are attached by its central carbon (C8), as shown in **Figure 1**. More recently, Gotlieb (1978) proposed that micromolecules with two phenylpropanoid units coupled in other manners, like C5-C5´ for example should be named "neolignans" (Umezawa, 2003). According to Gordaliza et al (2004), lignans can be found in more than 60 families of vascular plants and have been isolated from different plant parts, exudates and resins.

Biological activity	Reference
Antiviral	(Charlton, 1998; Cos et al., 2008; McRae & Towers, 1984; Yousefzadi et al., 2010)
Anticancer	(McRae & Towers, 1984; Pan et al., 2009; Saleem et al., 2005; Yousefzadi et al., 2010)
Cancer prevention	(Huang et al., 2010; Webb & McCullough, 2005)
Anti-inflammatory	(Saleem et al., 2005)
antimicrobial	(Saleem et al., 2005)
antioxidant	(Fauré et al., 1990; Pan et al., 2009; Saleem et al., 2005)
immunosuppressive	(Saleem et al., 2005)
Hepatoprotective	(Negi et al., 2008)
Osteoporosis prevention	(Habauzit & Horcajada, 2008)

Table 1. Main biological activities of lignans

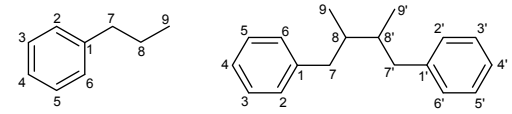

Fig. 1. Phenylpropanoid unit and lignan structure

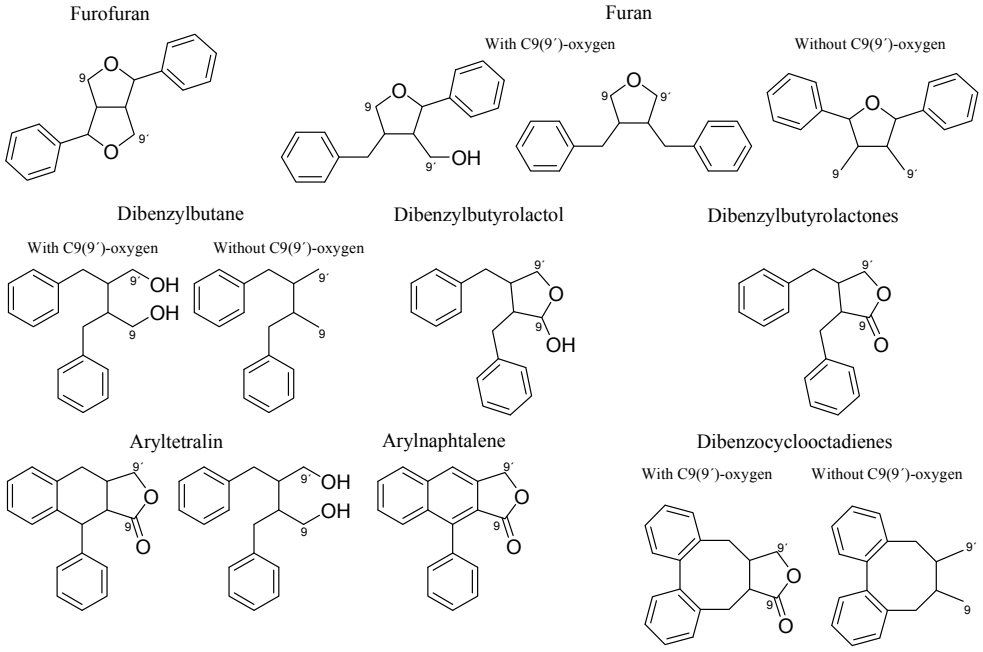

Fig. 2. Main subclasses of lignans. Adapted from Suzuki & Umezawa (2007).

Most of the known natural lignans are oxidized at C9 and C9´ and, based upon the way in which oxygen is incorporated into the skeleton and on the cyclization patterns, a wide range of lignans of very different structural types can be formed. Due to this fact, lignans are classified in eight subgroups and (Chang et al., 2005; Suzuki & Umezawa, 2007), among these subgroups, the furan, dibenzylbutane and dibenzocyclooctadiene lignans can be further classified in "lignans with C9 (9´)-oxygen" and "lignans without C9 (9´)-oxygen". **Figure 2** displays the main classes of lignans, as well as their subgroups. It is noteworthy that, despite its structural variation, lignans also display a substantial variation on its enantiomeric composition (Umezawa et al., 1997). In this sense, these metabolites can be found as pure enantiomers and as enantiomeric compositions, including racemates (Macias et al., 2004).

2. Chemical aspects of lignans

As mentioned before, lignins and lignans are both originated from C_6-C_3 units, thus indicating that these metabolites are biosynthesized through the same pathway in the earlier steps. As seen in **Figure 3**, aromatic aminoacids *L*-phenylalanine and *L*-tyrosine are produced from shikimic acid pathway, and then converted in a series of cinnamic acid

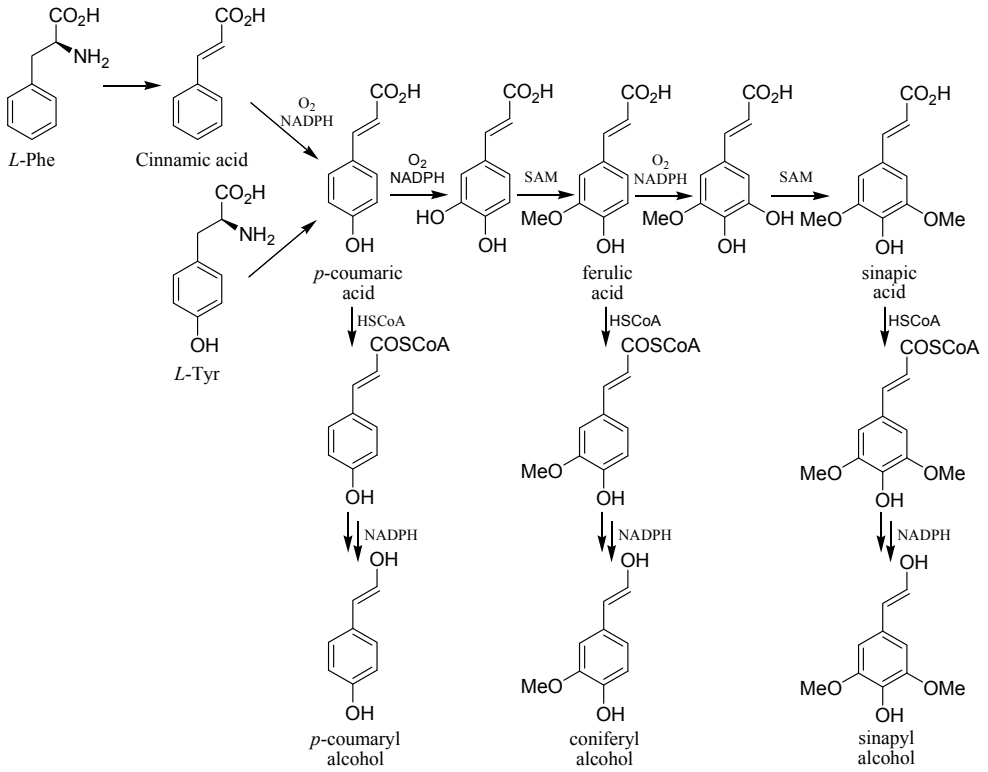

Fig. 3. Biosynthesis of hydroxycinamyl alcohol monomers, the precursors of lignans according to Dewick (2002).

derivatives. The reduction of these acids via coenzyme A of related esters and aldehydes forms three alcohols (*p*-coumaryl alcohol, coniferyl alcohol and sinalpyl alcohol) that are the main precursors of all lignins and lignans.

The peroxidase induces one-electron oxidation of the phenol group allowing the delocalization of the unpaired electron through resonance forms. In these hydroxycinamyl alcohols, conjugation allows the unpaired electron to be delocalized also into the side chain. After this point, radical pairing of these resonance structures originates reactive dimeric systems susceptible to nucleophilic attack from hydroxyl groups, leading to a wide range of lignans, as shown in **Figure 2**.

Among these subgroups, the biosynthesis of C9 (9´)-oxygen lignans is the most well known. This type of lignan is formed through the enantioseletive dimerization of two coniferyl alcohol monomeric units (D resonance form of coniferyl alcohol radical, **Figure 4**) into pinoresinol via intermolecular 8,8´ oxidative coupling with the aid of dirigent protein (Dewick, 2002; Suzuki & Umezawa, 2007).

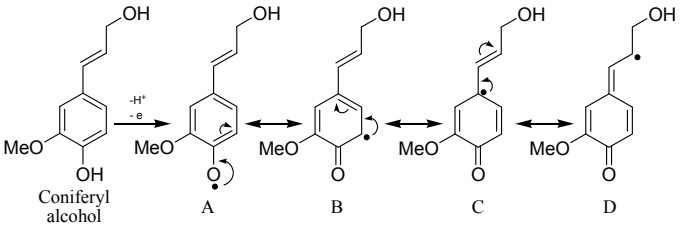

Fig. 4. Resonance forms of coniferyl alcohol radical

The following steps involve sequencial stereoselective enzymatic reduction of pinosresinol by pinoresinol/lariciresinol reductase to generate lariciresinol and then secoisolariciresinol by secoisolariciresinol dehydrogenase. The main steps of this biosynthetic proposal are depicted in **Figure 5**. Secoisolariciresinol gives the presumably common precursor of all dibenzylbutyrolactol lignans and, through the formation of matairesinol and yatein, also forms the aryltetralin lignans. These subclasses of lignans includes some important bioactive compounds such as cubebin (**1**) and podophyllotoxin (Canel et al., 2000; de Souza et al., 2005; Gordaliza et al., 2004; Saraiva et al., 2007; Silva et al., 2007; Silva et al., 2009; Srivastava et al., 2005; You, 2005; Yousefzadi et al., 2010).

3. Podophyllotoxin: chemical and biological approaches

Podophyllotoxin (**Figure 6**), a naturally occurring aryltetralin lignin, is one of the most important compound due to its high toxicity and current use as a local antiviral agent (Yousefzadi et al., 2010). Moreover, this metabolite has been used to obtain structural analogues which are employed as anticancer drugs (Ayres & Loike, 1990; Yousefzadi et al., 2010) and several semi-synthetic podophyllotoxin-related derivatives showed to be topoisomerase II inhibitor, acting as an antimitotic compound (You, 2005; Yousefzadi et al., 2010). **Figure 6** also shows the clinically valuable anticancer agents, etoposide, teniposide and etoposide phosphate, obtained from podophyllotoxin.

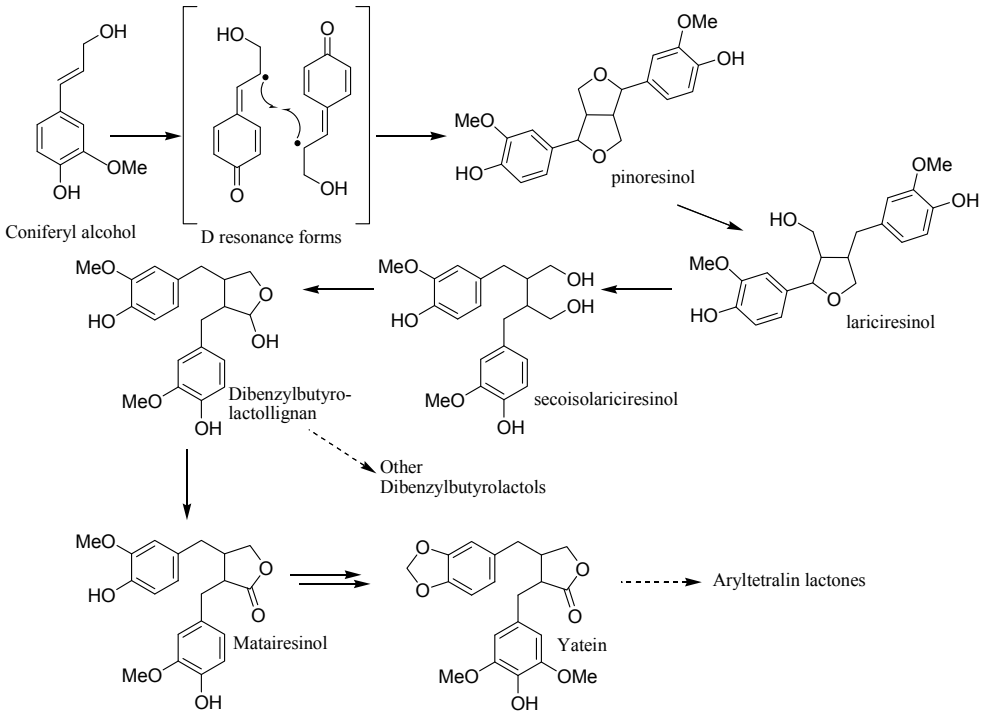

Fig. 5. Biosynthesis of dibenzylbutyrolactols and aryltetralin lactones (Canel et al., 2000).

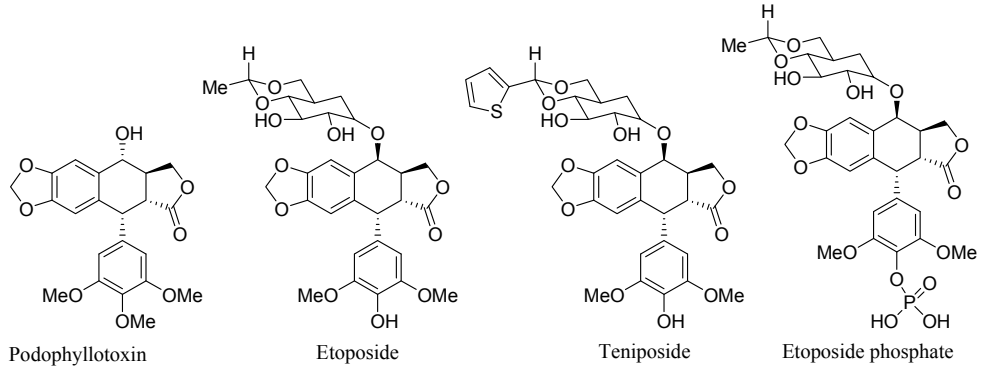

Fig. 6. Chemical structures of clinically available podophyllotoxin derivatives.

According to You (2005), podophyllotoxin still can be considered a hot prototype for discovery and development of novel anticancer agents, even in the 21st century. This leading compound has been isolated from the roots of *Podophyllum* species and more recently from other genus, such as *Linum* (Yousefzadi et al., 2010). Due to its importance in anticancer therapy, several biotechnological approaches including the use of cell cultures, biotransformation processes and metabolic engineering techniques to manipulate the

biosynthetic pathway (**Figure 7)**, have been currently developed and are alternatives for the production of podophyllotoxin.

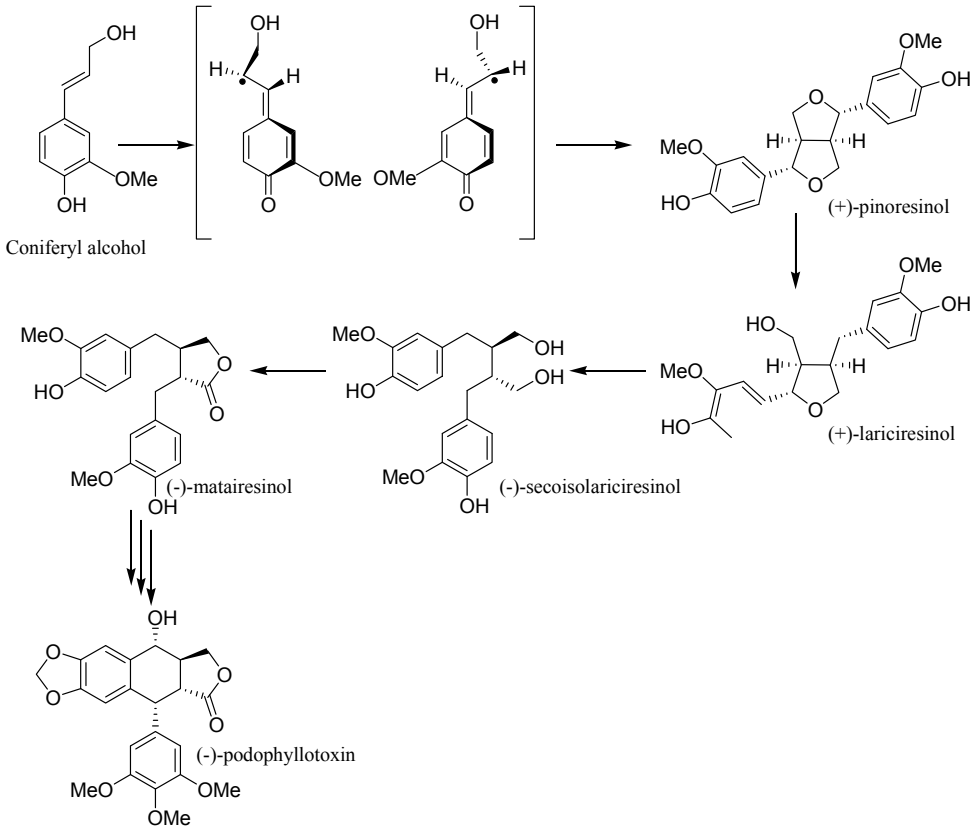

Fig. 7. Biosynthesis proposal of podophyllotoxin according to Canel et al., 2000.

Despite the fact that etoposide, teniposide and etoposide phosphate are clinically valuable anticancer agents, several adverse effects and drug resistance have been associated with the use of these drugs (You, 2005). In this sense, several studies focusing to prepare novel derivatives and to understand the structure-activity relationship (SAR) of podophyllotoxins have been published (You, 2005). Based on these data a great number of potential drug candidates were synthesized (You, 2005; Yousefzadi et al., 2010). **Figure 8** shows the structures of new antineoplasic candidates developed from podophyllotoxin chemical skeleton.

In order to better explore the biological potential of this class of metabolites, our research group has concentrated efforts to investigate the biological activity of some dibenzylbutyrolactone lignans, mainly cubebin (**Figure 9**, **1**) and its semi-synthetic derivatives. In this sense, the most significant achievements in our investigations are described in the following sections.

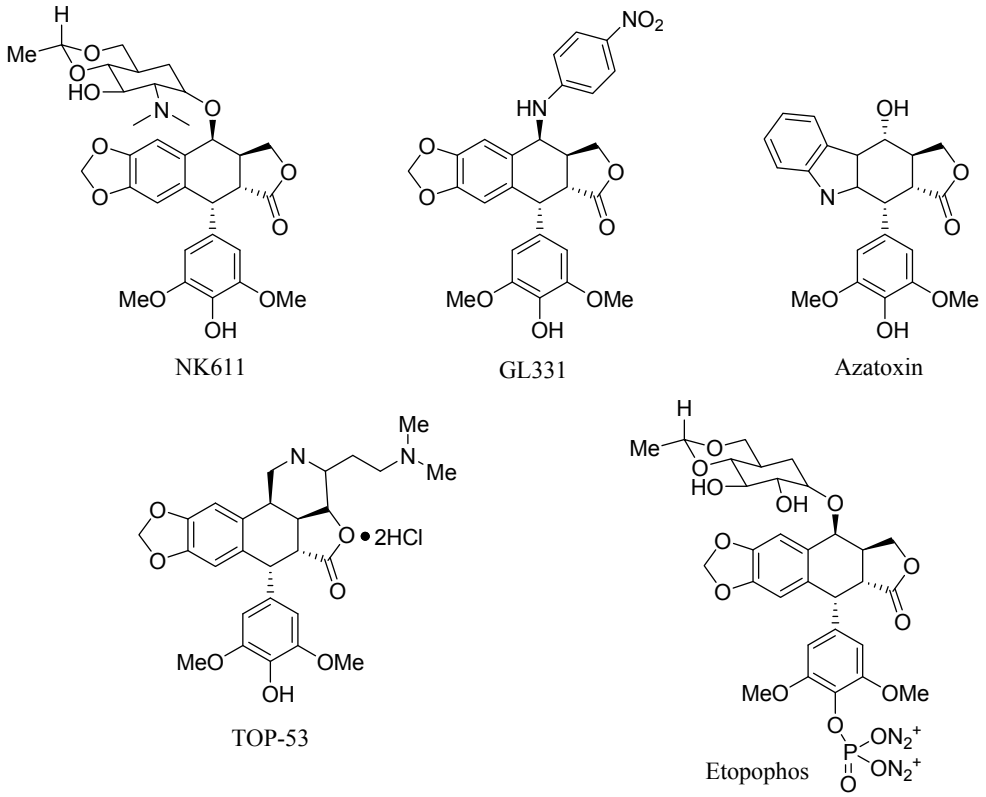

Fig. 8. Examples of antineoplasic candidates developed from podophyllotoxin chemical skeleton.

4. Trypanocidal activity of cubebin and its derivatives

The Chagas' disease, or American trypanosomiasis, is endemic in Central and South America and it is estimated that 16–18 million people are currently infected with the protozoan flagellate *Trypanosoma cruzi* (Molfetta et al., 2005) and more than 100 million are exposed to the risk of infection (Takeara et al., 2003).

Since it was discovery in 1909, Chagas´ disease infection has been difficult to control due to its multiple characteristics (de Souza et al., 2005). One of the main causes of these difficulties are to find an efficient compound to combat the aetiologic agent (*T. cruzi*) is directly linked to the morphologic characteristics of its strains, mainly due to the occurrence of various sub-populations of the parasite, leading to a different host tissue´s tropism (de Souza et al., 2005).

Clinical treatment of infected patients is relied on two nitroheterocyclic drugs, the nifrofuran nifurtimox, Lampit®, which production has now been discontinued, and the 2-nitroimidazole benznidazole, Rochagan® (Paulino et al., 2005). Both drugs, if administered during the acute phase of the disease, could cure 50-70% of the patients. However, these

drugs display limited efficacy in the treatment of the chronic phase of the disease and are quite toxic for the patients (de Souza et al., 2005). Therefore, there is an urgent demand for the discovery and development of novel therapeutic compounds to treat Chagas´ disease

De Souza et al. (2005) (de Souza et al., 2005) have reported the trypanocidal activity of cubebin (**1**) and its semi-synthetic derivatives against free amastigote forms of *T. cruzi*. **Figure 9** also shows the compounds obtained by partial synthesis from cubebin (**1**), as well as the reagents and conditions used in these reactions.

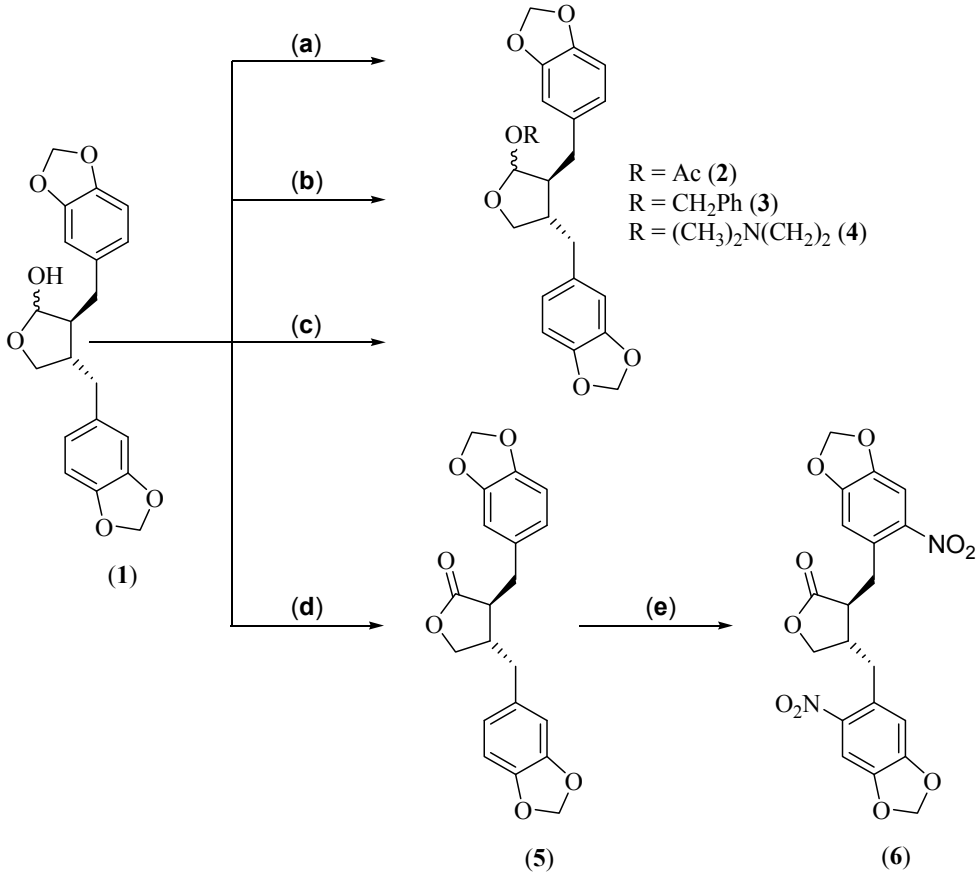

Fig. 9. Reagents and conditions: (a) Ac_2O, Py, room temperature, 24 h; (b) NaH, BnBr, THF, room temperature, 24h; (c) EtONa, $(CH_3)_2CH_2Cl$, EtOH, reflux, 6h; (d) PCC, CH_2Cl_2, room temperature, 12h; (e) HNO_3, 2h, -10° C.

The natural cubebin (**1**), used as the starting compound to obtain the evaluated dibenzylbutyrolactone derivatives, did not display activity against trypomastigote forms of *T. cruzi* (Bastos et al., 1999). Hence, the biological evaluation against amastigote forms was undertaken only for lignans **2**, **3**, **4**, **5** and **6**. Cubebin was selected as starting compound because of its availability, being easily isolated in large amounts from the seeds of *Piper*

cubeba (de Souza et al., 2005). **Table 2** shows the results of the trypanocidal activity evaluation of compounds **2**, **3**, **4**, **5**, **6** and benznidazole, against amastigote forms of Y strain of *T. cruzi*.

Compounds	Concentration (μM) X % of lyse (± SD)				IC_{50} (μM)
	0.5	2.0	8.0	32.0	
2	14.5 ± 1.9	26.0 ± 2.5	29.0 ± 5.2	26.4 ± 1.2	1.5 X 10^4
3	37.0 ± 1.4	38.0 ± 7.0	46.8 ± 6.4	68.8 ± 2.9	5.7
4	32.6 ± 2.6	55.4 ± 4.3	50.8 ± 1.0	57.8 ± 8.0	4.7
5	47.6 ± 9.5	57.0 ± 1.1	57.6 ± 8.9	63.6 ± 5.2	0.7
6	34.6 ± 7.9	48.7 ± 1.4	38.9 ± 2.0	48.5 ± 6.1	95.3
Benznidazole	38.4 ± 3.0	67.0 ± 7.2	69.0 ± 4.0	68.6 ± 1.7	0.8

Table 2. Results of the trypanocidal activity evaluation of compounds **2**, **3**, **4**, **5**, **6** and benznidazole, against amastigote forms of Y strain of *T. cruzi* (de Souza et al., 2005).

The production of compound **2** by substitution of the lactol hydrogen of cubebin by an acetyl group led to a strong reduction of its trypanocidal ctivity, in comparison with all other evaluated compounds belonging to the same group (IC_{50} = 1.5 X 10^4 μM; **Table 2**). Furthermore, the comparison of compounds **2** and **3** indicate that the biological activity against the amastigote forms of *T. cruzi* was significantly affected by the nature of the substituting group at position C-9, which played an important role in the reduction of the calculated IC_{50} value for compound **3** (IC_{50} = 5.7 μM; **Table 2**). Likewise, cubebin derivative **4**, bearing an amino group at the lactol ring, displayed an activity quite similar to compound **3**.

Analysis of the obtained results, displayed in **Table 2**, indicate that compound 5 was the most active, with an IC_{50} value of 0.7 μM similar to that displayed by benznidazole (IC_{50} = 0.8 μM), a standard drug used as the positive control. On the other hand, most of the other evaluated compounds displayed much lower activity, with the exception of compounds **3** (IC_{50} = 5.7 μM) and **4** (IC_{50} = 4.7 μM), which showed significant activity.

In this study, De Souza et al. (2005) also pointed out that hinokinin (**HK**, **5**) is a promising compound to continue examining, since at 0.5 μM it displayed higher activity than benznidazole and at the other assayed concentrations (2.0, 8.0 and 32.0 μM) it showed similar activity.

In view of higher trypanocidal activity displayed by **HK** (**5**) against free amastigote forms of *T. cruzi* (**Table 2**; (de Souza et al., 2005), this lignan was selected to be assayed against epimastigote and intracellular amastigote forms of *T. cruzi*, both in vitro and *in vivo* assays (Saraiva et al., 2007). The results of the trypanocidal activity against epimastigote and intracellular amastigote forms of *T. cruzi* are shown in **Tables 3 and 4**, respectively.

Compounds	Concentration (μM) X % of lyse (± SD)					IC_{50} (μM)
	0.5	2.0	8.0	32.0	128.0	
HK	21.79±1.82	99.03±0.15	100.0±0.35	100.0±0.35	100.0±0.64	0.67
Nifurtimox	11.78±13.92	49.38±6.71	65.46±5.36	81.54±2.71	97.54±1.80	3.08
benznidazole	0	1.23±5.87	26.18±10.71	51.13±5.23	76.09±2.74	30.89

Table 3. Results of the trypanocidal activity evaluation of **HK**, benznidazole and nifurtimox against epimastigote forms of CL strain of *T. cruzi* (Saraiva et al., 2007).

Compounds	Concentration (μM) X % of lyse (± SD)				IC_{50} (μM)
	2.0	8.0	32.0	128.0	
HK	25.84±1.09	31.92±9.29	61.72±9.17	100.0±0.40	18.36
Nifurtimox	44.6±0.99	63.78±1.25	83.31±0.79	90.63±1.13	3.54
benznidazole	14.33±2.65	35.81±0.65	57.28±1.99	78.75±0.67	20.00

Table 4. Results of the trypanocidal activity evaluation of HK, benznidazole and nifurtimox against intracellular amastigote forms of CL strain of *T. cruzi* (Saraiva et al., 2007).

As it can be observed in **Table 3**, **HK** showed a very significant activity against epimastigote forms of *T. cruzi*, displaying IC_{50} value (0.67 μM) much lower than benznidazole and nifurtimox, used as positive controls (Saraiva et al., 2007). **HK**, also showed to be very active against intracellular amastigote forms, displaying IC_{50} value of 18.36 μM, which was similar to benznidazole (IC_{50} = 20.0 μM, **Table 4**).

The *in vivo* assays (Saraiva et al., 2007) were performed using five groups of five BALB/c males, weighing approximately 20 g each. The groups were as follows: group 1, animals without infection; group 2, control infected animals; group 3, animals treated with solvent; group 4, animals treated with benznidazole 40 mg kg^{-1} day^{-1}; group 5, animals treated with **HK** 40 mg kg^{-1} day^{-1}. The animals were inoculated with 2 X 10^4 trypomastigote forms of *T. cruzi* (Y strain). The treatment was initiated 48 h after infection and maintained for 20 days. The animals were treated twice a day with 20 mg kg^{-1} benznidazole and **HK** orally. The results obtained showed that the treatment with **HK** promoted 70.8% of parasitaemia reduction in the parasitaemic peak, while benznidazole displayed approximately 29.0% of parasite reduction (Saraiva et al., 2007). In addition, **HK** was able to reduce the number of parasites more than benznidazole not only in the parasitaemic peak, but also in all curse of infection (Saraiva et al., 2007).

Moreover, it was observed that the groups treated with **HK** displayed better survival rates than the group treated with benznidazole, with survival until the 22nd and 16th day after the beginning of the infection, respectively (Saraiva et al., 2007). Despite the obtained significant results for the *in vivo* assays, the treatment with **HK** or benznidazole did not cause parasitological cure. Overall, considering the promising results displayed by **HK** against both the epimastigote and amastigote forms of the parasite in the *in vitro* assay, as well as the good result displayed in the *in vivo* assay, this lignan has been considered as a lead compound for the development of new drugs for the treatment of Chagas´disease (Saraiva et al., 2007).

In order to obtain better efficacy of **HK** towards the intracellular forms of the parasite, our research group prepared and investigated the effect of **HK** load poly($_{D,L}$-lactide-co-glycolide) microparticules.

5. Hinokinin-load poly($_{d,l}$-lactide-co-glycolide) microparticles for Chagas´ disease

The drug delivery system were developed for the purposes of bringing, uptaking, retaining, releasing, activating, localizing, and targeting the drugs at the right timing, period, dose, and place (Ueda & Tabata, 2003). The use of biodegradable polymers, as poly($_{D,L}$-lactic-co-glycolic acid; PLGA), for the controlled release of therapeutic agents is now well established.

These systems have been extensively utilized for oral and parenteral administration (Saraiva et al., 2010). The physical properties and the Food and Drug Administration approval of poly(lactide-co-glycosides) make them the most extensively studied commercially available biodegradable polymers (Birnbaum et al., 2000).

The microparticles can be able to sustain the release of the drug for a considerable period of time, to reduce the required frequency of administration increasing patient compliance, to avoid plasmatic fluctuations, to decrease side effects, and to facilitate dosage administration (Hans & Lowman, 2002). In this sense, our research group prepared **HK**-loaded PLGA microparticles to protect HK of biological interactions and promote its sustained release for treatment of Chagas´ disease. Moreover, the trypanocidal effect of microparticles containing **HK** was evaluated *in vivo*.

The **HK**-loaded PLGA microparticles were prepared with success (Saraiva et al., 2010) and presented narrow distribution size and a mean diameter of 0.862 μm, with PDI of 0.072 mm. Scanning electron micrographs of PLGA microparticles obtained showed that **HK** loaded microparticles presented, smooth and spherical surface. Due to their small diameter, the **HK** microparticles obtained are better suited for parenteral delivery (Cegnar et al., 2005).

The trypanocidal *in vivo* experiments were performed using Female Swiss mice (weigh, 20-22 g) which were infected intraperitoneally with 2 X 10^4 trypomastigotes forms of *T. cruzi*. The treatment (20 days) was performed through subcutaneous route and initiated 48 h after infection, according to Saraiva et al. (2010).

The treatment of infected mice with 40 mg kg^{-1} of **HK**-loaded microparticles each 2 days was able to provoke significant decrease in parasitemia levels compared with those recorded in untreated controls ($P<0.05$ at days 12, 14, 16, 19 and 21 post-infection with *T. cruzi*). The treatment with an equivalent amount of empty microparticles (without **HK**) had no effect on the parasitemia compared to untreated controls (Saraiva et al., 2010). Moreover, administration of **HK**-loaded microparticles was able to reduce the number of parasites more than the treatment with 20 mg kg^{-1} day^{-1} of **HK** not only in the parasitemic peak, but also in the course of infection ($P<0.05$ at days 14, 16, 19 and 21 post-infection with *T. cruzi*) (Saraiva et al., 2010). The use of PLGA micropartiles as vehicle for HK delivery can improve HK trypanocidal activity. It may be attributed to the fact that it can protect HK of biological interactions and promote its sustained release, with maintenance of its plasmatic concentration in therapeutic levels (Saraiva et al., 2010).

The **HK**-loaded microparticles developed by our research group can be considerable a promising system for sustained release of **HK** for therapeutic use and could be used in future clinical studies (Saraiva et al., 2010). Also, it is very important to point out that other *in vivo* assays have been developed by our research group in order to evaluate the parasitological cure of infection and the activity of this delivery system coating **HK** against other strains of *T. cruzi*.

6. Influences of stereochemistry on trypanocidal activity of dibenzylbutyrolactone lignans

We have been reporting the significant trypanocidal activity of dibenzylbutyrolactone lignans, mainly the semi-synthetic **HK**. Such results aroused the interest within our group

to study the effect of stereochemistry in this biological property. For this purpose, methylpluviatolide, one of the most powerful compounds regarding trypanocidal activity (Bastos et al., 1999) was synthesized in its *trans* and *cis* racemic forms. Thus, allowing us to evaluate the trypanocidal activity not only of a mixture of these two stereoisomers, but also of the pure enantiomers, which were separated by chiral HPLC (da Silva et al., 2008).

Trans (tM) and *cis* (cM)racemic forms of methylpluviatolide were prepared by a procedure described by Landais et al. (1991) and Charlton and Chee (1997) (**Figure 10**).

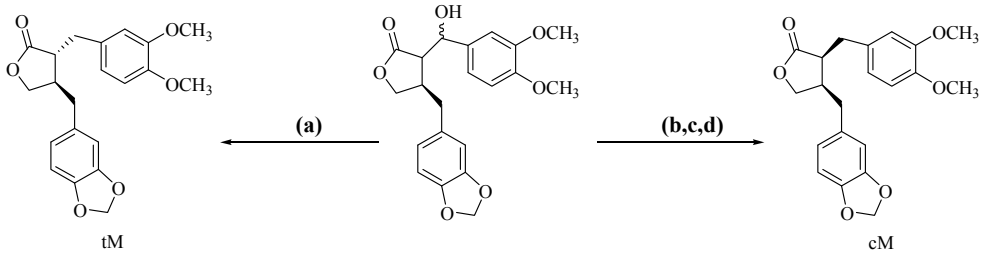

Fig. 10. Reagents and conditions: (a) H_2, 4 atm, Pd/C, ETOH, $HClO_4$, 60h, room temperature; (b) THF, AC_2O, Et_3N, DMAP, 2h, room temperature; (c) DBU, CH_2Cl_2, 5h, room temperature; (d) H_2, 4 atm, Pd/C, ETOH, 60h, room temperature.

The results obtained for the racemic mixture of *trans* and *cis* methylpluviatolide against *T. cruzi* showed that racemic *cis*-stereoisomer (**2**) is inactive, while the racemic *trans*-stereoisomer (**1**) display significant trypanocidal activity, with an IC_{50} of 89.3 µM (da Silva et al., 2008).

On the basis of these results, a separation of the *trans*-stereoisomer from the racemic mixture was undertaken by chiral HPLC using an analytical Chiracel OJ (4.6 x 250 mm) column, aiming to evaluate the trypanocidal activity of each enantiomer separately. The chromatogram gave a well resolved peak separation, allowing the isolation of both enantiomers (da Silva et al., 2008), which were evaluated against trypomastigote forms of the Y strain of *T. cruzi*. **Table 5** shows the results of the trypanocidal activity evaluation of (+)-*trans*- methylpluviatolide (**+tM**) and (-)-*trans*- methylpluviatolide (**-tM**) against trypomastigote forms of the Y strain of *T. cruzi*.

Compounds	Concentration (µM) X % of lyse (± SD)			IC_{50} (µM)
	8.0	32.0	128.0	
+tM	5.3±2.5	7.6±3.4	9.9±2.5	1.3 X 10^6
-tM	40.6±3.6	52.3±4.4	79.7±0.0	18.7

Table 5. Results of the trypanocidal activity evaluation of **+tM** or **-tM** against trypomastigotes forms of the Y strain of *T. cruzi*. (da Silva et al., 2008).

The results show that **+tM** is completely inative, whereas the **–tM** displayed good activity, with an IC_{50} of 18.7 µM (da Silva et al., 2008). These results indicate that despite being completely inactive, the **+tM** blocks the action of the **–tM** when they are present in a racemic mixture. It should be taken into consideration that the **+tM** might bind to the active sites as a

competitive antagonist, which may be confirmed by comparison of the IC_{50} value of the racemic mixture with that of the **–tM** itself (da Silva et al., 2008).

In conclusion, this study pointed the importance of the stereochemistry on trypanocidal activity of dibenzylbutyrolactone lignans and brings new perspective in the importance to understand the trypanocidal structure-activity relationship for this class of natural compounds.

7. Antimicrobial potential of some natural and semi-synthetic lignans against *Mycobacteria* and oral pathogens

The lignans possess a wide spectrum of biological activities, including antimicrobial (Saleem et al., 2005). Considering this fact, our research group also decided to investigate the potential of some natural and semi-synthetic lignans against mycobateria and oral pathogens (Silva et al., 2007; Silva et al., 2009).

Tuberculosis is a severe infectious disease caused by mycobacteria belonging to the *Mycobacterium tuberculosis* complex. According to WHO, tubercolosis affects nearly 30% of the world´s population and is responsible for 3 million deaths worldwide each year, mainly in developing countries (Raviglione, 2003). The current chemotherapy of this pathology has been based on the use of combined drug therapy with rifampicin, isonizid, and pyrazinamide. However, the incorrect use and long drug administration, as well as the high cost and countless side-effects have led people to abandon the treatment before being completely cured, leading to resistant bacilli (Timmins & Deretic, 2006). In addition, the existence of drug-resistant tuberculosis reinforces the need to develop new safe and effective antimycobacterial drugs. In this sense, our research group evaluated the antimycobacterial activity of several lignans obtained from cubebin (Silva et al., 2009).

As shown in **Figure 11**, (-)-cubebin (**1**) was isolated from powdered seeds of *Piper cubeba* and then submitted to various semi-synthetic procedures to obtain hinokinin (**HK**, **5**), (-)-O-acetyl-cubebin (**2**), (-)-O-methyl-cubebin (**7**), (-)-O-(N,N-dimethylamine-ethyl)-cubebin (**4**) and (-)-6,6´-dinitrohinokinin (**6**). All these compounds were assayed *in vitro* by the microdilution technique on a Resazurin microtiter assay (REMA) plate, using a procedure adapted from (Palomino et al., 2002).

Cubebin (**1**) did not display any activity against the investigated strains (**Table 6**). **HK** (**5**) was moderately active against *Mycobacterium tuberculosis*, with a MIC value equal to 62.5 μg mL^{-1}. Compound (**2**), whose lactol group is acetylated, displayed activity against *M. tuberculosis* (MIC = 125 μg mL^{-1}) and *M. avium* (MIC = 62.5 μg mL^{-1}). The best result was achieved with compound **7**, whose lactol group is methylated, leading to a MIC value equal to 31.25 μg mL^{-1} against *M. avium*. The other compounds were not active against any of the studied mycobacteria. In the case of the lactol-containing compounds evaluated here, it seems to be essential that the lactol group is absent, and the substituent of this group should be small, as in the case of **2** and **7**. *M. kansaii* (ATCC 12478) was the most resistant mycobacterium concerning the evaluated compounds, with MIC values varying between 1000 and 2000 μg mL^{-1}.

To sum up, cubebin and hinokinin semi-synthetic derivatives were prepared and evaluated for their antimycobacterial activity. Some derivatives were active against *M. tuberculosis* and *M. avium*, suggesting that this class of compounds may lead to a new generation of antituberculosis agents (Silva et al, 2009).

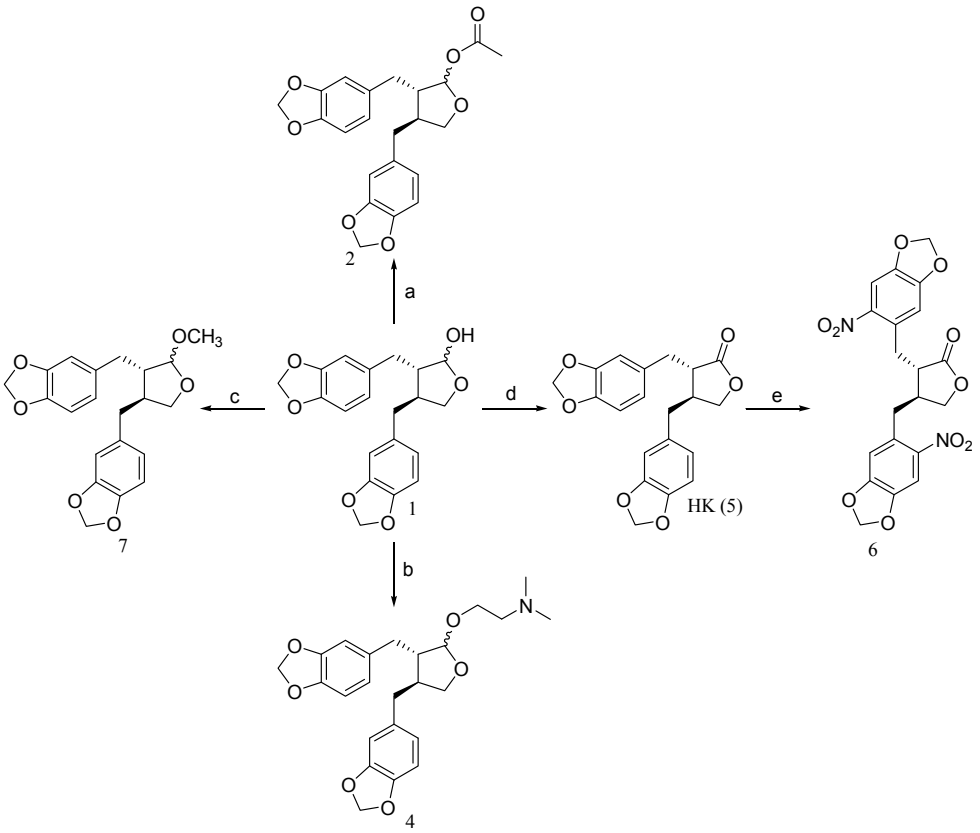

Fig. 11. Chemical structures and conditions of the reactions (a) Acetic anhydride, room temperature, 24h. (b) Dimethyllethylammonium chloride, EtONa, dry THF, room temperature, N_2 atmosphere, 6h. (c) Methyl iodide, NaH, dry THF, room temperature, 6h. (d) PCC (pyridinium chlorochromate) in dry methylene chloride, 24h, in an ice bath with continuous stirring. (e) HNO_3, chloroform, -6°C, 2h.

Compound	MIC [μg mL^{-1}]		
	M. tuberculosis	*M. kansaii*	*M. avium*
1	500	2000	1000
2	125	1000	62.5
4	250	2000	250
5	62.5	2000	500
6	1000	2000	1000
7	250	2000	31.25
Rifampicin[a]	0.031	0.015	0.062

[a] Standard antibiotic

Table 6. Minimal inhibitory concentration (MIC) of cubebin (**1**) and its derivatives against *M. tuberculosis*, *M. kansasii*, and *M. avium*.

Recently, our research group also investigated the antimicrobial activity of cubebin and related derivatives against oral pathogens, mainly those responsible for caries disease, which are intimately related with the dental plaque formation.

Dental plaque is defined as a biofilm consisting of cariogenic bacteria adhered on the tooth surface and plays an important role in the development of dental caries (Chung et al., 2006; Xie et al., 2008), one of the main oral diseases that affect humankind (More et al., 2008; Souza et al., 2010). This destructive infection of the dental hard tissues can progress and if untreated, lead to the death of vital pulp tissue and tooth loss (Allaker & Douglas, 2009). Bacteria from the genus *Streptococci* are commonly isolated from the oral cavity (Hirasawa & Takada, 2002) and have been responsible for this infectious disease. Among them, *Streptococcus mutans* is considered one of the main cariogenic microorganisms, due to its ability to synthesize extracellular polysaccharides from sucrose, mainly water-insoluble glucan, and initiate plaque formation (Koo et al., 2000). Other aerobic bacteria such as *Enterococcus faecalis*, *Lactobacillus casei*, *Streptococcus mitis*, *S. sanguinis*, *S. sobrinus* and *S. salivarius* are also important in the latter formation of the dental biofilm (Chung et al., 2006).

The mechanical removal of the dental plaque is the most efficient procedure to prevent caries, but the majority of the population does not perform this removal efficiently (Ambrosio et al., 2008). Moreover, dental treatment is often very expensive and not readily accessible, especially in developing countries (More et al., 2008). In this sense, the use of chemicals as a complementary measure is necessary and has demonstrated to be of great value in the prevention of the formation and in the decreasing of the tooth surface biofilm (Furiga et al., 2008).

Extensive efforts have been made toward the search for anticariogenic compounds that can be incorporated into dental products, aiming at complementing the mechanical removal. Several antibiotics, such as ampicillin, chlorhexidine, sanguinarine, metronidazole, phenolic-antiseptics and quaternary ammonium-antiseptics have been used to prevent dental caries. Among these compounds, chlorhexidine is considered a gold standard anticariogenic and has received the approval of the American Dental Association Council on Dental Therapeutics (Ambrosio et al., 2008). However, the regular use of oral care products containing this chemical are often associated with tooth and restoration staining, changes in the taste of food, and a burning sensation at the tip of the tongue (Greenberg et al., 2008; More et al., 2008; Porto et al., 2009b). In addition, chlorhexidine is much less effective in reducing the levels of *Lactobacillus* species, which are strongly related to caries evolution (Ambrosio et al., 2008). All these problems, therefore, denote that finding new, safe and effective anticariogenic coumpounds is still needed.

Thus, our research group tested compounds **1**, **4**, **5**, and **6** (**Figure 11**) and another semi-synthetic derivative (O-benzyl cubebin, **8**, **Figure 12**)using the broth microdilution method (Andrews, 2001) against the following microorganisms: *Enterecoccus faecalis* (ATCC 4082), *Streptococcus salivarius* (ATCC 25975), *Streptococcus mitis* (ATCC 49456), *Streptococcus mutans* (ATCC 25275), *Streptococcus sobrinus* (ATCC 33478), *Streptococcus sanguinis* (ATCC 10556) and *Candida albicans* (ATCC 28366) (Silva et al. 2007). **Table 7** displays the minimum inhibitory concentration values obtained for these compounds

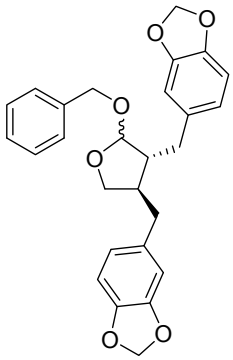

Fig. 12. Structure of O-benzyl cubebin (compound **8**)

Compound	Microorganism						
	E. faecalis	*S. salivarius*	*S. sanguinis*	*S. mitis*	*S. mutans*	*S. sobrinus*	*C. albicans*
1	0.35	0.25	0.22	0.20	0.32	0.27	0.28
4	0.31	0.21	0.21	0.19	0.28	0.23	0.23
5	0.38	0.25	0.25	0.25	0.32	0.28	0.28
6	0.30	0.20	0.21	0.18	0.27	0.23	0.23
8	0.31	0.20	0.23	0.18	0.29	0.23	0.28
CHD[a]	$5.9x10^{-3}$	$1.7 x10^{-3}$	$3.9x10^{-3}$	$5.9x10^{-3}$	$5.9x10^{-3}$	$1.5x10^{-3}$	$7.9x10^{-3}$

a Chlorhexidine

Table 7. Values of minimum inhibitory concentrations (in milimolar) of cubebin and its semi-synthetic derivatives against oral pathogens

The semi-synthetic derivative **6** was the most active one against all the evaluated microorganisms (**Table 7**). Compounds **5** and **6** are lignan-lactones and differ from cubebin by the presence of a carbonyl group at C9 **(Figure 11**). Analysis of the obtained results suggested that the presence of the carbonyl group at C9 with introduction of polar groups in the aromatic rings is beneficial for the antimicrobial activity.

The obtained results for antimicrobial activity are in accordance to those obtained for anti-inflammatory and analgesic activities. Compounds possessing a lactone ring bearing two methylendioxyaryl groups display significant anti-inflammatory and analgesic activities, and the introduction of polar groups in the aromatic rings is advantageous for these activities . However, with regard to trypanocidal activity, the introduction of nitro groups at the aromatic rings is harmful for this activity. Besides, the lignan-lactone **HK** (**5**) was the most active compound against *T. cruzi* (de Souza et al., 2005).

8. Future perspectives

Despite of the wide spectrum of biological activities related to lignans, the literature used to emphasize the antioxidant properties and the role of these metabolites in cancer treatment and prevention. (Fauré et al., 1990; McRae & Towers, 1984; Pan et al., 2009; Saleem et al., 2005; Yousefzadi et al., 2010). However, in recent years our research group pointed out the importance of such metabolites, specially cubebin and their semi-synthetic derivatives, as potential antichagasic agents (da Silva et al., 2008; de Souza et al., 2005; Saraiva et al., 2010; Saraiva et al., 2007). The very promising results obtained against *T. cruzi* suggested that further investigations of these lignans against other parasitic diseases should be performed. In this sense, our group is now focusing the evaluation of such compounds against, for example, *Schistossoma mansoni* and *Fasciola hepatica*, as well as the obtainment of new cubebin-related semi-synthetic derivatives.

In addition, our results on the antimicrobial activities of these metabolites also highlighted their potential as new antimicrobial agents (Silva et al., 2007; Silva et al., 2009). In this context, the literature also reports additional experiments with the objective of investigating other features of the antimicrobial activity, such as the time-kill curve experiments based on D'Arrigo et al (2010) and investigations about a possible synergistic effect between the most effective tested lignans and the current used antimicrobial agents (White et al., 1996).

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11

The Genus *Galanthus*: A Source of Bioactive Compounds

Strahil Berkov[1], Carles Codina[2] and Jaume Bastida[2]
[1]*AgroBioInstitute, Sofia,*
[2]*Departament de Productes Naturals, Biologia Vegetal i Edafologia, Facultat de Farmàcia, Universitat de Barcelona, Barcelona, Catalonia,*
[1]*Bulgaria*
[2]*Spain*

1. Introduction

The Amaryllidaceae family is one of the 20 most important alkaloid-containing plant families (Zhong, 2005). It comprises about 1100 perennial bulbous species classified in 85 genera, distributed throughout the tropics and warm temperate regions of the world (Willis, 1988). The specific alkaloids produced by the amaryllidaceous plants have attracted considerable attention due to their interesting pharmacological activities. One of them, galanthamine, is a long acting, selective, reversible and competitive inhibitor of the acetylcholinesterase enzyme (Thomsen *et al.,* 1998), which is marketed as a hydrobromide salt under the name of Razadyne® (formerly Reminyl®) and Nivalin® for the treatment of Alzheimer's disease, poliomyelitis and other neurological diseases (Heinrich and Teoh, 2004). After its discovery in *Galanthus woronowii* by Proskurina and co-authors in 1955 (Proskurina *et al.,* 1955), the pharmacological properties of galanthamine soon attracted the attention of the pharmaceutical industry. It was first produced by Sopharma (Bulgaria) under the name of Nivalin® from *G. nivalis* in the early 1960s, but due to the small plant size and variability of galanthamine content, this species was soon replaced by other plant sources (Berkov *et al.,* 2009*b*).

The genus *Galanthus* (Snowdrop; Greek *gála* "milk", *ánthos* "flower") comprises about 19 species (World Checklist of Selected Plant Families), and to our knowledge 11 have been investigated for their alkaloid content. Although the genus has only been partially studied, phytochemical work has revealed an exceptional diversity of alkaloid structures, many of them reported for the first time and with still unknown bioactivity. The present article provides a brief overview of the phytochemical studies within the genus *Galanthus*.

2. Geographical distribution, taxonomical aspects and ecology of *Galanthus*

The genus *Galanthus* L. is distributed around Europe, Asia Minor and the Caucasus region. The limits of its area of distribution are the Pyrenees in the west, the Caucasus and Iran in the east, and Sicily, the Peloponnese and Lebanon in the south. The northern distribution limit cannot be assessed due to human introduction and cultivation (Davis, 1999). Some

species are widespread, while others are restricted to small areas. *G. nivalis,* for example, is native to a large area of Europe, stretching from the Pyrenees to Italy, Northern Greece, Ukraine, and European Turkey, while *G. trojanus* is a rare plant in the wild, found in a single location (an area less than 10 km²) in western Turkey (Davis and Ozhatay, 2001). Turkey is the country where most species (14) are geographically concentrated (Ünver, 2007).

All species of *Galanthus* are perennial, herbaceous plants that grow from bulbs. They have two or three linear leaves and an erect, leafless scape. The scape bears a pair of bract-like spathe valves at the top, from which emerges a solitary, bell-shaped white flower, held on a slender pedicel. The flower of *Galanthus* consists of six tepals, the outer three being larger and more convex than the inner series. The inner flower segments are marked with a green, or greenish-yellow, bridge-shaped mark at the tip of each tepal. The ovary is three-celled, ripening into a three-celled capsule. Each whitish seed has a small, fleshy tail (elaiosome) containing substances attractive to ants, which distribute the seeds (Davis, 1999). The genus *Galanthus* is closely related to the genus *Leucojum* L. but its plants can be easily distinguished because *Leucojum* has flowers with six equal tepals, from 2 to 6-7 flowers per scape and several leaves (Meerow and Snijman, 1998).

Species of the genus *Galanthus* L. (Amaryllidaceae) are difficult to distinguish and classify because of a lack of clearly definable morphological characteristics and a high level of variability. The search for other useful systematic information has produced little consensus in the enumeration of the species, divisions within the genus and relationships among their various components (Davis and Barnet, 1997). Besides morphological features, cariological (Kamari, 1981), anatomical (Davis and Barnet, 1997) and DNA (Zonneveld *et al.,* 2003) methods have been used to clarify the taxonomy of the genus.

It is generally accepted that the genus *Galanthus* comprises 19 species, 6 varieties and 2 natural interspecies hybrids (World Cheklist of Selected Plant Families):

1. *Galanthus alpinus* Sosn., Vestn. Tiflissk. Bot. Sada 19: 26 (1911).
   Galanthus alpinus var. alpinus.
   Galanthus alpinus var. bortkewitschianus (Koss) A.P.Davis, Kew Bull. 51: 750 (1996).
2. *Galanthus angustifolius* Koss, Bot. Mater. Gerb. Bot. Inst. Komarova Akad. Nauk S.S.S.R. 14: 134 (1951).
3. *Galanthus cilicicus* Baker, Gard. Chron. 1897(1): 214 (1897).
4. *Galanthus elwesii* Hook.f., Bot. Mag. 101: t. 6166 (1875), nom. cons.
   Galanthus elwesii var. elwesii
   Galanthus elwesii var. monostictus P.D.Sell in P.D. Sell & G.Murrell, Fl. Great Britain Ireland 5: 363 (1996).
5. *Galanthus fosteri* Baker, Gard. Chron., III, 5: 458 (1889).
6. *Galanthus gracilis* Celak., Sitzungsber. Königl. Böhm. Ges. Wiss., Math.-Naturwiss. Cl. 1891(1): 195 (1891).
7. *Galanthus ikariae* Baker, Gard. Chron. 1893(1): 506 (1893).
8. *Galanthus koenenianus* Lobin, C.D.Brickell & A.P.Davis, Kew Bull. 48: 161 (1993).
9. *Galanthus krasnovii* Khokhr., Byull. Moskovsk. Obshch. Isp. Prir., Otd. Biol., n.s., 68(4): 140 (1963).
10. *Galanthus lagodechianus* Kem.-Nath., Zametki Sist. Geogr. Rast. 13: 6 (1947).
11. *Galanthus nivalis* L., Sp. Pl.: 288 (1753).
12. *Galanthus peshmenii* A.P.Davis & C.D.Brickell, New Plantsman 1: 17 (1994).

13. *Galanthus platyphyllus* Traub & Moldenke, Herbertia 14: 110 (1948).
14. *Galanthus plicatus* M.Bieb., Fl. Taur.-Caucas., Suppl.: 225 (1819).
15. *Galanthus reginae-olgae* Orph., Atti Congr. Int. Bot. Firenze 1874: 214 (1876).
    Galanthus reginae-olgae subsp. reginae-olgae.
    Galanthus reginae-olgae subsp. vernalis Kamari, Bot. Jahrb. Syst. 103: 116 (1982).
16. *Galanthus rizehensis* Stern, Snowdrops & Snowflakes: 37 (1956).
17. *Galanthus transcaucasicus* Fomin, Opred. Rast. Kavk. Kryma 1: 281 (1909).
18. *Galanthus trojanus* A.P.Davis & Özhatay, Bot. J. Linn. Soc. 137: 409 (2001).
19. *Galanthus woronowii* Losinsk. in V.L.Komarov (ed.), Fl. URSS 4: 749 (1935).
20. *Galanthus* × *allenii* Baker, (*G. alpinus* × *G. woronowii*) Gard. Chron., III, 9: 298 (1891).
21. *Galanthus* × *valentinei* Beck, (*G. plicatus* × *G. nivalis*) Wiener Ill. Gart.-Zeitung 19: 57 (1894).

The habitats of *Galanthus* species are varied, ranging from undisturbed broad-leaved or coniferous woodlands of, for example oak (*Quercus* spp.), beech (*Fagus orientalis*), maple (*Acer* spp.), pines (*Pinus* spp.), Cilician fir (*Abies cilicia*), and cedar of Lebanon (*Cedrus libani*), woodland edges, river banks, scrub, grassland, amongst large rocks, and pockets of soil on rocks and cliff faces. *G. peshmenii* can sometimes be found only 10 m from the sea-shore on Kastellorhizo, a typical hot and dry Aegean island. In contrast, *G. platyphyllus* is a plant of the subalpine to alpine zone, and occurs mainly at altitudes of 2,000 - 2,700 m in alpine grasslands and meadows above the tree-line and at the edges of high-altitude woodlands (Davis, 1999). Typically, the *Galanthus* species are winter-to-spring flowering plants, but some species, like *G. cilicicus*, *G. peshmenii* and *G. reginae-olgae*, flower in autumn.

G. nivalis and *G. elwesii* are two of the best known and most frequently cultivated bulbous plants. Their popularity is due to their beauty, longevity and because they flower when little else is in season. A vast number of cultivars and clones are available (Davis, 1999). Huge numbers of wild-collected bulbs are exported annually from Turkey. In the early 1980s onwards this trade increased, with many millions of *G. elwesii* bulbs being exported via the Netherlands. The large numbers of *Galanthus* bulbs coming into commerce caused great concern because it was uncertain whether the collection of bulbs in such high numbers was sustainable. For this reason, *Galanthus* was placed on Appendix II of CITES in 1990. The wild harvesting of *G. elwesii* bulbs is now carefully controlled and monitored, and export quotas are set each year. Some snowdrop species are threatened in their wild habitats, and in most countries it is now illegal to collect bulbs from the wild. Under CITES regulations, international trade in any quantity of *Galanthus*, whether bulbs or plants, live or dead, is illegal without a CITES permit. This applies to hybrids and named cultivars as well as species. CITES does, however, allow a limited trade in wild-collected bulbs of just three species (*G. nivalis*, *G, elwesii* and *G. woronowii*) from Turkey.

3. Biosynthesis and structural types of Amaryllidaceae alkaloids

A particular characteristic of the Amaryllidaceae plant family is a consistent presence of an exclusive group of isoquinoline alkaloids, which have been isolated from plants of all the genera of this family. As a result of extensive phytochemical studies, over 500 alkaloids have been isolated from the amaryllidaceous plants (Zhong, 2005). The Amaryllidaceae type alkaloids have been structurally classified into nine main subgroups, namely lycorine, crinine, haemanthamine, narciclasine, galanthamine, tazettine, homolycorine, montanine

and norbelladine (Bastida *et al.*, 2006). In the genus *Galanthus*, however, two new structural subgroups, graciline and plicamine type alkaloids, have been found (Ünver, 2007). The following new subgroups have also been reported: specific augustamine-type structures in *Crinum kirkii* (Machocho *et al.*, 2004), a carboline alkaloid in *Hippeastrum vittatum* (Youssef, 2001), mesembrane (*Sceletium*)-type compounds in *Narcissus pallidulus* and *N. triandrus* (Bastida *et al.*, 2006), and phtalideisoquinoline-, benzyltetrahydroisoquinoline- and aporphine-type alkaloids in *G. trojanus* (Kaya *et al.*, 2004*b*, 2011). Mesembrane-type compounds are typical of the genus *Sceletium* of the Aizoaceae, while phtalideisoquinoline-, benzyltetrahydroisoquinoline- and aporphine-type alkaloids are found in the Papaveraceae, both families being dicotyledonous. Tyramine-type protoalkaloids, which are biosynthesized in Poaceae, Cactaceae, some algae and fungi, have also been found in *Leucojum* and *Galanthus* species (Berkov *et al.*, 2009*a*, 2011).

Amaryllidaceae alkaloids are formed biogenetically by intramolecular oxidative coupling of norbelladines derived from the amino acids L-phenylalanine and L-tyrosine (Bastida *et al.*, 2006). The key intermediate metabolite is *O*-methylnorbelladine. *Ortho-para´* phenol oxidative coupling of *O*-methylnorbelladine results in the formation of a lycorine-type skeleton, from which homolycorine-type compounds proceed. The galanthamine-type skeleton originates from *para-ortho´* phenol oxidative coupling. *Para-para´* phenol oxidative coupling leads to the formation of crinine, haemanthamine, tazettine, narciclasine and montanine structures (Bastida *et al.*, 2006). In the present article, for the structures reported by different authors we have adopted the numbering system according to Bastida *et al.*, (2006, Fig. 1).

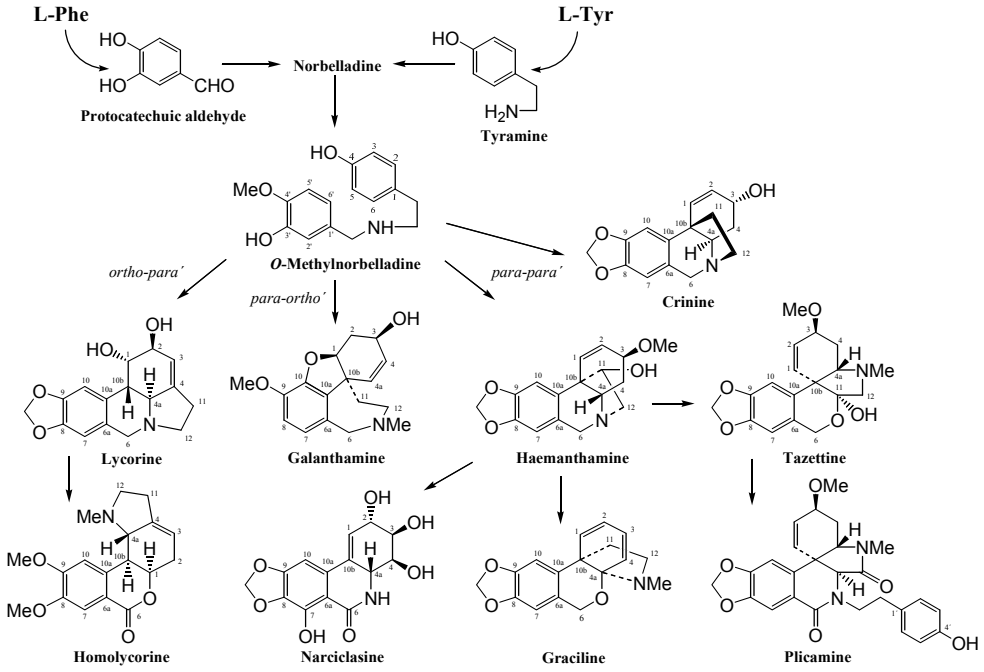


Fig. 1. Biosynthetic pathway of *Galanthus* alkaloids with representative compounds.

The biogenetic pathway of gracilines possibly originates from the 6-hydroxy derivatives of haemanthamine-type species (Noyan *et al.,* 1998), while plicamine-type alkaloids most probably proceed from tazettine-type compounds, considering their structural similarities (Ünver *et al.,* 1999*a*).

4. Distribution of alkaloids in the genus *Galanthus*

The phytochemical studies of the genus *Galanthus* started in the early fifties of the last century. Two of the first alkaloids reported for the genus were galanthine (Proskurina and Ordzhonikidze, 1953) and galanthamine (Proskurina *et al.,* 1955), which were isolated from *G. voronowii*. To the best of our knowledge, eleven species from the genus *Galanthus* have been phytochemically studied to date and ninety alkaloids have been found and classified in 11 structural types (Table 1, Fig.2).

Until recently, the distribution of alkaloids within the genus has been studied by classical phytochemical approaches. The collected biomass is extracted with alcohol, the neutral compounds removed at low pH and the alkaloids fractionated after basification of the extract. Individual alkaloids have been separated by column chromatography, preparative TLC, prep. HPLC, etc., and identified by spectroscopy, mainly 1D and 2D NMR. The GC-MS technique has proved to be very effective for rapid separation and identification of complex mixtures of Amaryllidaceae alkaloids obtained from low mass samples (Kreh *et al.,* 1995). Thus, the assessment of alkaloid distribution at species, populational and individual levels and the detection of new compounds have become much easier and faster (Berkov *et al.,* 2007*a*, 2009*c*, 2011).

An overview of the literature indicates that the genus *Galanthus* is a very rich source of novel compounds. Thirty-seven alkaloids (namely **12**, **22**, **26**, **29**, **34-39**, **46-49, 53, 56-58, 62, 67, 69-75, 77-86**) or *ca*. 40% of all identified compounds from the genus have been isolated for the first time from *Galanthus*. What is more, the biochemical evolution of the genus has led to the occurrence of two specific subgroups, namely graciline- and plicamine-type alkaloids.

The most studied species are *G. nivalis* and *G. elwesii*. Due to taxonomical changes over the years, the information on the alkaloids of *G. nivalis* is confusing. Thus, until 1966, only one *Galanthus* species had been recognized in Bulgaria, namely *G. nivalis* L. (Jordanov, 1964). This taxon was subsequently separated into *G. nivalis* L. and *G. elwesii* Hook. (Kozuharov, 1992). At present, it is unclear which plant species the alkaloids isolated in the early sixties from Bulgarian *G. nivalis* can be attributed to (Valkova, 1961; Bubeva-Ivanova and Pavlova, 1965). Kaya *et al.* (2004*b*) have reported five alkaloids for *G. nivalis* L. subsp. *silicicus* (Baker) Guttl.-Tann., a taxon regarded as a synonym of *G. silicicus* Baker by other authors (Davis and Barnett, 1997; Davis, 1999). A recent revelation has substantiated that *G. nivalis* subsp. *cilicicus* is identical to the newly introduced species, G*. trojanus* A. P. Davis and N. Özhatay, a plant species endemic to Northwestern Turkey (Davis and Özhatay, 2001).

Latvala *et al.,* (1995) isolated 18 alkaloids (6 new) from *G. elwesii* in addition to the already reported flexinine, elwesine, tazettine and haemanthamine (Boit and Ehmke, 1955; Boit and Döpke, 1961). The occurrence of elwesine (**26**) in the genus is particularly interesting. This compound displays a β-configuration of its 5,10b-ethano bridge, which is typical of the South African representatives of the family (Viladomat *et al.,* 1997). Although widely

accepted that *G. nivalis* was the industrial source of galanthamine (in Bulgaria) during the 1960s (Heinrich and Teoh, 2004), later studies on 32 Bulgarian populations of *G. nivalis* and *G. elwesii* indicate that the distribution of this important compound is limited to a few populations of *G. elwesii*, while just one population of *G. nivalis* has been found to contain galanthamine and only as a minor alkaloid (Sidjimova *et al.,* 2003; Berkov *et al.,* 2011). These studies, however, have also shown a great intra-species diversity of alkaloid synthesis in *G. nivalis* and *G. elwesii.* The populations displayed between 6 and 31 alkaloids in their alkaloid patterns and about 70 compounds have been detected in total. Many of them were left unidentified due to the lack of reference spectra, possibly indicating new structures. This biochemical diversity has led to the isolation of eight more new alkaloids from these well-studied species, after the collection of plant material from populations proven by GC-MS to be a rich source of unknown compounds (Berkov *et al.,* 2007*a*, 2009*c*). Interestingly, many of the *G. elwesii* populations have accumulated the tyramine-type protoalkaloids as major compounds (up to 99 % of all alkaloids). In addition to the tyramine chemotype, homolycorine, lycorine haemanthamine and galanthamine chemotypes have also been found in the studied populations of *G. elwesii*. A galanthamine chemotype population was also found for *G. nivalis*, but in contrast with *G. elwesii*, this *G. nivalis* population accumulated the 4,4a-dihydrogenated derivatives of galanthamine (**12**), lycoramine (**16)** and its isomer (**17**) (Berkov *et al.,* 2011).

As well as a high level of alkaloid diversity and the existence of different chemotypes among the species populations, *G. elwesii* and *G. nivalis* have also shown some important differences in their alkaloid patterns, at least in the studied Bulgarian populations. A study of sympatric populations, and 32 populations from both species showed that the alkaloid pattern of *G. nivalis* is dominated by compounds coming from a *para–para´* oxidative coupling of *O*-methylnorbelladine (haemanthamine- and tazettine-type alkaloids, Fig. 1). The conjugated and free lycorine-type alkaloids proceeding from an *ortho–para´* oxidative coupling were relatively less abundant. Homolycorine-type alkaloids were not detected in this plant species. In contrast to *G. nivalis*, the alkaloid pattern of *G. elwesii* was dominated mainly by compounds coming from *ortho–para´* oxidative coupling: free lycorine- and homolycorine-type alkaloids. The synthesis of *para–para´* oxidative products in *G. elwesii* is relatively weak (only haemanthamine- and no tazettine-type compounds, Berkov *et al.,* 2008, 2011). In total, 46 and 38 alkaloids have been identified in *G. elwesii* and *G. nivalis,* respectively.

In a study on sympatric *G. nivalis* and *G. elwesii* populations, it was found that the organs of the plants presented different alkaloid patterns (Berkov *et al.,* 2008). Thus, the predominant alkaloids of *G. nivalis* roots were found to belong to the lycorine and tazettine structural types, bulbs were dominated by tazettine, leaves by lycorine and flowers by haemanthamine-type alkaloids. The predominant alkaloids in *G. elwesii* roots, bulbs and leaves were those of the homolycorine type, whereas the flowers accumulated mainly tyramine-type compounds. To the best of our knowledge, no studies of the dynamics of the alkaloid patterns during ontogenesis have been reported for either of these two species or any other *Galanthus* species. Such studies, however, may contribute to the understanding of the chemoecological role of the alkaloids in the genus *Galanthus* and the Amaryllidaceae as a whole. A remarkably high number of alkaloids conjugated with 3-hydroxybutyryl moieties occur in *G. nivalis*. Co-existence of free and conjugated alkaloids in the plant implies that the latter may have a chemoecological role. Such conjugated alkaloids have rarely been reported for Amaryllidaceae plants.

Compound	*G. elwesii*	*G. nivalis*	*G. plicatus*	*G. gracilis*	*G. woronowii*	*G. caucasicus*	*G. ikariae*	*G. krasnovii*	*G. reginae-olgae*	*G. trojanus*	*G. rizehensis*
I. Tyramine type											
Tyramine (**1**)	+[1]	+[1]								+[25]	
Methyltyramine (**2**)	+[1]	+[1]									
Hordenine (**3**)	+[1,2]	+[1]	+[10]								
N-feruloyltyramine (**4**)	+[2]										
II. Norbelladine											
O-Methylnorbelladine (**5**)										+[25]	
III. Narciclasine type											
Ismine (**6**)	+[1]	+[1,7]	+[11]								
N-Formylismine (**7**)			+[12]								
Trisphaeridine (**8**)	+[1]	+[1]	+[13]								
5,6-Dihydrobicolorine (**9**)			+[13]	+[15]							
Arolycoricidine (**10**)										+[25]	+[27]
Narciprimine (**11**)											+[27]
IV. Galanthamine type											
Galanthamine (**12**)	+[1,2]	+[1]			+[19]	+[22]	+[4]	+[23]	+[24]		
3-Epigalanthamine (**13**)	+[1]										
Narwedine (**14**)	+[1,2]										
N-Demethylgalanthamine (**15**)	+[1,2]										
Lycoramine (**16**)	+[1]	+[1]									
3-Epilycoramine (**17**)	+[1]	+[1]									
Sanguinine (**18**)	+[2]										
N-Formylnorlgalanthamine (**19**)	+[1]										
Leucotamine (**20**)	+[1,2]										
O-Methylleucotamine (**21**)	+[2]										
Nivalidine (**22**)	+[3]	+[8]									
V. Haemanthamine type											
Buphanisine (**23**)	+[1,4]										
Vittatine/crinine (**24**)	+[1,5]								+[24]	+[26]	
Flexinine (**25**)	+[6]										
Elwesine (**26**)	+[6]										
Hamayne (**27**, 3-Epihydroxybulbispermine)	+[7]	+[7,9]					+[4]				
11-Hydroxyvittatine (**28**)	+[2]		+[10]							+[26]	
11-Hydroxyvittatine *N*-oxide (**29**)										+[25]	
Maritidine (**30**)	+[1]										
8-*O*-Demethylmaritidine (**31**)										+[25]	
Narcidine (**32**)										+[25]	
Haemanthamine (**33**)	+[1]	+[1]								+[25]	
11-*O*-(3′-Hydroxybutanoyl)hamayne (**34**)	+[1,7]	+[9]									
3,11-*O*-(3′,3′′-Dihydroxybutanoyl)hamayne (**35**)		+[9]									
3-*O*-(2′′-Butenoyl)-11-*O*-(3′-hydroxybutanoyl)hamayne (**36**)		+[9]									
3,11,3′-*O*-(3′, 3′′, 3′′′- Trihydroxybutanoyl)-hamayne (**37**)		+[9]									
3,3′-*O*-(3′,3′′-Dihydroxybutanoyl)hamayne (**38**)		+[7]									

Compound	*G. elwesii*	*G. nivalis*	*G. plicatus*	*G. gracilis*	*G. woronowii*	*G. caucasicus*	*G. ikariae*	*G. krasnovii*	*G. reginae-olgae*	*G. trojanus*	*G. rizehensis*
11,3′-*O*-(3′,3′′-Dihydroxybutanoyl)hamayne (**39**)		+[7]									
VI. Tazettine type											
11-Deoxytazettine (**40**)	+[1]	+[1]									
6-*O*-Methylpretazettine (**41**)	+[1]	+[1]									
Tazettine (**42**)	+[1]	+[7]	+[11]	+[15]	+[20]	+[22]	+[4]		+[24]		
Criwelline (**43**)		+[6]									
Macronine (**44**)		+[1]									
Epimacronine (**45**)		+[7]	+[11]	+[15]							
3-*O*-Demethyl-3-epimacronine (**46**)			+[13]								
3-*O*-Demethylmacronine (**47**)				+[13]							
3-*O*-(3′-Hydroxybutanoyl)tazettinol (**48**)			+[12]								
Isotazettinol (**49**)				+[13]							
VII. Lycorine type											
Anhydrolycorine (**50**)	+[1]	+[1]									
11,12-Dehydroanhydrolycorine (**51**)	+[1]	+[1]									
Caranine (**52**)		+[5]									
Galanthine (**53**)	+[1,2]	+[1]			+[21]	+[22]					
Lycorine (**54**)	+[1,2]	+[1,7]	+[14]	+[14]	+[21]	+[22]			+[24]	+[26]	
Incartine (**55**)	+[1]	+[1]									
2-*O*-(3′-Hydroxybutanoyl)lycorine (**56**)	+[1,7]	+[1]									
2?-*O*-(3′-Hydroxybutanoyl)lycorine isomer (**57**)		+[5]									
2-*O*-(3′-Acetoxybutanoyl)lycorine (**58**)		+[1,9]									
Ungeremine (**59**)		+[9]									
8-*O*-Demethylvasconine (**60**)		+[7]									
Nartazine (**61**)		+[6]									
8-*O*-Methyldihydrosternbergine *N*-oxide (**62**)										+[25]	
Dihydrolycorine (**63**)										+[25]	
VIII. Homolycorine type											
Homolycorine (**64**)	+[1]										
8-*O*-Demethylhomolycorine (**65**)	+[1,2]		+[15]	+[15]		+[22]	+[4]				
Masonine (**66**)	+[5]	+[6]									
2-Methoxy-8-*O*-demethylhomolycorine (**67**)	+[1,2]										
Hippeastrine (**68**)	+[1]	+[8]									
Galwesine (**69**)	+[1,2]										
8-*O*-Demethylgalwesine (**70**)	+[2]										
8-*O*-Demethyl-10b-hydroxygalwesine (**71**)	+[2]										
10b-Hydroxygalwesine (**72**)	+[2]										
Galasine (**73**)	+[2]										
2α-Hydroxyhomolycorine (**74**)	+[1]										
Galanthindole (**75**)			+[10]								
Neronine (**76**)									+[24]		
Galanthusine (**77**)						+[22]					
IX. Graciline type											
Graciline (**78**)				+[16]							
11-Acetoxygraciline (**79**)			+[16]								

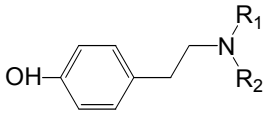
Compound	*G. elwesii*	*G. nivalis*	*G. plicatus*	*G. gracilis*	*G. woronowii*	*G. caucasicus*	*G. ikariae*	*G. krasnovii*	*G. reginae-olgae*	*G. trojanus*	*G. rizehensis*
3,4-Dihydro-3-hydroxygraciline (**80**)				+[12]							
3-Epi-3,4-dihydro-3-hydroxygraciline (**81**)				+[12]							
Digracine (**82**)				+[16]							
Gracilamine (**83**)				+[18]							
X. Plicamine type											
Plicamine (**84**)			+[17]								
Plicane (**85**)			+[12]								
Secoplicamine (**86**)			+[17]								
XI. Other											
Bulbocapnine (**87**)										+[26]	
Capnoidine (**88**)										+[26]	
Stylopine (**89**)										+[25]	
Protopine (**90**)										+[25]	

1) Berkov *et al.,* (2011); 2) Latvala *et al.,* (1995); 3) Bubeva-Ivanova and Pavlova (1965); 4) Sener *et al.,* (1998); 5) Berkov *et al.,* (2008); 6) Wildman, (1968); 7) Berkov *et al.,* (2009c); 8) Kalashnikov (1970); 9) Berkov *et al.,* (2007a); 10) Ünver *et al.,* (2003); 11) Akıneri and Günes (1998); 12) Ünver *et al.,* (2001); 13) Ünver *et al.,* (1999a); 14) Kaya *et al.,* (2004*a*); 15) Noyan (1999); 16) Noyan *et al.,* (1998); 17) Ünver *et al.,* (1999b); 18) Ünver and Kaya, (2005); 19) Proskurina *et al.,* (1955); 20) Yakovleva (1963); 21) Proskurina Ordzhonikidze (1953); 22) Tsakadze *et al.,* (1979); 23) Asoeva *et al.,* (1968); 24) Conforti *et al.,* (2010); 25) Kaya *et al.,* (2011); 26) Kaya *et al.,* (2004*b*); 27) Bozkurt *et al.,* (2010).

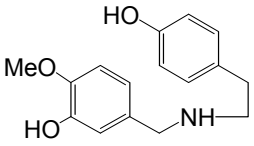
Table 1. Alkaloids reported in the genus *Galanthus*

Another two phytochemically interesting species from which a number of new alkaloids have been isolated are *G. gracilis* and *G. plicatus*. Phytochemical studies on *G. gracilis* resulted in the isolation of three novel monomeric alkaloids (**78**, **80**, **81**) and a dimeric compound (**82**) bearing a 10b,4a-ethanoiminodibenzo[b,d]pyrane skeleton, which represents a new subgroup of Amaryllidaceae alkaloids named gracilines (Fig. 1, Noyan *et al.,* 1998; Ünver *et al.,* 2001). An unusual pentacyclic dinitrogenous alkaloid, gracilamine (**83**), was also isolated from this species (Ünver and Kaya, 2005). Another new graciline-type alkaloid (**79**, Noyan *et al.,* 1998) has been isolated from *G. plicatus*, together with compounds **84**-**86** (Ünver et al., 1999*a*, 2001), representing a new subgroup of the Amaryllidaceae alkaloids where the oxygen atom at position 5 of a tazettine molecule is replaced by a nitrogen atom, conjugated with a 4-hydroxyphenethyl moiety. This new subgroup, named after the lead compound plicamine (**84**), was found later in another amaryllidaceous plant, *Cyrtanthus obliquus* (Brine *et al.,* 2002). Apart from plicamines, four new tazettine-type alkaloids (**46**-**49**) and a compound with a nonfused indole ring (**75**) have also been isolated in *G. plicatus* (Ünver *et al.,* 1999*b*, 2003). In total, 17 and 12 alkaloids have been reported for *G. plicatus* and *G. gracilis,* respectively (Table 1).

The other *Galanthus* species are relatively less studied. Four known alkaloids (**12**, **42**, **53**, and **54**), including galanthamine, have been reported for *G. woronovii* (Proskurina *et al.,* 1955; Proskurina and Ordzhonikidze, 1953; Yakovleva, 1963). A new compound, galanthusine (**78**),



1 R_1=R_2=H
2 R_1=H, R_2=Me
3 R_1=R_2=Me
4 R_1=H, R_2= feruloyl



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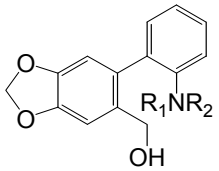
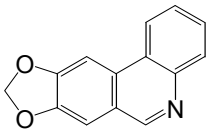


6 R_1=H, R_2=Me
7 R_1=CHO, R_2=Me



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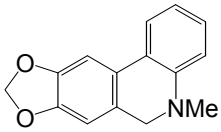

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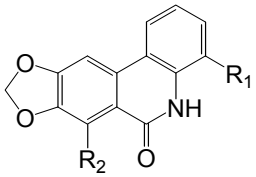
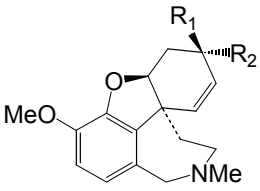
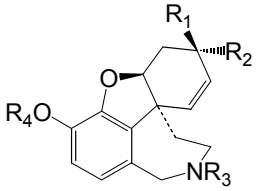


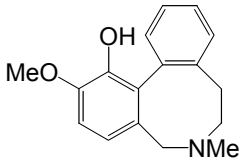
10 R_1=OH, R_2=H
11 R_1=R_2=OH



16 R_1=OH, R_2=H
17 R_1=H, R_2=OH



12 R_1=OH, R_2=H, R_3=R_4=Me
13 R_1=H, R_2=OH, R_3=R_4=Me
14 R_1+R_2=O, R_3=R_4=Me
15 R_1=OH, R_2=R_3=H, R_4=Me
18 R_1=OH, R_2=H, R_3=Me, R_4=H
19 R_1=OH, R_2=H, R_3=CHO, R_4=Me
20 R_1=OCOCH$_2$CHOHMe, R_2=H, R_3=Me, R_4=H
21 R_1=OCOCH$_2$CHOHMe, R_2=H, R_3=R_4=Me



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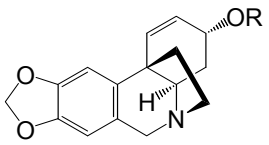
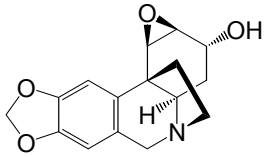


23 R=Me
24 R=H (crinine)



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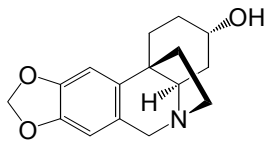

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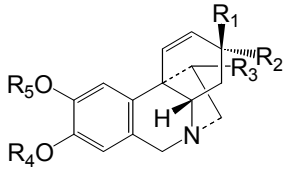
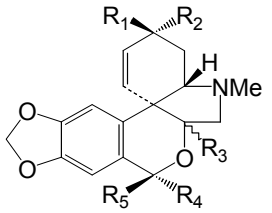
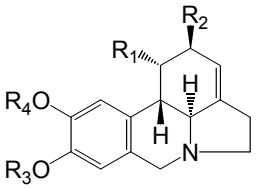


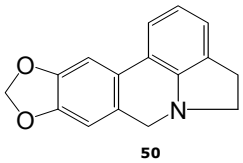
24 R_1=OH, R_2=R_3=H, R_4+R_5=CH$_2$(vittatine)
27 R_1=H, R_2=R_3=OH, R_4+R_5=CH$_2$
28 R_1=OH, R_2=H, R_3=OH, R_4+R_5=CH$_2$
30 R_1=OH, R_2=R_3=H, R_4=R_5=Me
31 R_1=OH, R_2=R_3=R_4=H, R_5=Me
32 R_1=OMe, R_2=H, R_3=OH, R_4=H, R_5=Me
33 R_1=OMe, R_2=H, R_3=OH, R_4+R_5=CH$_2$
34 R_1=H, R_2=OH, R_3=OCOCH$_2$CHOHMe, R_4+R_5=CH$_2$
35 R_1=H, R_2=R_3=OCOCH$_2$CHOHMe, R_4+R_5=CH$_2$
36 R_1=H, R_2=OCOCH=CHMe, R_3=OCOCH$_2$CHOHMe, R_4+R_5=CH$_2$
37 R_1=H, R_2=OCOCH$_2$CH(Me)OCOCH$_2$CHOHMe, R_3=OCOCH$_2$CHOHMe, R_4+R_5=CH$_2$
38 R_1=H, R_2=OCOCH$_2$CH(Me)OCOCH$_2$CHOHMe, R_3=OH, R_4+R_5=CH$_2$
39 R_1=H, R_2=OH, R_3=OCOCH$_2$CH(Me)OCOCH$_2$CHOHMe, R_4+R_5=CH$_2$



40 R_1=OMe, R_2=H, R_3=αH, R_4=R_5=H
41 R_1=OMe, R_2=H, R_3=βH, R_4=H, R_5=OMe
42 R_1=OMe, R_2=H, R_3=αOH, R_4=R_5
43 R_1=H, R_2=OMe, R_3=αOH, R_4=R_5=H
44 R_1=H, R_2=OMe, R_3=βH, R_4+R_5=O
45 R_1=OMe, R_2=H, R_3=βH, R_4+R_5=O
46 R_1=OH, R_2=H, R_3=βH, R_4+R_5=O
47 R_1=H, R_2=OH, R_3=βH, R_4+R_5=O
48 R_1=$OCOCH_2CHOHMe$, R_2=H, R_3=αOH, R_4=R_5=H
49 R_1=H, R_2=OH, R_3=αOH, R_4=R_5=H



52 R_1=OH, R_2=H, R_3+R_4=CH_2
53 R_1=OH, R_2=OMe, R_3=R_4=Me
54 R_1=R_2=OH, R_3+R_4=CH_2
56 R_1=OH, R_2=$OCOCH_2CHOHMe$, R_3+R_4=CH_2
58 R_1=OH, R_2=$OCOCH_2CHOAcMe$, R_3+R_4=CH_2



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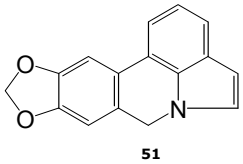

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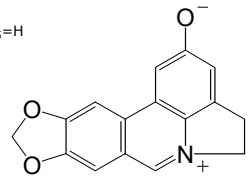

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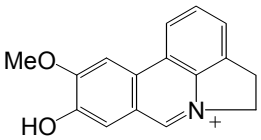

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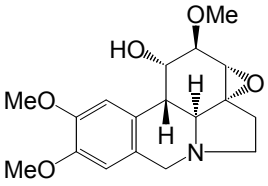

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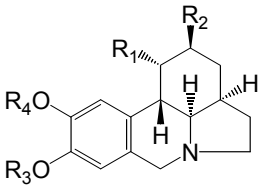
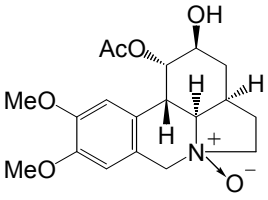


61 R_1=R_2=OAc, R_3+R_4=CH_2
63 R_1=R_2=OH, R_3+R_4=CH_2



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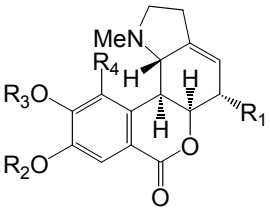
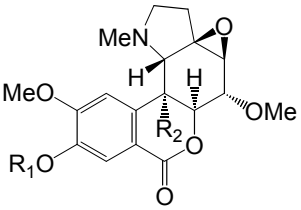
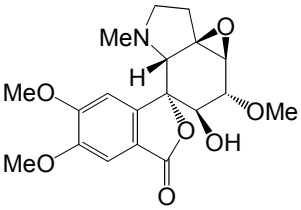


64 R_1=H, R_2=R_3=Me, R_4=H
65 R_1=R_2=H, R_3=Me, R_4=H
66 R_1=H, R_2+R_3=CH_2,R_4=H
67 R_1=OMe, R_2=H, R_3=Me, R_4=H
68 R_1=OH, R_2+R_3=CH_2, R_4=H
74 R_1=OH, R_2=R_3=Me, R_4=H
76 R_1=OH, R_2+R_3=CH_2, R_4=OMe



69 R_1=Me, R_2=H
70 R_1=R_2=H
71 R_1=H, R_2=OH
72 R_1=Me, R_2=OH



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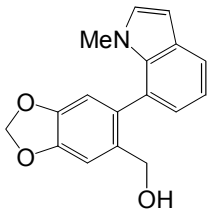

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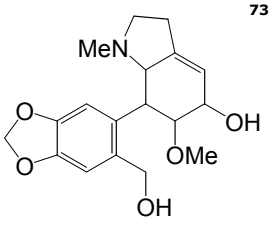

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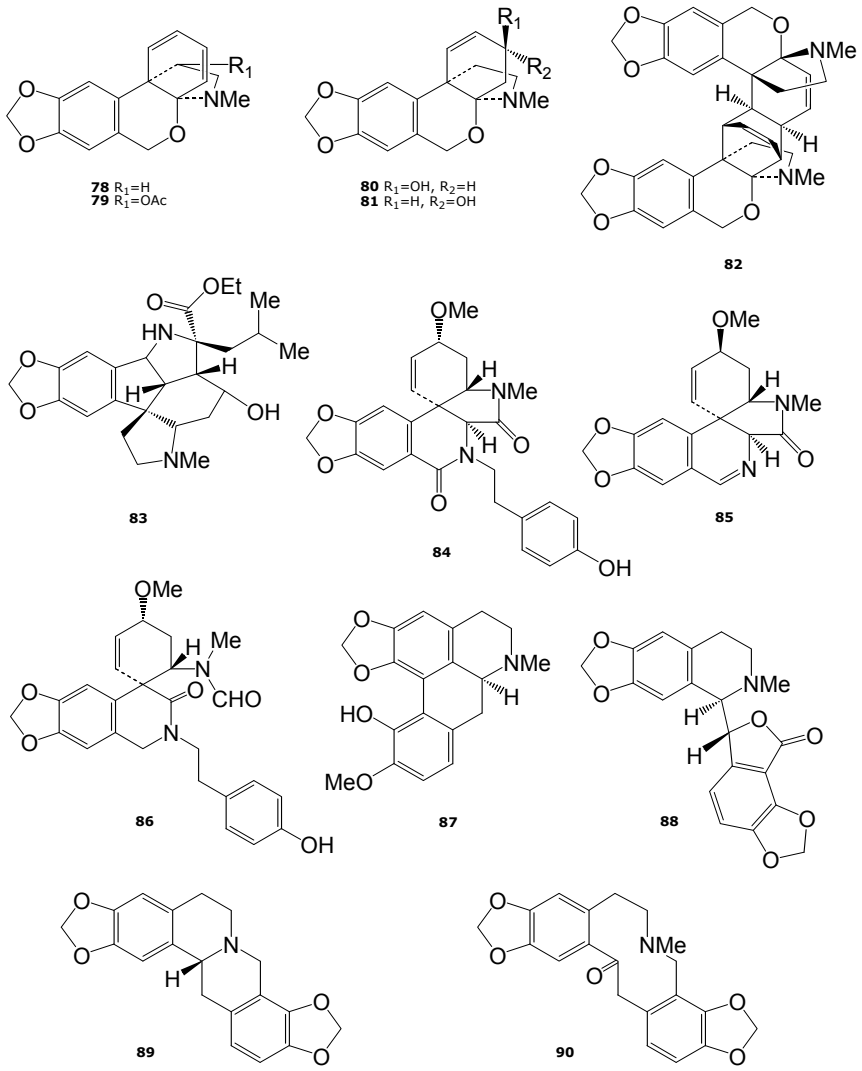

Fig. 2. Structures of the alkaloids found in the genus *Galanthus*

has been found in *G. caucasicus*, along with five known alkaloids (**12**, **42**, **53**, **54**, and **65;** Tsakadze *et al.*, 1979). Only galanthamine has been reported for *G. krasnovii* (Asoeva *et al.*, 1968). *G. ikariae* has furnished four known alkaloids (**12**, **27, 42**, and **65;** Sener *et al.*, 1998). A recent GC-MS report on *G. reginae-olgae* resulted in the identification of compounds **12**, **24**, **42**, and **66** (Conforti, *et al.*, 2010)**.** The presence of crinine (with the 5,10b-ethano bridge at the β-position) in this species, as well as in *G. elwesii*, as reported in our earlier GC-MS studies (Berkov *et al.*, 2004), is debatable because the absolute configuration of the 5,10b-ethano bridge cannot be established by GC-MS alone. Later phytochemical studies on *Galanthus* resulted in the isolation of crinane-3-ol derivatives with a *a*-configuration of their 5,10b-

ethano bridges, including vittatine (Kaya *et al.,* 2004*b*), which is the optical isomer of crinine, 11-hydroxyvittatine (Latvala *et al.,* 1995; Kaya *et al.,* 2004*b*); Ünver *et al.,* 2003) and hamayne (Berkov *et al.,* 2007*a*; 2009c). On the other hand, elwesine (**26**, 2,3-dihydrocrinine) and buphanisine (**23**) display a *β*-configuration of the 5,10b-ethano bridge (Wildman, 1968, Capo and Saa, 1989). Recently initiated phytochemical studies on *G. rizehensis* (Bozkurt *et al.,* 2010) have identified two narciclasine-type compounds, arolycoricidine (**10**) and narciprimine (**11**). An interesting example of biochemical convergence is the presence of bulbocapnine (**87**), capnoidine (**88**), stilopine (**89**) and protopine (**90**) in *G. trojanus* (studied as *G. nivalis* subsp. *silicicus* (Baker) Gottlieb-Tannenhain). Two new alkaloids, the *N*-oxides of 9-*O*-methyldihydrosternbergine (**62**) and 11-hydroxyvittatine (**29**), were also isolated, along with several known alkaloids **2, 5**, **10, 24, 28, 29, 31-33, 54, 62** and **63** (Kaya *et al.,* 2004*b*; Ünver 2007). Compounds **84-90** are benzyltetrahydroisoquinoline-, aporphine- and phthalide-type isoquinolines, found in dicotyledonous plants of the Fumariaceae and Papaveraceae families (Kametani and Honda 1985; MacLean, 1985).

5. Biological and pharmacological activities of the alkaloid found in *Galanthus*

Alkaloids are important for the well-being of the producing organism. One of their main functions is to provide a chemical defence against herbivores, predators or microorganisms (Wink, 2008). The biological roles of the numerous alkaloids found in the genus *Galanthus* remain largely unknown and only a few have been studied for their pharmacological activities.

Galanthamine-type

The most studied *Galanthus* alkaloid, galanthamine (**12**), is a long-acting, selective, reversible and competitive inhibitor of acetylcholinesterase (AChE) and an allosteric modulator of the neuronal nicotinic receptor for acetylcholine. AChE is responsible for the degradation of acetylcholine at the neuromuscular junction, in peripheral and central cholinergic synapses. Galanthamine has the ability to cross the blood-brain barrier and to act within the central nervous system (Bastida *et al.,* 2006; Heinrich and Teoh, 2006). Owing to its AChE inhibitory activity, galanthamine is used and marketed under the name of Razadine®, formerly Reminyl®, in the USA, for the treatment of certain stages of Alzheimer's Disease (AD). According to data presented by the Alzheimer's Association in 2007, the prevalence of Alzheimer's disease will quadruple by 2050. Galanthamine hydrobromide has superior pharmacological profiles and higher tolerance as compared to the original AChE inhibitors, physostigmine or tacrine (Grutzendler and Morris, 2001).

Epigalanthamine (**13**), with a hydroxylgroup at *a*-position, and narwedine (**14**), with a keto group at C3, are also active AChE inhibitors, but about 130-times less than galanthamine (Thomsen *et al.,* 1998). The loss of the methyl group at the *N* atom, as in *N*-demethylgalanthamine (**15**), decreases the activity 10-fold. On the other hand, sanguinine (**18**), which has a hydroxylgroup at C9 instead of a methoxyl group, is *ca*. 10 times more active than galanthamine. Hydrogenation of the C4-C4a, as in lycoramine (**16**), results in a complete loss of AChE inhibitory activity (López *et al.,* 2002). It is suggested that in plants AChE inhibitors act as pesticides. The synthetic pesticides such as phosphoorganic compounds are non-reversible AChE inhibitors (Hougton *et al.,* 2006).

Tyramine-type

Compounds **1**-**4** can be attributed to the group of the phenolic amines that impact the hypothalamic-pituitary-adrenal axis (Vera-Avila *et al.,* 1996) due to their structural similarity to adrenaline (epinefrine). The consequent release of adrenocorticotropic hormone and cortisol results in sympathomimetic action with toxic effects in animals (Clement *et al.,* 1998). Hordenine (**3**) possesses diuretic, disinfectant and antihypotensive properties, and acts as a feeding repellent against grasshoppers (Dictionary of Natural Products).

Narciclasine-type

Trisphaeridine (**8**) has a high retroviral activity but a low therapeutic index. Ismine (**6**) shows a significant hypotensive effect on rats and cytotoxicity against Molt 4 lymphoid and LMTK fibroblastic cell lines (Bastida *et al.,* 2006). A recent study revealed that arolycoricidine (**10**) and narciprimine (**11**) were considerably effective in DNA topoisomerase reactions in a dose-dependent manner. Topoisomerase-interfering ability of these alkaloids partially correlated with cytostatic assays, using HeLa (cervix adenocarcinoma), MCF7 (breast adenocarcinoma) and A431 (skin epidermoid carcinoma) cells (Bozkurt *et al.,* 2010). Arolycoricidine showed inhibitory activity against African trypanosomes, (*Trypanosoma brucei rhodesiense)* at micromolar levels (Kaya *et al.,* 2011).

Haemanthamine type

Haemanthamine **(33)** has been shown to be a potent inducer of apoptosis in tumour cells at micromolar concentrations (McNulty *et al.,* 2007). This compound also possesses antimalarial activity against strains of chloroquine-sensitive *Plasmodium falciparum,* hypotensive effects and antiretroviral activity (Bastida *et al.,* 2006; Kaya *et al.,* 2011). Vittatine (**24**) and maritidine (**30)** have shown cytotoxic activity against HT29 colon adenocarcinoma, lung carcinoma and RXF393 renal cell carcinoma (Bastida *et al.,* 2006; Silva *et al.,* 2008). Antibacterial activity against Gram-positive *Staphylococcus aureus* and Gram-negative *E. coli* have been reported for vittatine (**24**) and 11-hydroxyvittatine (**28**) (Kornienko and Evidente, 2008). Data about the bioactivity of recently isolated compounds **34**-**39** is still lacking.

Tazettine-type

Moderate cytotoxic activity has been reported for tazettine (**42**), and epimacronine (**45**) (Weniger *et al.,* 1995). Tazettine, however, is an isolation artefact of chemically labile pretazettine, which is indeed present in plants. This compound has shown remarkable cytotoxicity against a number of tumor cell lines, being therapeutically effective against advanced Rauscher leucemia, Ehrlich ascites carcinoma, spontaneous AKR lymphocytic leukaemia, and Lewis lung carcinoma (Bastida *et al.,* 2006).

Lycorine-type

Lycorine (**54**), one of the most frequently occurring alkaloids in Amaryllidaceae plants, possesses a vast array of biological properties. It has been reported as a potent inhibitor of ascorbic acid synthesis, cell growth and division and organogenesis in higher plants, algae, and yeasts, inhibiting the cell cycle during the interphase (Bastida *et al.,* 2006). Additionally, lycorine exhibits antiviral (against poliovirus, vaccine smallpox virus and SARS-associated coronavirus), antifungal (*Saccharomyces cerevisiae, Candida albicans*), and anti-protozoan (*Trypanosoma brucei*) activities (McNulty *et al.,* 2009), and is more potent than indomethacin

as an anti-inflammatory agent (Citoglu *et al.,* 1998). Lycorine has also been shown to have insect antifeedant activity (Evidente *et al.,* 1986). As a potential chemotherapeutic drug, this compound has been studied as an antiproliferative agent against a number of cancer cell lines (Likhitwitayawuid *et al.,* 1993). The *in vitro* mode of action in a HL-60 leukemia cell line model is associated with suppressing tumor cell growth and reducing cell survival via cell cycle arrest and induction of apoptosis (Liu *et al.,* 2004). Further investigation showed that it is able to decrease tumor cell growth and increase survival rates with no observable adverse effects in treated animals (Liu *et al.,* 2007), thus being a good candidate for a therapeutic agent against leukaemia (Liu *et al.,* 2009).

Anhydrolycorine (**50**), in contrast to caranine (**52**), has shown a higher ability to inhibit ascorbic acid synthesis than lycorine (Evidente *et al.,* 1986). Analgesic and hypotensive effects have been reported for caranine and galanthine (**53**), the latter also being active against *Tripanosoma brucei rhodesiense* and *Plasmodium falciparum*. Some lycorine-type compounds such as caranine and ungeremine (**59**) have shown acetylcholinesterase inhibitory activity (Bastida *et al.,* 2006). Incartine was found to be cytotoxic and to weakly inhibit AChE (Berkov *et al.,* 2007).

Homolycorine-type

Cytotoxic activity has been demonstrated for homolycorine (**64**), 8-*O*-demethylhomolycorine (**65**), and hippeastrine (**68**). Homolycorine has shown high antiretroviral activity, while hippeastrine is active against *Herpes simplex* type 1. Homolycorine and 8-*O*-demethylhomolycorine have a hypotensive effect on normotensive rats. In addition, hippeastrine shows antifungal activity against *Candida albicans* and also possesses a weak insect antifeedant activity (Bastida *et al.,* 2006).

The bioactivity of the plicamine- and graciline-type alkaloids is largely unknown. Bulbocapline (**87**) and protopine (**90**) have been shown to act as inhibitors of acetylcholinesterase (Kim et al., 1999; Adsersen *et al.,* 2007) and dopamine biosynthesis (Shin *et al.,* 1998). Stylopine (**89**) suppresses the NO and PGE2 production in macrophages by inhibiting iNOS and COX-2 expression (Jang et al., 2004).

6. Conclusions

Although only some of the species of this phytochemically interesting genus have been studied, it has yielded a considerable number of new structures. Moreover, the high level of intraspecies diversity indicates that new compounds can be expected from already studied taxons. Only a few of the new alkaloids have been screened for their bio- and pharmacological activities, probably due to the small amounts isolated. Consequently, their synthesis or *in silico* studies will facilitate further bioactivity assessment.

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12

Silymarin, Natural Flavonolignans from Milk Thistle

Sameh AbouZid
Faculty of Pharmacy, University of Beni-Sueif, Egypt

1. Introduction

Plants are a valuable source of pharmaceuticals, food ingredients, agrochemicals, insecticides, flavors and pigments. These compounds are called secondary metabolites. These are compounds with a restricted occurrence in taxonomic groups that are not essential for an organism to live but play a role in the interaction of the organism with its environment, ensuring the survival of the organism in its ecosystem (Verpoorte and Alfermann, 2000).

Milk thistle or St. Mary's thistle [*Silybum marianum* (L.) Gaertn. (Syn. *Cardus marianum*) Asteraceae] is an annual or biennial herb. The plant is native to the Mediterranean and North African regions (Boulos, 2000). It grows wild throughout Europe, North Africa, Americas and Australia (Hamid et al., 1983). The plant reaches to heights 10 feet. It has a stem of 20-150 cm high, erect, ridged and branched in the upper part. A distinguishing characteristic of milk thistle is the white patches found along the veins of the dark green leaves (Fig. 1). The broad leaves are deeply lobed, 50 cm long and 25 cm wide. The leaf margins are yellow and tipped with woody spines (3-12 mm long). The leaves are alternate and clasping to the stem. Each stem ends with solitary composite flower heads, about 2 inches in diameter, consisting of purple disc florets. The flower heads of milk thistle differ from other thistles by the presence of leathery bracts that are also tipped with stiff spines. The fruits (Fig. 2) are hard skimmed achenes, 6-8 mm long flat, smooth and shiny dark brown in color. The fruits yield 1.5-3% of an isomeric mixture of flavonolignans collectively known as silymarin (Morazzoni and Bombardelli, 1995). Silymarin accumulates mainly in the external cover of the fruits of *S. marianum* (Madrid and Corchete, 2010).

2. Chemistry of flavonolignans

The principal components of silymarin are silybin A, silybin B, isosilybin A, isosilybin B, silychristin A, silychristin B and silydianin (Fig. 3). The first six compounds exist as equimolar mixtures as trans diastereoisomers. These diastereomers have very similar ^{1}H and ^{13}C NMR spectra and have no characteristic signals for facile identification of the individual isomers (Lee and Liu, 2003). A number of other chemically related compounds have been found in the fruits including dehydrosilybin, desoxysilychristin, desoxysilydianin, silandrin, silybinome, silyhermin and neosilymermin. The common feature of these

Fig. 1. Milk thistle with white patches along the veins of dark green leaves.

Fig. 2. *Silybum marianum* fruits.

compounds is a flavonolignan skeleton ($C_{25}H_{22}O_{10}$, mol wt 482). Basically, flavonolignan nucleus consists of the dihydroflavanol taxifolin linked to coniferyl alcohol moiety through an oxeran ring. The oxeran ring is responsible for the biological activity of silymarin, and opening of this ring results in loss of activity. Only silybins and isosilybins contain the 1,4-dioxane ring system in their structure. Silybin and isosilybin have the same trans conformation of C-2, C-3 and C-7′, C-8′. Silybin is considered the major and most active component in silymarin (Ligeret et al., 2008; Kim et al., 2009). The chemical structure of

silybin has been identified in 1975 using a degradative method (Lee and Liu, 2003). The first trials to synthesize silybin suffered from the problem of giving a product which is a mixture of regioisomers, silybin and isosilybin (57:43). Regioselective synthesis of diastereomeric silybin in 63% overall yield was achieved by synthesizing a key intermediate which was coupled with 2,4,6-trimethoxyacetophenone to form a chalcone intermediate. Epoxidation, deprotection and acidic cyclization were followed (Tanaka et al., 1985).

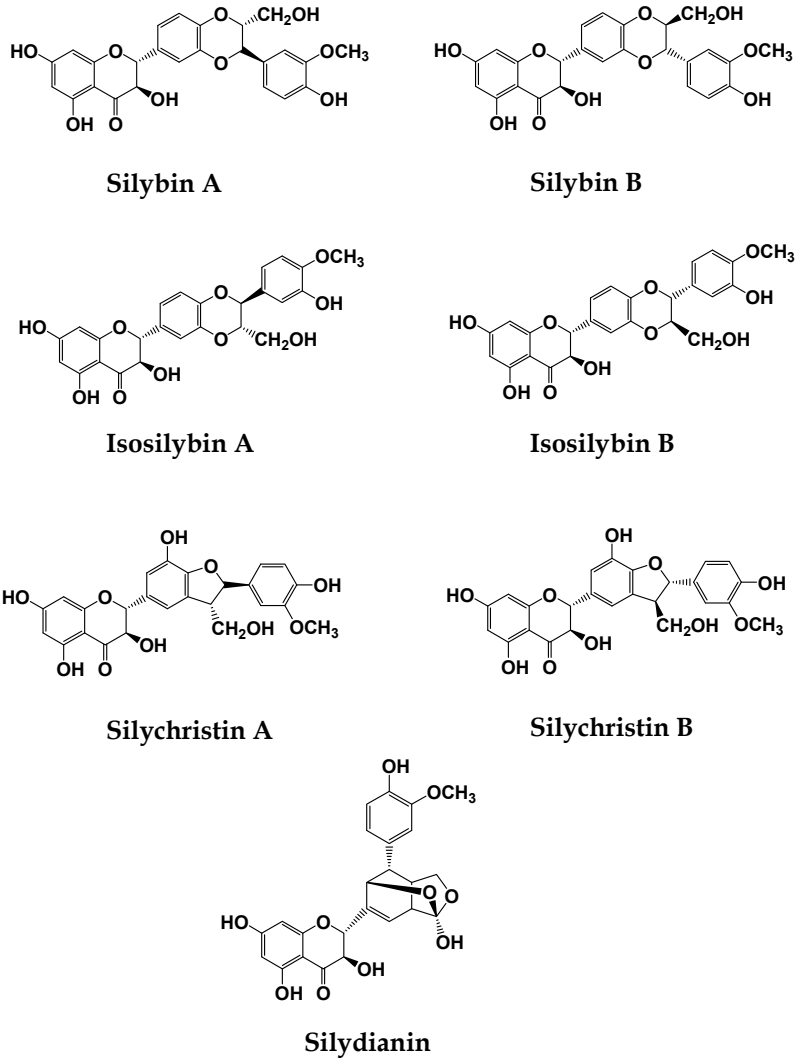

Fig. 3. Chemical structures of silymarin components.

3. Analysis of flavonolignans

Extract obtained from the fruits of *S. marianum* is available worldwide in the pharmaceutical market as antihepatotoxic drug under a variety of brand names. There are many products that contain silymarin either as a single component or in a mixture with other active constituents. The extract contains about 80% wt/wt of flavonolignans. Due to its poor water solubility and thus low bioavailability, silymarin is complexed with phosphatidylcholin, β-cyclodextrin or even given as glycosides, which have better water solubility and higher activity. A method for extraction of silymarin from plants on an industrial level has been reported (Madaus et al. 1983). In this method large part of the fruit oil is removed by cold pressing, the compressed mass is broken up, the pressed residue is extracted with ethyl acetate and the ethyl acetate extract is evaporated and processed. There is a need to have a selective and accurate analytical method for qualitative and quantitative determination of silymarin flavonolignan components during standardization of the extract. This is expressed as silymarin percentage and it corresponds to the sum of silybins, isosilybins, and silychristins and silydianin concentrations. It is important that the analytical method characterizes and quantifies each component in silymarin.

3.1 Thin layer chromatography analysis

Flavonolignans were analyzed by Thin Layer Chromatography (TLC) (Wagner et al., 2009). Chloroform-acetone-formic acid (75:16.5:8.5) was used as a solvent system and detection was done using natural products-polyethylene glycol reagent. Silymarin is characterized in UV-365 nm by two intense green-blue fluorescent zones of silybin/isosilybin (R_f = 0.6), silychristin (R_f = 0.35) and an orange zone of taxifolin (R_f = 0.4).

3.2 UV-visible spectrophotometry analysis

UV-visible spectrophotometry was proposed for the quantitative determination of flavonolignans (Famacopea Ufficiale Italiana, 1985). This spectrophotometric method is time consuming, shows a non-satisfactory repeatability and measures total and not individual flavonoligans. A fast, simple and sensitive spectrophotometric method for determination of silymarin in pure form and in pharmaceutical formulations was reported. This method was based on oxidation with potassium permanganate at pH 7. The reaction was followed spectrophotometrically by measuring the decrease in the absorbance at 530 nm (Rahman et al., 2004).

3.3 High performance liquid chromatography analysis

High Performance Liquid Chromatography (HPLC) was proposed as a method for determination of silymarin (Quaglia et al., 1999). Two reversed stationary phases, RP-18 and RP-8, were compared for resolution of all considered flavonolignans. The RP-18 stationary phase showed good separation among silybin and isosilybin, while silydianin and silychristin were not baseline resolved. The increase in water concentration in the mobile phase allowed the separation of two distereomers of silybin. RP-8 stationary phase, a more polar phase, improved the resolution of peaks related to all flavonolignans but did not allow the resolution of the two silybin diastereomers. Among the advantages of this method are precision, sensitivity, ability to measure individual constituents in a mixture, the good

separation of all compounds allowed the purity control of each peak, plotting of UV spectra, useful for the peak identification and a more correct quantification. However, time consumption, the need for pre-purification step and availability of pure reference compounds are the main disadvantages of HPLC. Analysis of silymarin components by HPLC on RP-18 in our laboratory only showed separation of the two diastereomers of silybin. However, the two peaks were not base-line resolved (Fig. 4).

3.4 Capillary electrophoresis analysis

Capillary zone electrophoresis has been proposed as a method for separation and determination of silymarin components (Kvasnička et al., 2003). Repeatability, accuracy, linearity and limit of detection were evaluated. The method was comparable to HPLC results. Shorter analysis time and better resolution of silydianin and silychristin from sample constituents were the main advantages of this method. High Performance Capillary Electrophoresis (HPCE) was used for determination of silymarin in the extract of *S. marianum* using borate buffer solution at pH 9. At this pH the flavonolignans having many phenolic groups in their structure were negatively charged (Quaglia et al., 1999). In these conditions isosilybin co-eluted together with silybin. Adding 12 mM dimethyl β–cyclodextrins solution to the running buffer, the separation of silybin from isosilybin was obtained.

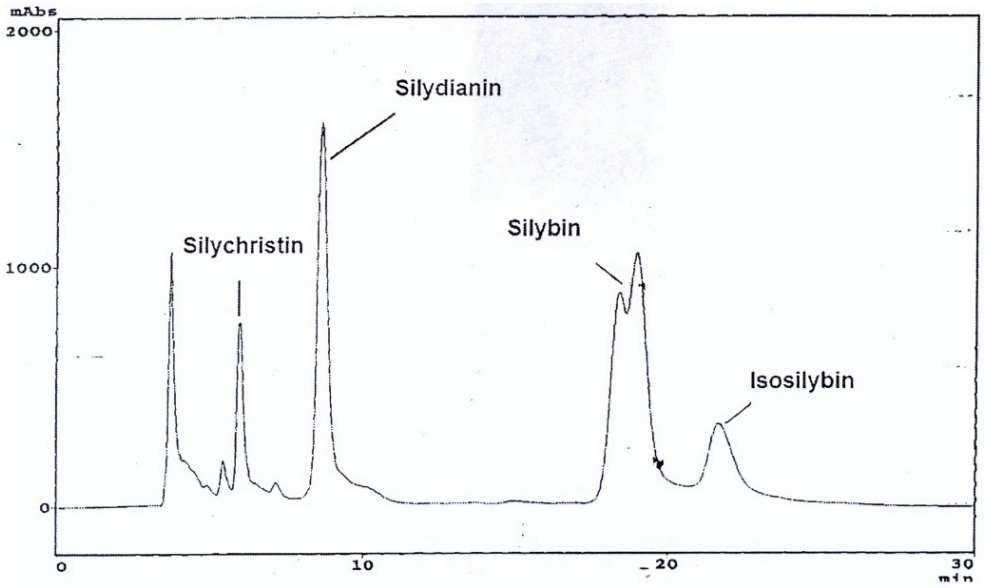

Fig. 5. Analysis of silymarin components by HPLC on RP18 (analysis was carried out in author laboratory).

3.5 Ultra performance liquid chromatography analysis

Ultra-Performance Liquid Chromatography (UPLC) offer many advantages over traditional HPLC for separation and quantification of multicomponent analytes such as silymarin

components. Among these advantages are short analysis time, maintaining the resolution and increasing peak capacity and sensitivity. Complete separation of the seven major active flavonolignans of silymarin by UPLC RP18 column was reported (Wang et al., 2010). In this study, the use of electrospray ionization tandem mass spectrometry allowed to obtain detailed analysis of fragmentation and distinguish between the seven flavonolignans for online identification. Advantages and disadvantages of different methods for quantitative analysis of flavonolignan components in silymarin are summarized in table 1.

Method	**Advantages**	**Disadvantages**
Spectrophotometric	• Fast and simple • Sensitive	• Individual flavonolignans are not quantified
HPLC	• Precise and sensitive • Individual flavonolignans are quantified • Peak identification • Purity control	• Not all flavonolignans are separated from each other • Time consuming • Needs pre-purification step • Pure reference compounds are needed
HPCE	• Shorter analysis • Less solvent consumption • Individual flavonolignans are quantified including diastereomers	• Needs pre-purification step
UPLC	• Short analysis time • Less solvent consumption • Increased resolution • Increased peak capacity and sensitivity	• Expensive • Needs pre-purification step • Needs calibration curve

Table 1. Advantages and disadvantages of different methods for quantitative analysis of flavonolignan components in silymarin.

4. Biosynthesis of flavonolignans in *Silybum marianum*

Flavonolignans are formed by combination of flavonoid and lignan structures. This occurs by oxidative coupling processes between a flavonoid and a phenylpropanoid, usually coniferyl alcohol (Dewich, 2002). Oxidative coupling occurs between free radical generated from the flavanol taxifolin and the free radical generated from coniferyl alcohol. This would lead to an adduct formation. This adduct could cyclize by attachment of the phenol nucleophile on to the quinine methide generated from coniferyl alcohol (Figure 5). The product in this case would be silybin. The fact that silybin exists in *S. marianum* in a mixture of two diastereomers reveals that the radical coupling reaction is not stereospecific. This is also true for isosilybin and silychristin. The latter flavonolignan originate from a mesomer of the taxifolin-derived free radical. Silydianin has a more complex structure and is formed by intramolecular cyclization of the coupling product. This is followed by hemiketal formation.

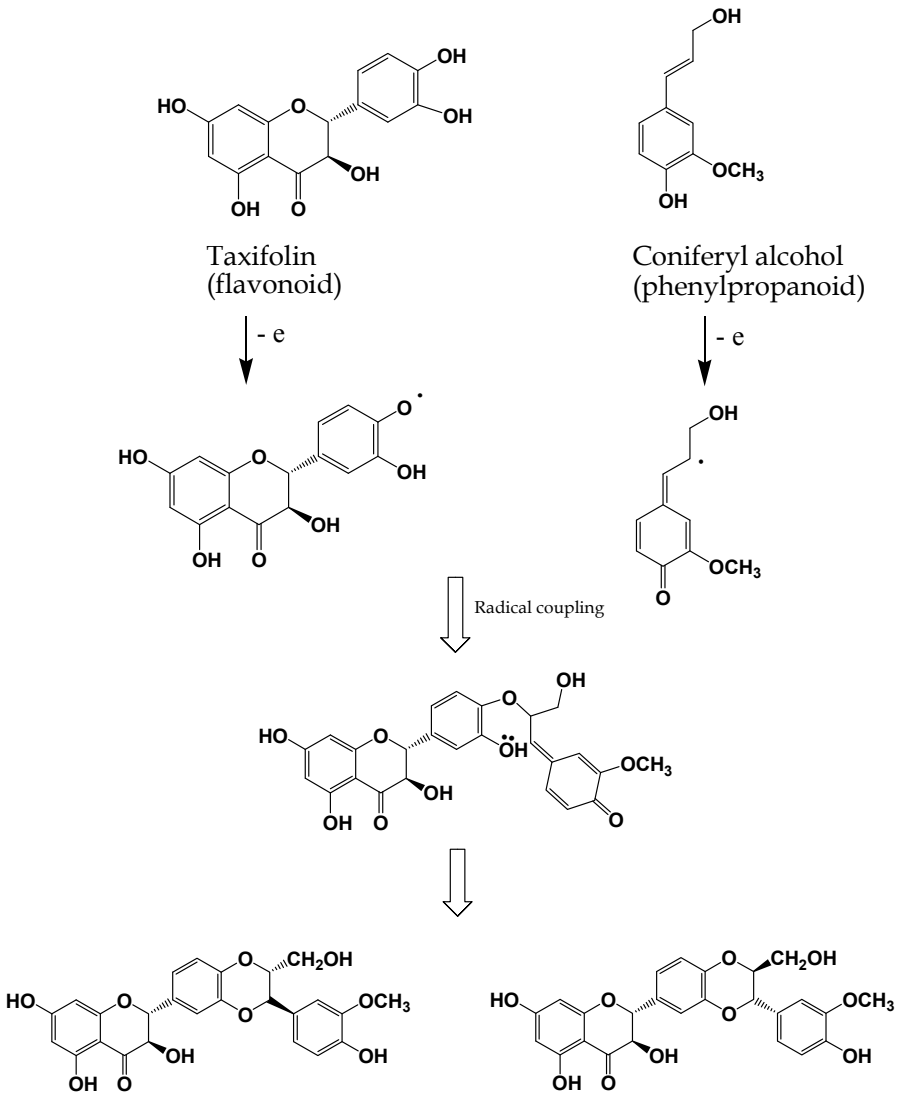

Fig. 5. Proposed biosynthetic pathway to silybin in *Silybum marianum*.

5. Biological activity of flavonolignans

Silymarin has been used for centuries to treat liver, spleen and gall bladder disorders (Shaker et al., 2010). It is known to possess hepatoprotective, antioxidant (Morazzoni and Bombardelli, 1995), anticancer (Zi et al., 1997), anti-inflammatory (De La Puerta, 1996) and anti-diabetic (Maghrani et al., 2004) properties. As a hepatoprotective agent, silymarin is used for oral treatment of toxic liver damage and for the therapy of chronic inflammatory liver diseases (Flora et al., 1998).

5.1 Hepatoprotective activity

Silymarin is one of the most investigated plant extracts with known mechanisms of action for oral treatment of toxic liver damage (Hiroshi et al., 1984). Silymarin is used as a protective treatment in acute and chronic liver diseases (Flora *et al.*, 1998). Silymarin supports the liver cells through multifactor action including binding to cell membrane to suppress toxin penetration into the hepatic cells, increasing superoxide dismutase activity (Feher and Vereckei, 1991), increasing glutathione tissue level (Pietrangelo *et al*., 1995), inhibition of lipid peroxidation (Bosisio *et al*., 1992; Carini *et al*., 1992) and enhancing hepatocyte protein synthesis (Takahara et al., 1986). The hepatoprotective activity of silymarin can be explained based on antioxidant properties due to the phenolic nature of flavonolignans. It also acts through stimulating liver cells regeneration and cell membrane stabilization to prevent hepatotoxic agents from entering hepatocytes (Fraschini et al., 2002). Recently it has been shown that flavonolignans inhibit leucotriene production; this inhibition explains their anti-inflammatory and antifibrotic activity (Dehmlow et al., 1996).

5.2 Anticancer activity of silymarin

Silymarin is also beneficial for reducing the chances for developing certain cancers (Deep et al., 2007; Zhao et al., 1999). The molecular targets of silymarin for cancer prevention have been studied (Ramasamy and Agrawal, 2008). Silymarin interfere with the expressions of cell cycle regulators and proteins involved in apoptosis to modulate the imbalance between cell survival and apoptosis. Sy-Cordero et al., 2010, isolated four key flavonolignan diastereoisomers (silybin A, silybin B, isosilybin A and isosilybin B) from *S. marianum* in gram scale. These compounds and other two related analogues, present in extremely minute quantities, were evaluated for antiproliferative/cytotoxic activity against human prostate cancer cell lines. Isosilybin B showed the most potent activity (Deep et al., 2007; Deep et al., 2008a; Deep et al., 2008b). The isolation of six isomers afforded a preliminary analysis of structure-activity relationship toward prostate cancer prevention. The results suggested that an *ortho* relationship for the hydroxyl and methoxy substituents in silybin A, silybin B, isosilybin A and isosilybin B was more favorable than the *meta* relationship for the same substituents in the minor flavonolignans. Silymarin suppressed UVA-induced oxidative stress that can induce skin damage (Svobodová et al., 2007). Therefore, topical application of silymarin can be a useful strategy for protecting against skin cancer.

5.3 Anti-inflammatory activity

Silymarin seems to possess anti-inflammatory properties by acting through different mechanisms such as its antioxidant action, membrane-stabilizing effect and inhibition of the production or release of inflammatory mediators such as arachidonic acid metabolites (Breschi et al., 2002). Gastric anti-ulcer activity of silymarin has been reported (Alarcon et al., 1992). This action was attributed to the inhibition of enzymatic peroxidation in the lipoxygenase pathway and free radical scavenging activity (Bauman et al., 1980). Silymarin exhibited significant anti-inflammatory and antiarthritic activities in the papaya latex induced model of inflammation and mycobacterial adjuvant induced arthritis in rats (Gupta et al., 2000). This action is mediated through inhibition of 5-lipoxygenase.

5.4 Effect on asthma

Activity of silymarin was examined against bronchial anaphylaxis and against post-anaphylactic, propranolol- or platelet activating factor-induced hyperreactivity in guinea-pigs (Breschi et al., 2002). Silymarin pretreatment reduced the bronchospasm induced by antigen-challenge in sensitized animals. This protective effect was due to indirect mechanism that reduces airway responsiveness to histamine, and consequently the immediate anaphylactic response. Therefore, silymarin can be used as protective agent in the management of asthmatic disorders.

5.5 Immunostimulatory activity

Several studies have reported the immunostimulatory actions of silymarin (Wilarusmee et al., 2002). The effect of treatment with silymarin was studied on glutathione level and proliferation of peripheral blood mononuclear cells of β–thalassemia major patients (Alidoost et al., 2006). In vitro treatment with 10 g/ml silymarin restored glutathione levels and enhanced cellular proliferation. This was explained by its antioxidant activity.

5.6 Treatment of obsessive-compulsive disorder

Many patients cannot tolerate the side effects of pharmaceutical agents available for treatment of obsessive-compulsive disorder, do not respond properly to the treatment or the medications lose their effectiveness after a period of treatment. An 8-week pilot double-blind randomized clinical trial on 35 adult patients was conducted to compare the efficacy of the extract of *S. marianum* with fluoxetine in the treatment of obsessive-compulsive disorder (Sayyah et al., 2010). The results showed that the extract of *S. marianum* has positive effects on obsession and compulsion starting from the fifth week. There were no any serious side effects accompanying *S. marianum* extract administration.

5.7 Hyperprolactinemic effect

S. marianum fruits have been traditionally used by nursing mothers for stimulating milk production (Newall et al., 1996). It was demonstrated that milk thistle increases lactation (Carotenuto and Di Pierro, 2005). The mechanism that led to the increase in lactation has been studied by measuring the concentration of circulating prolactin in female rats treated with silymarin (Capasso et al., 2009). It was shown that silymarin is able to produce a significant increase in circulating prolactin levels after oral administration. The levels of prolactin remains elevated for up to 66 days after silymarin discontinuation. Fig. 6 shows a summary of the wide range of biological activities attributed to silymain.

5.8 Toxicity of silymarin

An average daily dose of silymarin (420 mg/day for 41 months) was found to be non-toxic, relative to placebo, in clinical trials (Tamayo and Diamond, 2007). Drug-drug interaction and liver toxicity by interference with co-drugs by induction or inhibition of cytochrome-P450 is a major concern for the use of silymarin (Izzo and Ernst, 2009). Studies were performed to investigate the potential for hepatotoxicity, cytochrome-P450 isoenzymes induction and inhibition on dry extract from *S. marianum*, as contained in HEPAR-PASC® film-coated tablets (Doehmer et al., 2011). The results indicated that interference or

hepatotoxicity of the dry extract from *S. marianum* at the recommended maximum daily dose equivalent to 210 mg silybin is unlikely and is to be considered safe.

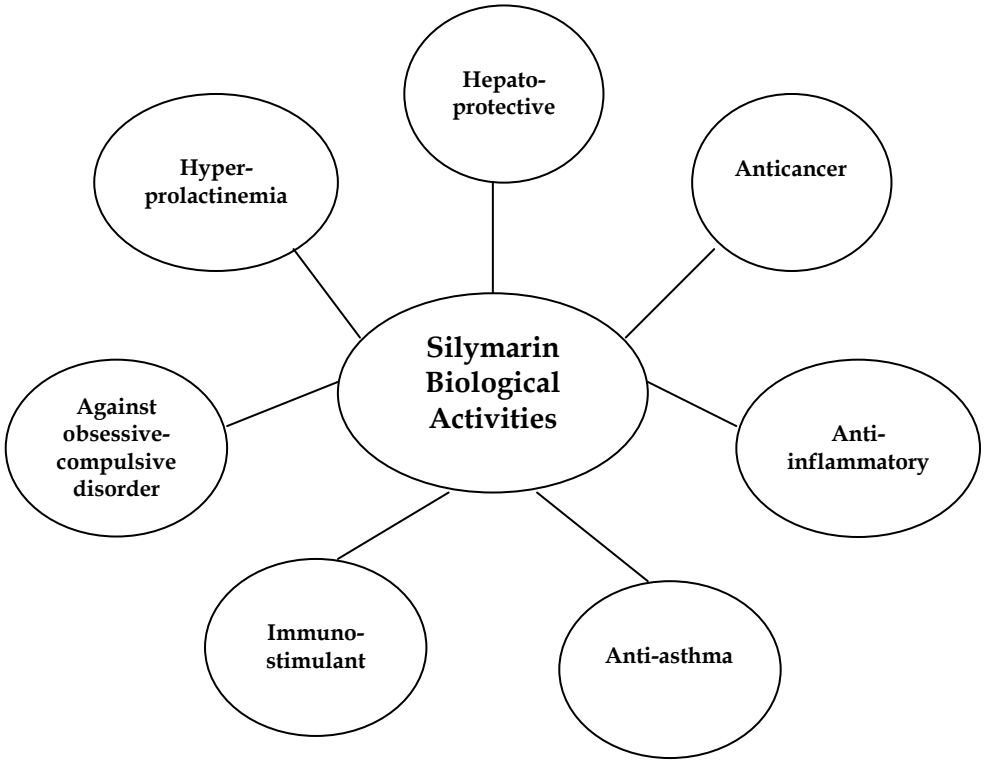

Fig. 6. Biological activities of silymarin.

6. Tissue culture studies

Plant tissue culture can be a potential source for important secondary metabolites (Misawa, 1994). This is based mainly on using plant cultures in a similar manner to microbial fermentation for factory-type production of pharmaceuticals and food additives. This technology has some advantages over conventional agricultural methods: production is independent of variation in crop quality or failure, yield of secondary metabolites would be constant and geared to demand, there is no difficulty in applying good manufacturing practice to the early stages of production, production would be possible anywhere under strictly controlled conditions, independent of political problems, free from risk of contamination with pesticides, herbicides or fertilizers and new methods of production can be patented (AbouZid et al., 2008). Cell suspension culture and hairy root culture were established from *S. marianum*. The former is established from callus tissue that developed on injured plant surface as a result of wounding or exogenous hormones (Fig. 7). The latter represent an approach to increase the yield of flavonolignans using morphologically differentiated/organized cultures.

6.1 Cell culture

In vitro cultured cells of *S. marianum* may offer an alternative and renewable source for this valuable natural product. However, the yield of silymarin was very low or sometimes not detectable in undifferentiated cultured cells (Becker and Schrall, 1977). In order to obtain silymarin in concentrations high enough for commercial manufacturing, many approaches have been made to stimulate the productivity of silymarin in cultured cells of *S. marianum*. These approaches compromise changes in the media composition (Cacho et al., 1999), treatment with elicitors such as yeast extract and methyl jasmonate (Sánchez-Sampedro et al., 2005a), addition of precursor (Tůmová et al., 2006) and morphological differentiation. Such approaches for improving silymarin production by manipulating plant cell cultures may also help in studying signal transduction pathways, cloning biosynthetic genes, studying metabolic flux and regulation of silymarin production (Zhao et al., 2005).

Fig. 7. Callus of *Silybum marianum* developed on explants.

Becker and Schrall, (1977) cultured cotyledon explants on MS media using different growth hormones for establishment of cell suspension culture. Typical flavonolignans of *S. marianum* were not detected. This was possible after feeding coniferyl alcohol and taxifolin

to cell suspension cultures (Schrall and Becker 1977). Feeding the culture medium with precursor of coniferyl alcohol offered enhancement of silydianin production but other components of silymarin were not influenced (Tůmová et al., 2006). Cacho et al. (1999) reported that callus and cell cultures of *S. marianum* could produce silymarin but to a lesser extent than that accumulates in the fruits. They also reported that elimination of calcium ion positively affected silymarin production. This point was further confirmed by Sánchez-Sampedro et al. (2005a), who also reported that silymarin accumulation was not altered by treatment of cultures with the calcium ionophore A23187. These results suggest that inhibition of external and internal calcium fluxes play a significant role in flavonolignans metabolism in *S. marianum* cell cultures. Sánchez-Sampedro et al. (2005b) reported that yeast extract and methyl jasmonate elicited the production of silymarin. Elicitation is one of the most effective approaches to enhance the yield of secondary metabolites in *in vitro* cultures (Namdeo, 2007). It has been shown that elicitors can affect level of secondary metabolites in medicinal plants by modulating the rates of biosynthesis, accumulation, and/or vacuolar transit, turnover and degradation (Barz et al., 1990). Jasmonic acid and its methyl ester are known to be involved in the plant defense response through altering the gene expression. The mechanism by which jasmonate induces gene expression was studied in *Catharanthus roseus* (van der Fits and Memelink, 2000). In this plant species induction occurs through an ORCA3 transcription factor with a conserved jasmonate–response domain. The use of methyl jasmonate as an elicitor has an advantage of being only one compound of well-defined chemical structure. The effect of elicitation with picloram, jasmonic acid and light on silymarin production was reported (Hasanloo et al., 2008). The greatest silymarin content (0.41 mg/g DW) was obtained with 3 mg/l picloram and 2 mg/l jasmonic acid in the dark after 28 days. The sequence of the signaling processes leading to stimulation of flavonolignan production by methyl jasmonate is not well-known. Madrid and Corchete, 2010, studied the possible involvement of a phospholipase D-mediated lipid signaling in the elicitation of flavonolignans. It was reported that methyl jasmonate increased the activity of phospholipase D. Mastoparan, a phospholipase D activity stimulator, caused a substantial increase in silymarin production. Phosphatidic acid, a product of phospholipase D activity, promoted silymarin accumulation. N-butanol which inhibits phospholipase D activity prevented silymarin elicitation by methyl jasmonate or mastoparan.

6.2 Root culture

Production of flavonolignans from root cultures (Fig. 8) of *S. marianum* was reported before (Alikaridis et al ., 2000). Silybin (1.79×10^{-3} % DW) and silychristin (0.81×10^{-3} % DW) were the major flavonolignans produced by the established root cultures. In the referred study hairy root cultures of *S. marianum* were established. Hairy root cultures are the roots obtained by genetic transformation of plant tissues with the pathogenic soil bacterium *Agrobacterium rhizogenes.* These roots can then be cultured on hormone-free media and have three main advantages: genetic and biochemical stability, cultivation without addition of growth regulators and ability to give high final biomasses from low inocula.

Salicylic acid was effective in increasing the flavonolignan content 2.42 times in hairy root cultures of *S. marianum* higher than control cultures (Khalili et al., 2009). Yeast extract stimulated flavonolignan production in hairy root cultures two-fold higher than the control cultures. Moreover, it was reported that yeast extract treatment induced the activity of

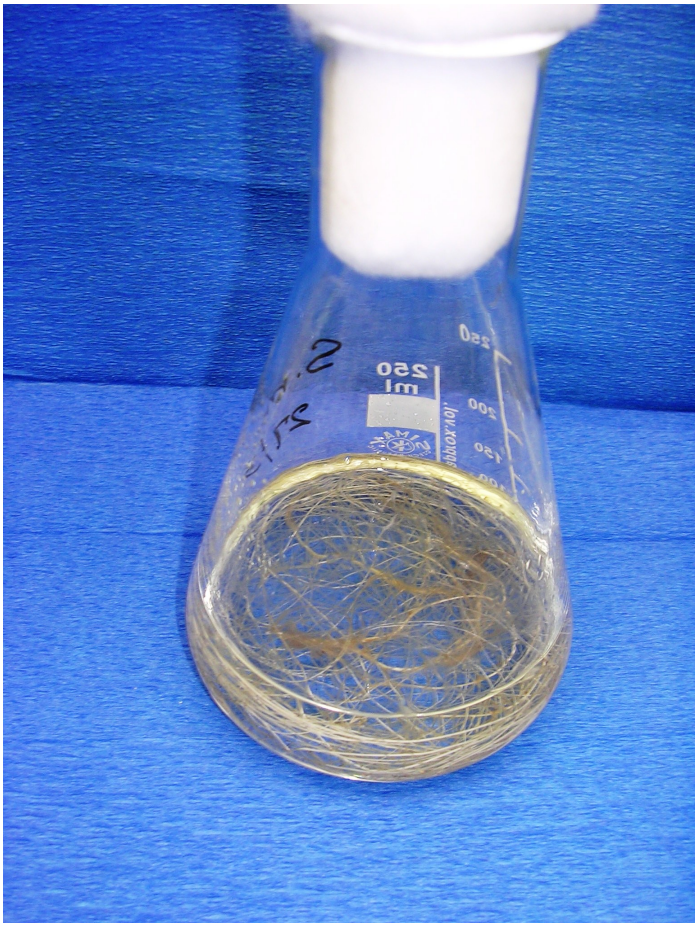

Fig. 8. Root culture of *Silybum marianum* growing in Murashige and Skoog medium.

lipoxygenase to allow for the production of jasmonate. It was concluded that jasmonate signaling is an integral part of the yeast extract signal transduction for the production of flavonolignans (Hasanloo et al., 2009).

7. Future directions

Plant tissue culture studies have contributed to our understanding of biosynthesis and regulation of silymarin in *S. marianum*. Using elicitation technology may offer an effective approach to improve silymarin production for industrial purpose. However, the possible signaling pathway that may be involved in accumulation of silymarin is still unknown. Understanding the basic components of this pathway is mandatory before these biotechnological methods can replace field crops as the basic source of pharmaceutical raw material. Establishment of plant tissue culture systems able to produce these biologically valuable compounds in high yield will facilitate such studies.

8. Conclusion

Milk thistle is an annual or biennial herb native to the Mediterranean and North African regions. The fruits of the plant contain an isomeric mixture of flavonolignans collectively known as silymarin. Basically, flavonolignan nucleus consists of the dihydroflavanol taxifolin linked to coniferyl alcohol moiety through an oxeran ring. Little is known about the coupling of coniferyl alcohol to taxifolin. Silymarin is widely used as a hepatoprotective agent for oral treatment of toxic liver damage and for the therapy of chronic inflammatory liver diseases. The hepatoprotective activity of silymarin is based on antioxidant properties, stimulating liver cells regeneration and cell membrane stabilization to prevent hepatotoxic agents from entering hepatocytes. It has been shown that flavonolignans exhibit wide range of biological activity including anticancer, anti-inflammatory, hyperprolactinemic properties. Various methods have been developed for analysis of the content and composition of main silymarin components in plant material and pharmaceuticals. Among these methods are thin layer chromatography, spectrophotometric, high performance liquid chromatography, capillary zone electrophoresis and ultra performance liquid chromatography. *In vitro* cultured cells of *S. marianum* may offer an alternative and renewable source for this valuable natural product. Flavonolignans production in cell and root cultures of *S. marianum* has been reported. Many approaches have been used to increase the yield of flavonolignans in *S. marianum* tissue culture including change in media composition, addition of precursors and elicitation.

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13

Phytocannabinoids

Afeef S. Husni and Stephen J. Cutler
University of Mississippi,
USA

1. Introduction

What is marijuana? Marijuana, also known as *Cannabis*, is defined as any preparation of the *Cannabis sativa* plant used to exploit psychoactive effects whether it is recreational or medicinal. According to the 2004 World Drug Report, 3.7% of the population 15-64 years of age consumed marijuana from 2001-2003 (World Drug Report, 2004). The use of marijuana is associated with numerous pharmacological effects; most, but not all may attributed to tetrahydrocannabinol (THC). The combination of THC and other compounds from *Cannabis sativa* may all exhibit specific pharmacological effects. These isolates from *Cannabis* are known as cannabinoids (ElSohly, 2010).

Cannabinoids are a chemical class of C_{21} terpenophenolic compounds that represent a group of compounds found in *Cannabis sativa* (Mechoulam & Gaoni, 1967). Phytocannabinoids are the naturally occurring cannabinoids from *Cannabis* sp (Pate, 1999). It is now known that at least 85 cannabinoids have been derived from *Cannabis sativa* (El-Alfy et al., 2010). It is also known that some of these compounds are of medical importance in today's society.

In order to gain a better understanding of the pharmacological effects of the phytocannabinoids, human and rodent receptors are used to evaluate binding affinity of these compounds to two cannabinoid receptors that have been reported in literature, CB_1 and CB_2. CB_1 receptors are located mainly in the brain, while CB_2 receptors are primarily

peripheral and found on mature B cells and macrophages within the tonsils and spleen (Raymon & Walls, 2010). When activated, the CB_1 receptors exhibit the psychoactive effects caused by *Cannabis* use. Since CB_1 receptors are not present in the medulla oblongata, part of the brain stem responsible for respiratory and cardiovascular functions, there is not a risk of overdose resulting in respiratory depression or cardiovascular failure that may be seen with abuse of other drugs, such as the opioids. CB_2 receptors are said to be responsible for anti-inflammatory effects.

2. Cannabinoid receptor function

Cannabinoid receptors are G-protein coupled receptors (Figure 1), which are a large family of seven member transmembrane receptors that act in a second messenger fashion. When cannabinoid receptors are activated, they inhibit the enzyme adenylate cyclase. Adenylate cyclase is responsible for breaking ATP to form cyclic AMP (cAMP). When a ligand binds to the extracellular surface of cannabinoid receptors, it causes a conformational change of the receptor. This change activates the second messenger by exchanging guanosine diphosphate (GDP) for guanosine triphosphate (GTP). Then, the G-protein's alpha subunit separates from the beta/gamma subunit to cause intracellular proteins to function properly. In CB_1 and CB_2 receptors, cAMP acts as the second messenger. When these receptors are activated, cAMP levels decrease within the cell. Therefore, the result of activating cannabinoid receptors leads to a decrease in cAMP levels, and in turn leads to an inhibition of function.

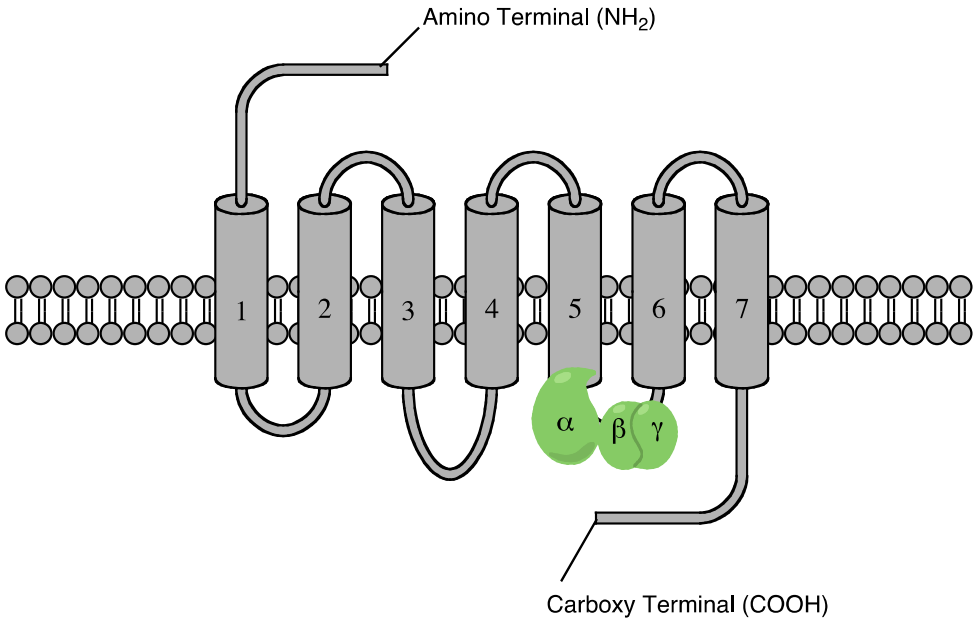

Fig. 1. Example of a G-Protein Coupled Receptor.

3. Endocannabinoids

Endogenous cannabinoids, or endocannabinoids, are substances produced in the body that activate the cannabinoid receptors. Generally, neurotransmitters are released presynaptically and activate the receptors on a postsynaptic cell. However, unlike most neurotransmitters, the endocannabinoids work in a reverse fashion. Endocannabinoids use retrograde signaling to achieve cannabinoid receptor activation. This means that the ligands are being produced postsynaptically, but acting presynaptically (Lambert, 2009). Another critical point in understanding the function of the endocannabinoids is that the endocannabinoid system can produce endocannabinoids "on demand" in response to an increase in intracellular calcium levels (Sugiura et al., 2006).

Shortly after the cloning of the cannabinoid receptors, researchers began searching for endogenous ligands that activate these receptors. The first endocannabinoid discovered was anandamide (Figure 2) in 1992 (Devane et al., 1992). Several years after the discovery of anandamide the second endogenous ligand, 2-arachadonoyl-glycerol (2-AG, Figure 3), was discovered (Sugiura et al., 2006). Anandamide and 2-AG act as a partial agonist and full agonist, respectively, at the CB_1 and CB_2 receptors. Although the structure of anandamide differs significantly from THC, both of these ligands have similar pharmacological profiles (Grotenhermen, 2002). Understanding the mechanism of how cannabinoids produce their effects is in part because of the discovery of the endocannabinoid system.

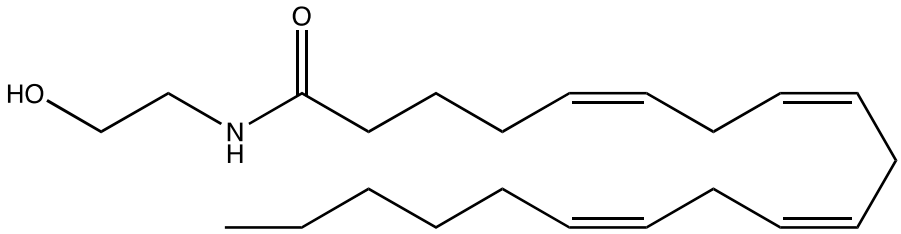

Fig. 2. Chemical structure of anandamide.

Although the physiological roles of the endocannabinoids are not fully defined, several pharmacological functions have been described. Studies suggest that these endogenous ligands may aid in pain relief, enhancement of appetite, blood pressure lowering during shock, embryonic development, and blocking of working memory (ElSohly, 2010).

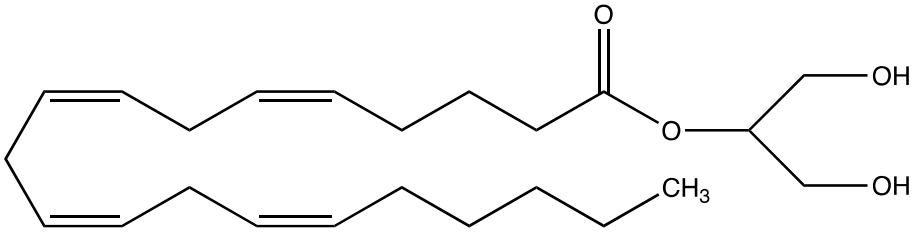

Fig. 3. Chemical structure of 2-AG.

4. Phytocannabinoids

The first cannabinoid identified was cannabigerol, and its precursor cannabigeric acid was shown to be the cannabinoid formed in the plant as well as endogenously (Yamauchi, 1975). Today, the most discussed phytocannabinoid is delta-9-tetrahydrocannabinol. In 1964, Gaoni and Mechoulam isolated and elucidated the chemical structure of THC from the leaves of *Cannabis sativa* (Mechoulam & Gaoni, 1964). THC is pharmacologically and toxicologically the best studied constituent of *Cannabis*, responsible for most of the psychoactive effects of natural *Cannabis* preparations (Grotenhermen, 2002). THC and cannabidiol (CBD) are the two most common naturally occurring cannabinoids.

As mentioned earlier, THC (Figure 4) is the main component of *Cannabis* responsible for the psychoactive effects. Other than *Cannabis* being abused to achieve a state of euphoria, it is now being used medicinally to aid in acquired immunodeficiency syndrome (AIDS) patients with wasting syndrome and for pain management, nausea, and vomiting associated with patients receiving cancer chemotherapy. Since THC is responsible for the psychoactive effects of *Cannabis*, people have learned how to genetically increase the concentration of THC within each plant to produce a stronger "high." Since 1980, the concentration of THC within marijuana has increased from less than 1.5% to approximately 20% (ElSohly et al., 2000). THC acts a partial agonist at the CB_1 and CB_2 receptors, but functions via interaction with the CB_1 receptor.

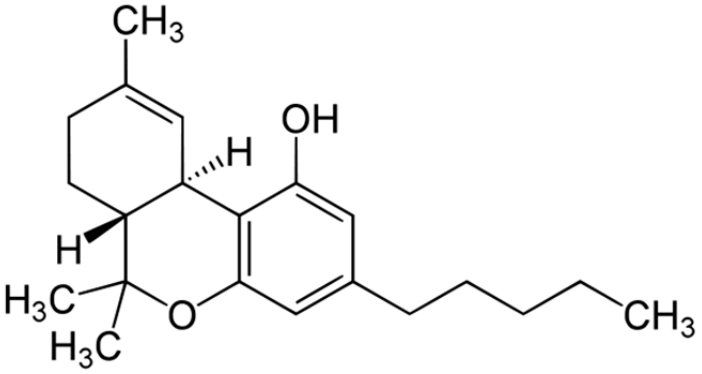

Fig. 4. Chemical structure of delta-9-THC.

The second major constituent of *Cannabis*, cannabidiol (CBD, Figure 5), is responsible for the anti-inflammatory effects due to its interactions with the human CB_2 receptor. CBD was first isolated in 1940 (Adams et al., 1940); however, it was not until 1963 that Mechoulam and Shvo elucidated its correct structure (Mechoulam & Shvo, 1963). At the human CB_2 receptor, CBD's mechanism of action shows inverse agonism activity (Pertwee et al., 2007). In 1995, Benet and colleagues show that cannabidiol is not only responsible for anti-inflammatory effects, but may also aid in reducing unpleasant side effects from THC, including reduced anxiety (Benet et al., 1995). They found that CBD inhibits cytochrome P450 3A11, which causes THC to change into its more potent metabolite 11-hydroxy-THC (Gallily et al., 2002).

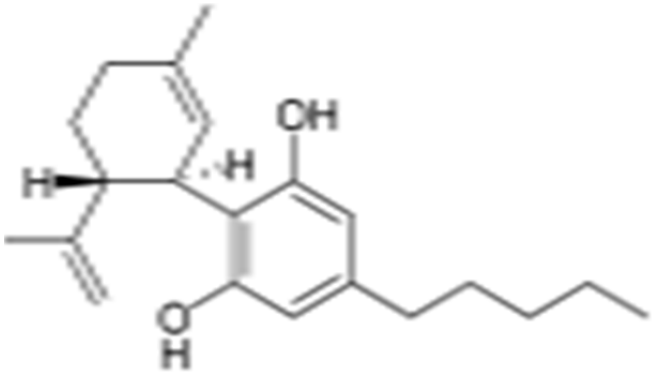

Fig. 5. Chemical structure of cannabidiol.

Tetrahydrocannabinol and cannabidiol are the two most discussed phytocannabinoids, but not the only ones known. ElSohly and co-investigators have divided the phytocannabinoids into ten subclasses: 1) Cannabigerol type – propyl side chains and monomethyl ether derivatives 2) Cannabichromene type – analogs present in the C-5 position 3) Cannabidiol type – analogs varying from C-1 to C-5 positions 4) Delta-9-tetrahydrocannabinol type – double bond in the C-9 position; responsible for psychoactive effects 5) Delta-8-tetrahydrocannabinol type – double bond in the C-8 position; thermodynamically more stable than delta-9-THC , however, 20% less active 6) Cannabicyclol type – five atom ring and C-1 bridge 7) Cannabielsoin type – artifacts formed from CBD 8) Cannabinol and Cannabinodiol types – A ring aromatization 9) Cannabitriol type – additional hydroxyl substitution 10) Miscellaneous types – ex: furano ring, carbonyl function, tetrahydroxy substitution (ElSohly, 2010).

Another phytocannabinoid that shows a significant amount of importance is cannabinol (CBN, Figure 6); it is a metabolite of tetrahydrocannabinol. It was the first cannabinoid identified from *Cannabis sativa*. (Wood et al., 1896). Along with THC, cannabinol is also a psychoactive component of *Cannabis* due to its interaction with CB_1 receptors. Compared to THC, it acts a weak agonist at both the CB_1 and CB_2 receptors.

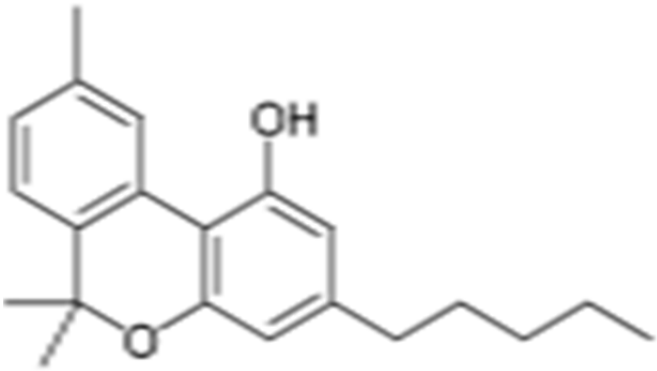

Fig. 6. Chemical structure of cannabinol.

Extracts that have been isolated from marijuana may be tested to see if they have affinity for each of the CB_1- or CB_2- type receptors. THC remains the best phytocannabinoid in terms of affinity for the cannabinoid receptors with a binding Ki of 14nM (Figure 7). Most of the compounds isolated from *Cannabis* show a sufficient amount of binding activity at both of the cannabinoid receptors. However, not all compounds isolated show interactions with either CB_1 or CB_2. For instance, even though cannabidiol is a major constituent of *Cannabis* and shows pharmacological effects, it has little or no activity for CB_1 or CB_2 receptors (Mechoulam & Rodriguez, 2007). To determine binding affinity and functional activity, in vitro assays are performed.

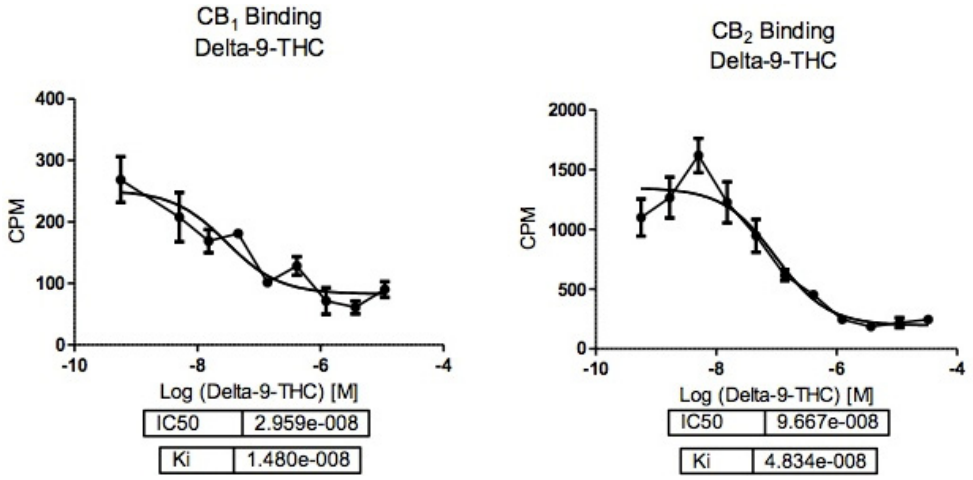

Fig. 7. Binding affinity of delta-9-THC at Cannabinoid Receptor 1 and Cannabinoid Receptor 2.

5. *In vitro* bioassays

In order to have success with in vitro assays, cultured cells containing the specific receptors must be developed. At the University of Mississippi HEK293 cells have been transfected with full length human CB_1 and human CB_2 DNA via electroporation. Once "shocked," the cells open and accept the human CB_1 and CB_2 cDNA with a linked specific antibiotic resistant plasmid. Since not all cells will receive the DNA, a selection process using the specific antibiotic is added to the cultured cells in order to kill off cells without the cDNA. After an allotted time period for growth, a single cell is selected and clonal colonies are grown in cell culture. The replication of a single cell containing either CB_1 or CB_2 DNA allows researchers to guarantee the over expression of cannabinoid receptors on the cell membrane. With this, mass subculture followed by "scraping" of the cells leads to the membrane with the receptors. Once the protein concentration is determined this membrane may be used for in vitro assays.

Phytocannabinoids may be tested for their binding affinity toward each of the cannabinoid receptors. A competitive binding assay is done to determine the binding affinity of each compound. The competition is between the chosen phytocannabinoid and a labeled ligand, such as ^3H- CP-55, 940. It is known that the labeled ligand will tightly bind to each of the cannabinoid receptors; therefore, if a test compound shows affinity for the receptors, the

amount of labeled ligand bound to the receptor will be low resulting in high binding affinity of the test compound. A compound showing strong binding affinity for either of the cannabinoid receptors, warrants testing to determine the functional activity.

A functional assay determines whether the compound is acting as an agonist, antagonist, or inverse agonist. As opposed to the binding assay, an in vitro functional assay is not based upon competitive binding, but rather "tracking" the amounts of guanosine triphosphate (GTP). When the membrane is not stimulated, there is a pool of guanosine diphosphate (GDP) associated with it. Upon stimulation, this pool of GDP is converted into GTP. To monitor this response, ^{35}S labeled GTP is added to the assay to bind to the receptors. Therefore, an increase in GTP is directly proportional to stimulation of the receptor by labeled ligand. An agonist compound is indicated by an increase in GTP. Delta-9-THC has a functional Ki of approximately 300nM, which means it is acting as a partial agonist, yet is still responsible for the psychoactive effects associated with *Cannabis* (Figure 8). To detect an antagonist, the compound must be tested in the presence of a known agonist at that specific receptor. The antagonist blocks the ability of the agonist to fully stimulate the receptor, thus resulting in a right shift of the agonist EC_{50}.

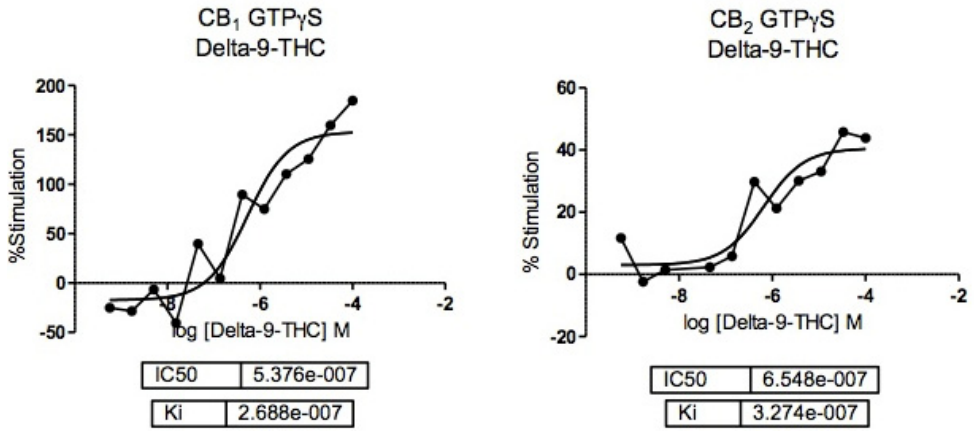

Fig. 8. Functional assay activity of delta-9-THC at Cannabinoid Receptor 1 and Cannabinoid Receptor 2.

6. *In Vivo* bioassays

Cannabinoids that show promising activity in the functional assay, whether acting as an agonist or antagonist, may be tested in vivo using the tetrad assay in mice. In the late 1980s, Little and his colleagues began testing rodents treated with cannabinoids in this tetrad assay. The term tetrad describes a series of four different tests to help evaluate the biological effects of a compound: 1) Locomotor activity 2) Catalepsy 3) Hypothermia and 4) Analgesia. The locomotor activity test allows a researcher to determine if the rodent is acting "lazy." The rodent is placed in a box with perpendicular gridlines, which are beams of light. The test determines the amount of times the beams are broken in an allotted time period, an increase in the number of times broken correlates with a decrease in locomotor activity. To determine if the drug causes cataleptic effects, a rodent is placed on a bar elevated off the ground surface. If

the rodent remains immobile, it is considered cataleptic. Hypothermia, also know as a rectal temperature assay, is simply a measure of the rodents rectal temperature after the drug has been administered. For the last part of the tetrad assay, there are two different methods of testing for analgesic effects. One method is the hot plate (Figure 9) assay. In this assay, a rodent is placed upon a hot plate and the time it takes for the rodent to react, usually a small jump, is recorded. The second method is known as the tail-flick assay. In this assay, the rodent is immobilized and a high temperature beam of light is sporadically placed on the tail. If the rodent feels pain, it will move its tail either left or right (Little, 1988).

Fig. 9. Analgesic portion of tetrad assay: hot plate test.

7. Medicinal uses of marijuana

According the United Nations, *Cannabis* "is the most widely used illicit substance in the world" (World Drug Report, 2010). There are people who use Cannabis medicinally, and there are others who abuse *Cannabis* in order get "high," or obtain a state of euphoria. Those who use marijuana regularly for medicinal purposes use strict, smaller amounts to control the strength and duration of the "high." However, those who abuse marijuana attempt to smoke or ingest as much as necessary to achieve their own personal state of euphoria. This abuse negatively affects the people who do need *Cannabis* to help with side effects of chemotherapy and AIDS. *Cannabis* is not only used to help those suffering from cancer chemotherapy and AIDS, but it also lowers intraocular eye pressure for those with glaucoma, acts a pain reliever, and more recently has been found to help with symptoms of multiple sclerosis and depression. Therefore, researchers are attempting to formulate a synthetic cannabinoid that resembles the compounds isolated from *Cannabis*, but do not exploit psychotropic properties.

The goal of research in this area is to synthesize a cannabinoid-like compound that warrants a high affinity for either CB_1 or CB_2 receptors, or both, and can help patients without causing some of the unwanted side effects of marijuana, such as the psychotropic effects associated with CB_1. With this said, studies show that *Cannabis* users have fewer psychological side effects than those users administering synthetic THC. There are two synthetic cannabinoid products available on the market in the United States, Nabilone and Dronabinol (Figure 10). Some of these side effects from synthetic cannabinoids include dysphoria, depersonalization, anxiety, and paranoia (Grinsponn & Bakalar, 1997). As previously

mentioned, CBD has shown to reduce anxiety and other unpleasant side effects caused by ingestion of pure THC (Zuardi et al., 1982). The preference of whole *Cannabis* over synthetic formulations of THC is due to the lack of extra side effects associated with the whole *Cannabis*. This opens the door for scientists to study what is actually causing all of the side effects associated with synthetic THC. This also shows that some of the compounds associated with *Cannabis sativa* may be working synergistically to alleviate unwanted effects from THC when used alone (McPartland & Russo, 2001). So, the ultimate goal in cannabinoid drug development would be to mimic the non-psychotropic effects associated with CB_1, mimic the beneficial effects associated with CB_2, and not deal with the negative side effects associated with marijuana or synthetic THC.

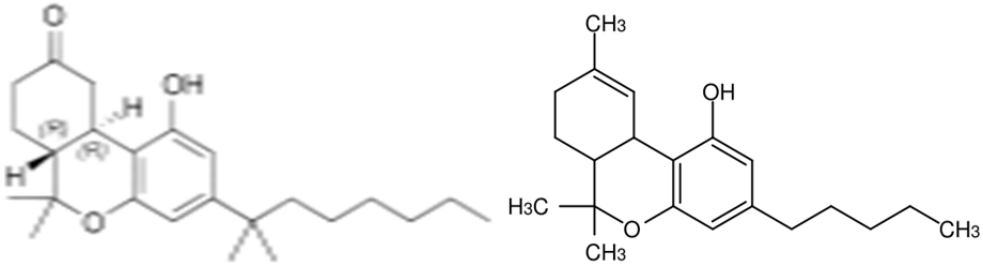

Fig. 10. Chemical structures of Nabilone (left) and Dronabinol (right).

8. Phytocannabinoids and depression

Depression may be described as a mood disorder associated with feeling down, sad, angry, or lost that interferes with everyday life. The most commonly associated drug categories for the treatment of depression include monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), selective-serotonin reuptake inhibitors (SSRIs), and serotonin-norepinephrine reuptake inhibitors (SNRIs). A new field of research involving *Cannabis* may be the link to the treatment of depression. However, studies show conflicting data as to whether *cannabis* is beneficial (Grinsponn & Balkar, 1998) or detrimental for the treatment of depression (Bovassa, 2001). Due to the conflicting results of these studies, Witkin switched the focus to the role of the endocannabinoid system and the treatment of depression from exogenously administered cannabinoids (Witkin et al., 2005). Since 2005, it has been concluded that the endocannabinoid system does play a role in the treatment of depression, but differs from minor depression to major depression.

New research has found that a common characteristic of *Cannabis*, mood elevation, may be the link to the treatment of depression. A study published by El-Alfy and co-investigators in 2010 describes the antidepressant effects associated with administration of phytocannabinoids. The objective of this study was to isolate the major cannabinoids from *Cannabis* and evaluate the antidepressant effects using the mouse forced swim test (FST), followed by the tail suspension test (TST). Typically in mice, when cannabinoids are administered they exert hypothermia and catalepsy, which means that a psychoactive state is being achieved. For these depression studies, only low dosages of these phytocannabinoids were administered so that the test subjects did not demonstrate psychoactive effects. The cannabinoids isolated and tested were cannabigerol (CBG), cannabinol (CBN), cannabichromene (CBC), cannabidiol (CBD), delta-8-THC, and delta-9-THC (THC) (Figure 12).

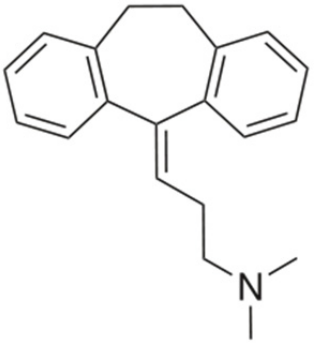

Fig. 11. Chemical structure of the tricyclic antidepressant, Amitriptyline.

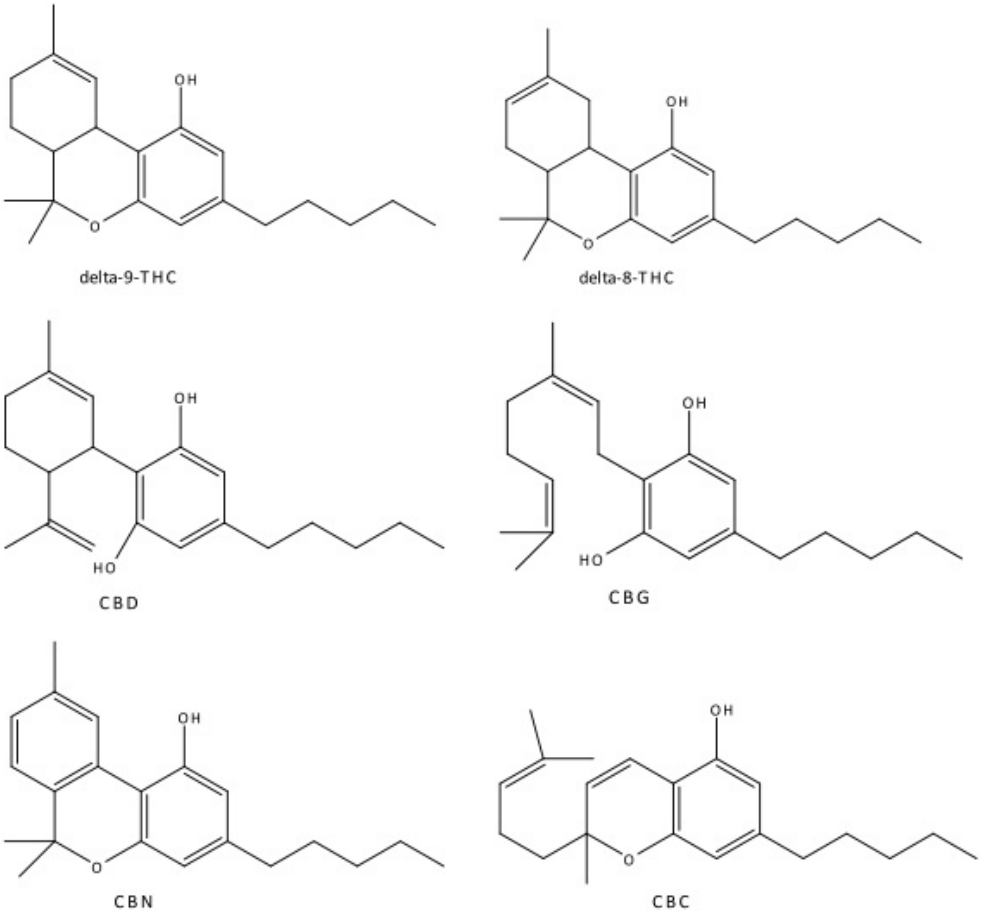

Fig. 12. The six phytocannabinoids tested for antidepressant-like effects (El-Alfy et al., 2010).

To assess that hypothermia and catalepsy were not achieved, the tetrad assay was completed after administration of each cannabinoid. Out of the six cannabinoids tested, only delta-8-THC and delta-9-THC showed a U-shaped dose response in the forced swim test. With this, only delta-9-THC showed significant antidepressant-like effects. Administration of the non-psychoactive components revealed that CBC and CBD displayed antidepressant-like effects in the forced swim test. However, a high dose of CBD was used to display these antidepressant-like effects.

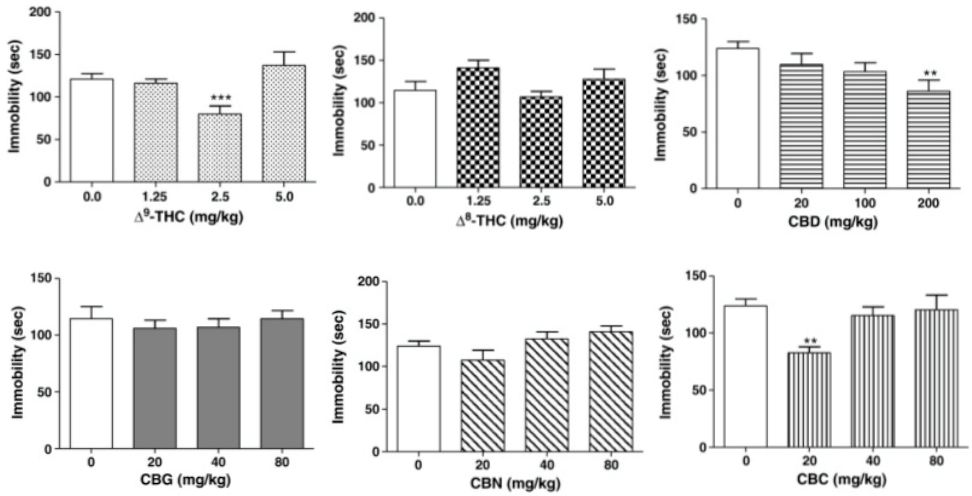

Fig. 13. Effects of each phytocannabinoid on immobility time in the mouse forced swim test (El-Alfy et al., 2010).

To further confirm these tests, delta-9-THC and CBC were evaluated in the tail suspension test. Between these two phytocannabinoids, only delta-9-THC continued to exhibit these antidepressant-like effects at low doses. Therefore, the results of this study show that delta-9-THC and other phytocannabinoids administered exogenously do indeed aid with the treatment of depression (El-Alfy et al., 2010).

9. Phytocannabinoids and appetite stimulation

Patients suffering from AIDS are now becoming the main target for the therapeutic use of *Cannabis*. Those with AIDS tend to lose their desire to eat regularly throughout the day. When this occurs, the patient becomes weak, agitated, tired, and anorexic; this occurrence in known as Wasting Syndrome. Research shows that at least 90% of patients who smoked marijuana had the desire to eat immediately after use (Haines & Green, 1970). With the use of *Cannabis* as a therapeutic drug to stimulate appetite, the suffering patients may be able to eat on a regular basis throughout the day, thus improving their quality of life. Several studies have shown that the use of marijuana does increase appetite, which also increases energy in daily life routines.

In a study conducted by Mattes and colleagues, the appetite stimulating effects of cannabinoids, specifically THC, were examined. A major focus in this study, for a means of clarification from previous research, was the route of administration of THC. The four

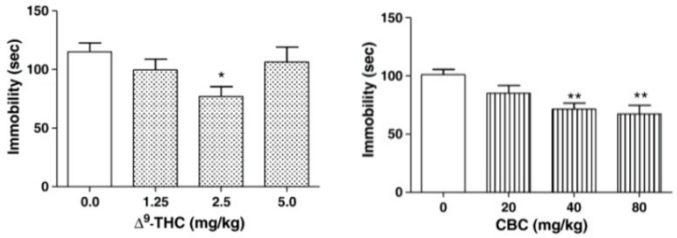

Fig. 14. Effects of THC and CBC on immobility time in the mouse tail suspension test (El-Alfy et al., 2010).

different ways in which THC was administered includes oral, inhaled, sublingual, and suppository. There are high levels of variability in determining if THC does actually stimulate appetite. Factors such as environment, age, gender, tolerance, dosage, and social influences play a role in the effect of THC on appetite. During one study, the suppository route of administration resulted in the highest energy intake when compared to oral, sublingual, and inhaled administration of THC (Figure 15).

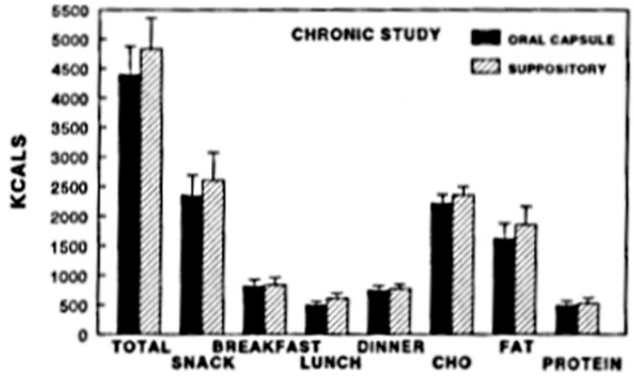

Fig. 15. Mean data from patients dosed orally and via suppository over a 72 hour time period (Mattes et al., 1994).

There is no single outcome on the effect of THC on appetite stimulation no matter the form of administration. The results vary from having no effect to the possibility of having major food cravings. In some circumstances, not only did the food cravings become increased, but during a meal the food seemed to also have an increased taste of delightfulness. The conclusion of this study indicates that THC as an appetite stimulant produces its highest effects on healthy, adult individuals who use low dosage amounts (Mattes et al., 1994).

10. Future directions

The growing population is becoming more aware of *Cannabis* as a medicinal plant, and not only a recreational drug. The first *Cannabis* publications date back to the early 1940's in which there was only one publication from 1940-1949. Today, when a search is performed there are over 7,000 journal articles that discuss anything associated with the words *Cannabis*, cannabinoids, or endocannabinoids. Over the last 50 years, marijuana has become

the most widely used illegal drug, along with one of the most widely studied plants. There are still many questions to be answered within the *Cannabis* field of study.

It is possible that the cannabinoid system has several other receptors that may explain the mechanism of action of compounds that exhibit cannabinoid-like effects when there is little or no affinity for CB_1 or CB_2. GPR55 and GPR119, both G-protein coupled receptors, are said to be novel cannabinoid receptors. All cannabinoid receptor antagonists appear to act as inverse agonists instead of neutral antagonists. There are few ligands starting to appear in literature as being neutral antagonists. Interest in this area could is important to help develop pharmacological tools to aid in finding neutral antagonists. These findings may possess unknown therapeutic advantages over receptor antagonists that act as inverse agonists (Pertwee, 2005).

It is now known that phytocannabinoids interact with the CB_1 and CB_2 receptors, and that the human body consists of an endocannabinoid system that activates these two receptors. However, what these receptors look like remains a mystery. A general structure-activity relationship has been determined for the cannabinoids, but there is no limitation to synthesizing new compounds that will interact strongly with these receptors. In *vitro* and in *vivo* bioassays play a crucial role in determining the affinities and functions of compounds associated with the CB_1 and CB_2 receptors. The information determined from these bioassays will continue to help develop novel therapeutic drugs that potentially have pharmacological effects related to *Cannabis* without the deleterious side effects.

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14

Alkaloids and Anthraquinones from Malaysian Flora

Nor Hadiani Ismail, Asmah Alias and Che Puteh Osman
Universiti Teknologi MARA, Malaysia

1. Introduction

The flora of Malaysia is one of the richest flora in the world due to the constantly warm and uniformly humid climate. Malaysia is listed as 12th most diverse nation (Abd Aziz, 2003) in the world and mainly covered by tropical rainsforests. Tropical rainforests cover only 12% of earth's land area; however they constitute about 50% to 90% of world species. At least 25% of all modern drugs originate from rainforests even though only less than 1% of world's tropical rainforest plant species have been evaluated for pharmacological properties (Kong, *et al.*, 2003). The huge diversity of Malaysian flora with about 12 000 species of flowering plants offers huge chemical diversities for numerous biological targets. Malaysian flora is a rich source of numerous class of natural compounds such as alkaloids, anthraquinones and phenolic compounds. Plants are usually investigated based on their ethnobotanical use. The phytochemical study of several well-known plants in folklore medicine such as *Eurycoma longifolia, Labisia pumila*, *Andrographis paniculata*, *Morinda citrifolia* and *Phyllanthus niruri* yielded many bioactive phytochemicals. This review describes our work on the alkaloids of *Fissistigma latifolium* and *Meiogyne virgata* from family Annonaceae and anthraquinones of *Renellia* and *Morinda* from Rubiaceae family.

2. The family *Annonaceae* as source of alkaloids

Annonaceae, known as *Mempisang* in Malaysia (Kamarudin, 1988) is a family of flowering plants consisiting of trees, shrubs or woody lianas. This family is the largest family in the Magnoliales consisting of more than 130 genera with about 2300 to 2500 species. Plants of the family Annonaceae are well known as source of a variety of alkaloids (Cordell, 1981). Many alkaloids have important physiological effects on human and exhibit marked pharmacological activity which is useful as medicine. For examples, atropine is used widely as an antidote to cholinesterase inhibitors such as physostigmine. Morphine and codeine are narcotic analgesics and antitusive agent while caffeine, which occurs in coffee, tea and cocoa is a central nervous system stimulant. Caffeine is also used as cardiac and respiratory stimulant andbesides as an antidote to barbiturate and morphine poisoning (Parker, 1997). The first report on phytochemical studies of alkaloids from Malaysian Annonaceae plants was on the leaves of *Desmos dasymachalus* which has led to the isolation of new 7-hydroxyaporphine, dasymachaline (Chan & Toh, 1985).

The phytochemical investigation of Malaysian Annoaceous plants for their alkoloidal content continue to flourish. Phytochemical survey of the flora of the Peninsula Malaysia and Sabah, with systematic screening for alkaloids resulted in reports on chemical constituents of several plants from Annonaceae illustrating great interest in this field (Teo, *et al*., 1990). Lavault *et al.,* (1981) analysed the alkaloid content of three Annonaceae plants; *Disepalum pulchrum*, *Polyalthia macropoda* and *Polyalthia stenopetala* which led to the isolation of several isoquinoline compounds. Isolation of two new 7,7'-bisdehydroaporphine alkaloids; 7,7'-bisdehydro-*O*-methylisopiline and 7-dehydronornuciferine-7'-dehydro-*O*-methylisopiline from bark of *Polyalthia bullata* was reported by Connolly *et al*., (1996). Kam (1999) reviewed the alkaloids derived from Malaysian flora in a book entitled chemical and biological approach of alkaloids.

In Malaysia, eight species of *Fissistigma* are known. They are *F. mobiforme, F. cylindrium, F. fulgens, F. kingii, F. lanuginosum, F. latifolium, F. munubriatum* and *F. kinabaluensis* (Nik Idris *et al*., 1994). Not much has been reported on the phytochemical studies of *Fissistigma* species. The studies on the alkaloids from *Fissistigma fulgens* have led to the isolation of aporphine, oxoaporphine and protoberberine alkaloids. Liriodenine, anonaine, argentinine, discretamine and kikemanine were found from this species (Awang, *et al*., 2000). The phytochemical work on alkaloidal composition of the Malaysian *Fissistigma manubriatum* by Saaid and Awang (2005) yielded two oxoaporphines, lanuginosine and liriodenine together with two tetrahydroprotoberberines, tetrahydropalmatine and discretine. We studied the alkaloids of *Fissistigma latifolium* and reported the isolation of nine alkaloids including a new aporphine compound (Alias *et al*., 2010).

Meiogyne cylindrocarpa, *Meiogyne monosperma* and *Meiogyne virgata* are the only three *Meiogyne* species found in Malaysia. Only *Meiogyne virgata* was studied by Tadic *et al.* (1987). The sample collected from Mount Kinabalu, Sabah was reported to contain azafluorene alkaloid, kinabaline, together with liriodenine, cleistopholine and other aporphine alkaloids. Our work on *Meiogyne virgata* from Hulu Terengganu yielded nine alkaloids from aporphine, oxoaporphines and azaanthracene groups.

2.1 Alkaloids of *Fissistigma latifolium* and *Meiogyne virgata*

Since the last three decades, a large number of alkaloidal compounds have been isolated from some Annonaceae species. Tertiary and quaternary isoquinoline and quinoline alkaloids are pharmacologically important compounds commonly found in Annonaceae plants. Continuing our interest on this family of plants, we pursued phytochemical investigation on *Fissistigma latifolium* and *Meiogyne virgata.*

2.1.1 Alkaloids of *Fissistigma latifolium*

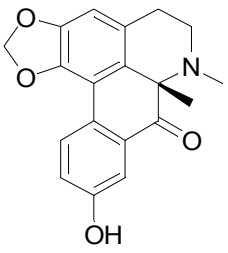
Fissistigma latifolium (Dunal) Merr. from the genus *Fissistigma* is a climbing shrub found in lowland forest of Malaysia, Sumatra, Borneo and Philippines (Verdout, 1976). The genus *Fissistigma* (Annonaceae) consists of about 80 species and is widely distributed in Asia and Australia (Sinclair, 1955). Several species of the genus *Fissistigma* have been used in Southeast Asia as traditional medicines (Perry, 1980). They have been used for muscular atrophy, hepatomegaly and hepatosplenomegaly (Kan, 1979). In Malaysia, the medicinal uses of *Fissistigma* species was briefly mentioned by Burkill as the treatment for childbirth, malaria, wounds, ulcer and rheumatism (Kamarudin, 1988).

Fig. 1. *Fissistigma latifolium*

Previous studies on *F. fulgens* and *F. manubriatum* have resulted in the isolation of aporphine, oxoaporphine and protoberberine alkaloids. Similarly, the studies on alkaloids from *Fissistigma latifolium* led to the isolation of a new aporphine alkaloid, (-)-*N*-methylguattescidine **1** (Alias, *et al*., 2010). This alkaloid, together with eight known alkaloids, namely liriodenine **2**, lanuginosine **3**, (-)-asimilobine **4**, dimethyltryptamine **5**, (-)-remerine **6**, (-)-anonaine **7**, columbamine **8** and lysicamine **9**, were obtained from the methanol extract of the bark of the plant. The new compound was characterized by analysis of spectroscopic methods such as NMR (Nuclear Magnetic Resonance), IR (Infrared) and GC-MS (Gas-Chromatography-Mass Spectrometry).

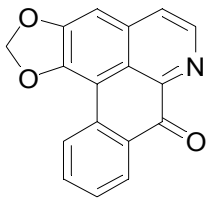
(-)-*N*-Methylguattescidine **1** exhibited a molecular formula of $C_{19}H_{17}O_4N$ based on the HRESIMS spectrum (positive mode), which showed a pseudomolecular ion at *m/z* 324.3581 $[M+H]^+$ (calcd. 324.3595). The UV spectrum showed an absorption band at 310 nm, suggesting the compound was an aporphine alkaloid with substitutions at position 1 and 2. The IR spectrum indicated the presence of C-H aromatic at 3056, C-O at 1266 and OH at 3409 cm^{-1}, respectively. The absorption of methyl group appeared at 2945 and 2833. The ^{13}C-NMR spectrum showed presence of 19 carbons. The signal at δ 198.0 ppm confirmed the presence of the carbonyl group, while the signal at δ 153.1 ppm is evidence for the oxygenated aromatic carbon. The DEPT spectrum revealed three methylene carbons at δ 26.9 ppm, 41.4 ppm and 96.9 ppm. Signal at δ 96.9 ppm is indicative of a methylenedioxy carbon. This is consistent with two doublets at δ 5.99 ppm (*J* = 1.2 Hz) and δ 6.07 ppm (*J* = 1.2 Hz) in the ^{1}H-NMR spectrum for the protons of methylenedioxy group which is typically located at positions 1 and 2. The characteristic ABD aromatic signals of H-11, H-10 and H-8 of aporphine alkaloid were observed at δ 8.24 ppm (*d, J* = 8.7 Hz), δ 7.13 ppm (*dd, J* = 8.7, 2.7 Hz) and δ 7.39 ppm (*d, J* = 2.7 Hz), respectively. The ^{1}H-NMR spectrum also exhibited an *N*-methyl signal at δ 2.34 ppm and another methyl group attached to C-6a gave a singlet at δ 1.52 ppm. The assignment of this methyl group at the 6a position is confirmed through its HMBC correlation with C-6a at δ 62.7 ppm, C-1b at δ 118.3 ppm and C-7 at δ 198.0 ppm. HMQC spectrum shows two cross peaks at δ 26.9 ppm (C-4) axis, represented the correlations of C-4 to H-4 (δ 2.55 ppm) and H-4′ (δ 3.00 ppm). At δ 41.4 ppm (C-5) axis, two

cross peaks showed the correlations between C-5 and H-5 (δ 2.99 ppm) and H-5′ (δ 3.01 ppm). The quaternary carbon signals were assigned based on HMBC experiment. C-1a at δ 108.9 ppm, C-7a at δ 126.0 ppm and C-9 at δ 153.1 ppm were assigned based on their correlations with H-11 at δ 8.24 ppm, while C-1b at δ 118.3 ppm and C-2 at δ 143.2 ppm showed correlations with H-3 at δ 6.54 ppm. (-)-*N*-methylguattescidine, is a rare 6a-methylated-7-oxo-aporphine alkaloid, having only been previously reported by Reynald *et al*. in 1982. Presented below are structures and spectroscopic data of the isolated compounds.



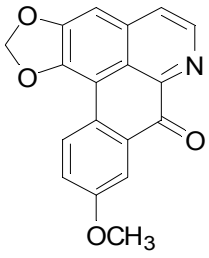
(1)

(-)-N-Methylguattescidine (**1**). yellow amorphous solid; [α] $^{30}_{D}$: -20° (c = 0.1 mg mL^{-1}, $CHCl_3$); MS *m/z*: 324.1242, $C_{19}H_{17}O_4N$; UV λ_{max} nm EtOH: 235, 310; IR υ_{max} cm^{-1}: 3409, 1710, 1266; ^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm : 8.24 (1H, d, *J* = 8.7 Hz, H-11), 7.39 (1H, d, *J* = 2.7 Hz, H-8), 7.13 (1H, dd, J_o = 8.7 Hz; J_m = 2.7 Hz, H-10), 6.54 (1H, s, H-3), 6.07 (1H, d, *J* = 1.2 Hz, H-2), 5.99 (1H, d, *J* = 1.2 Hz, H-1), 3.52 (1H, m, H-11a), 3.01 (1H, m, H-5), 3.00 (1H, m, H-4), 2.99 (1H, m, H-5′), 2.55 (1H, m, H-4′); ^{13}C NMR ($CDCl_3$, 75 MHz) δppm : 153.1 (C-9), 143.2 (C-2), 138.8 (C-1), 126.0 (C-7a), 125.3 (C-3a), 123.1 (C-11a), 122.7 (C-11), 122.2 (C-10), 118.3 (C-1b), 110.3 (C-8), 108.9 (C-1a), 103.9 (C-3), 96.9 (O-CH_2-O), 62.7 (C-6a), 41.4 (C-5), 34.1 (N-CH_3), 26.9 (C-4), 25.0 (CH_3).



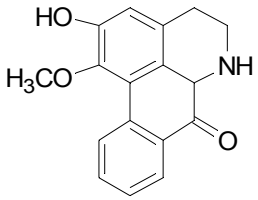
(2)

Liriodenine (**2**), yellow needles; MS m/z : 275, $C_{17}H_9O_3N$; UV λ_{max} nm EtOH : 215, 246, 268, 395, 412; IR υ_{max} cm^{-1} : 3054, 1726, 1421, 1265; ^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm : 8.9 (1H, d, *J* = 5.1 Hz, H-5), 8.66 (1H, dd, J_o = 7.2 Hz; J_m = 1.2 Hz, H-11), 8.59 (1H, dd, J_o = 7.8 Hz; J_m = 1.2Hz, H-8), 7.79 (1H, d, *J* = 5.1 Hz, H-4), 7.76 (1H, td, J_o = 7.8 Hz; 7.2 Hz; J_m = 1.5 Hz, H-10), 7.59 (1H, td, J_o = 7.8 Hz;7.2; J_m = 1.2 Hz, H-9), 7.16 (1H, s, H-3), 6.40 (2H, s, O-CH_2-O); ^{13}C NMR ($CDCl_3$, 75 MHz) δppm : 151.7 (C-2), 147.9 (C-1), 146 (C-6a), 145.4 (C-3a), 144.9 (C-5), 135.7 (C-1a), 133.9 (C-10), 132.9 (C-7a), 131.3 (C-11a), 128.8(C-8), 128.6 (C-9), 127.4 (C-11), 124.2 (C-4), 108.2 (C-1b), 103.3 (C-3), 102.4 (O-CH_2-O), 182.4 (C-7).



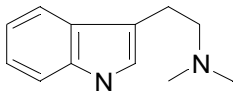
(3)

Lanuginosine (**3**), yellow needles; MS m/z : 305, $C_{18}H_{11}O_4N$; UV λ_{max} nm EtOH : 246, 271, 315, 258, 283, 334; IR υ_{max} cm^{-1} : 3055, 2987, 2306, 1712, 1635, 1363, 1265, 1046, 896; ^{1}H NMR ($CDCl_3$, 300MHz) δ ppm : 8.85 (1H, d, *J* = 5.4 Hz, H-5), 8.58 (1H, d, J_o = 9.0 Hz, H-11), 8.04 (1H, d, *J* = 3 Hz, H-8), 7.79 (1H, d, *J* = 5.4 Hz, H-4), 7.32 (1H, dd, J_o = 9.0 Hz; J_m = 3 Hz, H-10), 7.17 (1H, s, H-3), 6.47 (2H, s, O – CH_2 – O); ^{13}C NMR ($CDCl_3$, 75MHz) δ ppm : 158.0 (C-9), 151.0 (C-2), 146.0 (C-1), 144.9 (C-5), 144.0 (C-6a), 136.0 (C-3a), 133.0 (C-7a), 131.9 (C-1b), 129.1 (C-11), 126.2 (C-11a), 124.3 (C-4), 122.6 (C-10), 110.2 (C-8), 109.0 (C-1a), 102.3 (C-3), 55.8 (OCH_3), 102.5 (O – CH_2 – O), 182.0 (C-7).



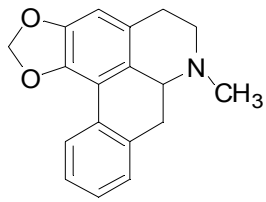
(4)

Asimilobine (**4**), brownish amorphous; MS m/z : 267, $C_{17}H_{17}O_2N$; UV λ_{max} nm EtOH : 274, 308; IR υ_{max} cm^{-1} : 3390, 1675, 1600, 1225; ^1H NMR ($CDCl_3$, 300MHz) δ ppm : 8.30 (1H, d, *J* = 7.8 Hz, H-11), 7.36 – 7.25 (3H, m, H-8, H-9, H-10), 6.73 (1H, s, H-3), 3.92 (1H, m, H-6a), 3.50 (1H, m, H-5′), 3.08 (1H, d, H-4′), 3.04 (1H, d, H-5), 2.99 (1H, m, H7), 2.85 (1H, m, H7), 2.74 (1H, d, H-4), 3.61 (3H, s, OCH_3), 2.00 (1H, s, N-H); ^{13}C NMR ($CDCl_3$, 75MHz) δ ppm : 148.6 (C-2), 143.0 (C-1), 135.6 (C-7a), 131.7 (C-11a), 129.4 (C-16), 128.1 (C-3a), 127.7 (C-8), 127.4 (C-10), 127.3 (C-9), 127.2 (C-11), 125.5 (C-1a), 114.6 (C-3), 53.4 (C-6a), 42.8 (C-5), 36.7 (C-7), 28.2 (C-4), 60.4 (OCH_3).



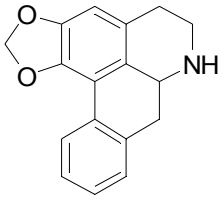
(5)

Dimethyltryptamine (**5**), reddish amorphous; MS m/z : 188, $C_{12}H_{16}N_2$; UV λ_{max} nm EtOH : 240, 252; IR υ_{max} cm^{-1} : 3945, 3055, 2305, 1634, 1422, 1265, 1046, 896; ^1H NMR ($CDCl_3$, 300MHz) δ ppm : 7.60 (1H, d, J_o = 5.7 Hz, H-7), 7.38 (1H, d, J_o = 7.1 Hz, H-4), 7.20 (1H, td, J_o = 6.9Hz; J_m0.9 Hz, H-5), 7.12 (1H, td, J_o = 6.9 Hz; J_m = 0.9 Hz, H-6), 3.03 (2H, m, H-8), 2.80 (2H, m, H-9), 8.28 (1H, brs, N-H), 2.49 (6H, s, 2(CH_3); ^{13}CNMR ($CDCl_3$, 75MHz) δ ppm : 136.0 (C-7), 127.0 (C-3a), 122.0 (C-6), 121.7 (C-2), 119.2 (C-5), 118.7 (C-4), 59.4 (C-9), 44 (C-3), 22.9 (C-8), 44.9 ($2CH_3$).



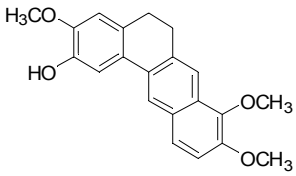
(6)

Remerine (**6**), yellow amorphous; MS m/z : 279, $C_{18}H_{17}O_2N$; UV λ_{max} nm EtOH : 234, 264; IR υ_{max} cm^{-1} : 1401, 1361, 1053, 942; ^1H NMR ($CDCl_3$, 300MHz) δ ppm : 8.09 (1H, d, J_o = 7.5 Hz, H-11), 7.34 – 7.24 (3H, m, H-8, H-9, H-10), 6.59 (1H, s, H-3), 4.000 (1H, m, H-6a), 3.4 (1H, m, H-5′), 3.10 (1H, m, H-4′), 3.00 (1H, m, H-5), 2.90 (1H, m, H-7′), 2.80 (2H, m, H-7, H-4), 6.11 (1H, d, J_m = 1.2 Hz, CH-O), 5.96 (1H, d, J_o = 1.2 Hz, CH-O), 2.62 (3H, s, CH_3); ^{13}C NMR ($CDCl_3$, 75MHz) δppm : 146.7 (C-2), 142.8 (C-1), 136.3 (C-7a), 128.1 (C-8), 128.0 (C-1b), 127.6 (C-9), 127.0 (C-10), 127.0 (C-11), 125.4 (C-3a), 126.5 (C-1a), 126.0 (C-11a), 125.4 (C-3a), 62.4 (C-6a), 53.3 (C-6a), 43 (C-5), 36.9 (C-7), 28 (C-4), 100.7 (O – CH_2 – O), 39.0 (CH_3).



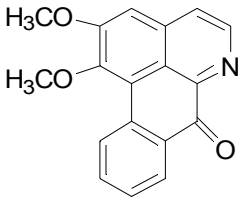
(7)

Anonaine (**7**)**,** yellow amorphous; MS m/z : 265, $C_{17}H_{13}O_2N$; UV λ_{max} nm EtOH : 234, 272, 315; IR υ_{max} cm^{-1} : 1040, 945; ^1H NMR ($CDCl_3$, 300MHz) δ ppm : 8.09 (1H, d, J_o = 7.5 Hz, H=11), 7.36 – 7.19 (3H, m, H-8, H-9, H-10), 6.6 (1H, s, H-3), 4.04 (1H, dd, H-6a), 3.48 (1H, m, H-5′), 3.1 (1H, m, H-4′), 3.07 (1H, m, H-5), 3.02 (1H, m, H-7′), 6.12 (1H, d, J_m = 1.5, CH – O), 5.97 (1H, d, J_m = 1.5, CH – O); ^{13}C NMR ($CDCl_3$, 75MHz) δ ppm : 147.0 (C-2), 143.0 (C-1), 135.4 (C-7a), 131.4 (C-11a), 129.0 (C-1b), 128.0 (C-3a), 127.8 (C-8), 127.7 (C-9), 127.0 (C-10), 126.1 (C-11), 116.3 (C-1a), 53.6 (C-6a), 43.6 (C-5), 37.4 (C-7), 29.6 (C-4), 100.6 (O –CH_2– O).



(8)

Columbamine (**8**), red amorphous solid; MS m/z : 338, $C_{20}H_{20}O_4N$; UV λ_{max} nm EtOH : 206, 225, 265, 345; IR υ_{max} cm^{-1} : 3390, 1600; ^1H NMR ($CDCl_3$, 300MHz) δ ppm : 9.00 (1H, s, H-8), 8.08 (1H, s, H-13), 7.65 (1H, d, J_o = 9.0 Hz, H-11), 7.61 (1H, d, J_o = 8.7 Hz, H-12), 7.27 (1H, s, H-1), 6.79 (1H, s, H-4), 4.68 (2H, t, J_o = 6.6 Hz; 6.3 Hz, H-6), 3.18 (2H, t, J_o = 6.0 Hz, H-5), 4.02 (3H, OCH_3), 3.99 (3H, OCH_3), 3.92 (3H, OCH_3). ; ^{13}C NMR ($CDCl_3$, 75MHz) δ ppm : 163.0 (C-10), 151.0 (C-3), 150.0 (C-4a), 149.0 (C-2), 142.0 (C-9), 140.0 (C-8), 139.0 (C-11), 132.0 (C-14), 129.0 (C-12a), 126.0 (C-1a), 123.3 (C-12), 120.0 (C-13), 119.0 (C-8a), 111.0 (C-4), 108.0 (C-1), 56.0 (C-6), 27.0 (C-5), 60.0 (OCH_3), 58.0 (OCH_3), 57.0 (OCH_3).



(9)

Lysicamine (**9**), yellow amorphous; MS m/z : 291, $C_{18}H_{13}O_3N$; UV λ_{max} nm EtOH : 214, 250, 255, 261, 319; IR υ_{max} cm^{-1} : 1675, 1600, 1225; ^1H NMR ($CDCl_3$, 300MHz) δ ppm : 9.10 (1H, d, J_o = 5.1 Hz, H-11), 8.70 (1H, d, J_o = 6.9 Hz, H-5) 8.48 (1H, dd, J_o = 7.5 Hz; J_m = 1.5 Hz, H-8), 7.76 (1H, td, J_o = 7.2 Hz; J_m = 1.5 Hz, H-10), 7.7 (1H, d, J_o = 6.9 Hz, H-4), 7.55 (1H, td, J_o = 7.2 Hz; J_m = 1.2 Hz, H-9), 7.24 (1H, s, H-3), 4.05 (3H, s, OCH_3), 3.97 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75MHz) δppm : 156.7 (C-6a), 152.0 (C-2), 145.2 (C-1), 139.0 (C-5), 135.3 (C-3a), 134.7 (C-11a), 132.0 (C-7a), 130.9 (C-10), 125.7 (C-9), 122.0 (C-1b), 119.6 (C-1a), 108.7 (C-3), 65.1 (OCH_3), 56.8 (OCH_3), 182.5 (C=O).

Table 1.

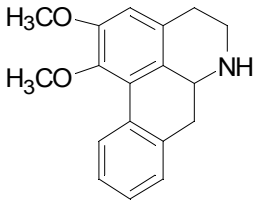
2.1.1 Alkaloids of *Meiogyne virgata*

Meiogyne virgata is a rainforest tree grows in Peninsular Malaysia, Borneo, Java and Sumatera. The genus *Meiogyne* (Annonaceae) consists of about 24 species and widely distributed in Indo-china, Thailand, Peninsular Malaysia, Sumatra, Java, Borneo and the Philippines. There is no formal report on the traditional uses of *Meiogyne virgata* in Malaysia. However, being an alkaloid rich species, it could be useful medicinally.

We have conducted phytochemical work on *Meiogyne virgata*. Six of the aporphine alkaloids in *Fissistigma latifolium* were also found in *Meiogyne virgata* collected from the Peninsular Malaysia. Isolation and purification of alkaloids from the bark of *Meiogyne virgata* afforded nine alkaloids; four oxoaporphines, liriodenine **2**, lanuginosine **3**, asimilobine **4** and lysicamine **9**; four aporphines, anonaine **7**, remerine **6**, nornuciferine **10** and norushinsunine **11**; and one azaanthracene alkaloid, cleistopholine **12**.

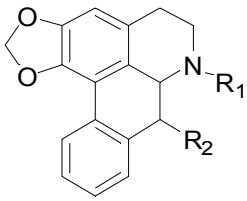
Most of the compounds are yellowish or colorless hygroscopic liquid at room temperature while impure samples will appear brownish. They have low solubility in water but dissolve well in methanol, chloroform, acetone, dichloromethane and other common organic solvent. They are also soluble in dilute acid as the protonated derivative. The melting point of these thype of compounds in range 100-300 ^oC.

Most of oxoaporphine and aporphine alkaloids showed IR spectra typified by the 7-oxo group with absorption band in the 1635-1660 cm^{-1} region. The UV spectra data for these type of compounds are quite characteristic for the skeletal type. There is indication that they may also be diagnostic for a particular oxygenation pattern. For example, 1, 2-methylenedioxy



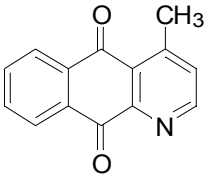
(**10**)

Nornuciferine (**10**), Colourless crystalline solid; MS m/z : 281 (M^+); UV λ_{max} nm EtOH : 234, 272, 315; IR υ_{max} cm^{-1} : 1040, 945
^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm : 8.39 (1H, *d*,*J*= 7.8 Hz, H-11), 7.33-7.20 (3H, *m*, H-8, H-9, H-10), 6.65 (1H, *s*, H-3), 3.98 (1H, *dd*, *J*= 13.4; 5.2 Hz, H-6a), 3.41 (1H, *dd*, *J*= 12.3; 6.3 Hz, H-5′), 3.90 (3H, *s*, OMe-2), 3.68 (3H, *s*, OMe-1), 3.08 (1H, *dd*, *J*=13.2 Hz, H-4), 3.04 (1H, *td*, *J*= 12.3; 5.1 Hz, H-5), 2.85 (1H, *dd*, *J*= 13.4 ; 5.2 Hz, H-7′), 2.68 (1H, *dd*, *J*= 13.2; 6.0 Hz, H-4′), 2.64 (1H, *t*, *J*= 13.4 Hz, H-7).; ^{13}C NMR ($CDCl_3$, 125 MHz) δ ppm : 152.3 (C-2), 145.2 (C-1), 135.0 (C-7a), 132.1 (C-1b), 132.1 (C-11a), 131.2 (C-3a), 128.4 (C-8), 127.8 (C-10), 127.4 (C-9), 127.1 (C-11), 126.6 (C-1a), 111.8 (C-3), 60.3 (OMe-1), 55.9 (OMe-2), 53.6 (C-6a), 43.0 (C-5), 37.2 (C-7), 29.7 (C-4).



(**11**)

Norushinsunine (**11**), Colourless crystalline solid; MS m/z : 281; UV λ_{max} nm EtOH : 217, 247, 252, 259, 273, 319; IR υ_{max} cm^{-1} : 3488, 3355, 1574, 1215; ^{1}H NMR ($CDCl_3$, 300MHz) δ ppm : 8.16 (1H, dd, *J* = 7.2;1.2 Hz, H=11), 7.45 (1H, td, *J* = 8.7;1.2 Hz, H-10), 7.40 (1H, dd, *J* = 8.1;0.9 Hz, H-8), 7.34 (1H, td, *J* = 7.2;1.2 Hz, H-9), 6.59 (1H, s, H-3), 6.11 (1H, d, *J* =1.5 Hz, O – CH_2 – O), 5.95 (1H, d, *J* = 1.2 Hz, O – CH_2 – O), 4.61 (1H, d, *J* = 3.0 Hz, H-7), 4.06 (1H, d, *J* = 3.3 Hz, CH – O). 3.37 (1H, ddd, *J* = 5.0;3.9;1.2 Hz, H-4', 2.68 (1H,dd, J=16.2;3.9 Hz, H-4); ^{13}C NMR ($CDCl_3$, 75MHz) δ ppm : 147.1 (C-1), 142.6 (C-2), 135.6 (C-1a), 130.3 (C-7a), 129.4 (C-9), 129.1 (C-3a), 123.6 (C-1b), 115.6 (C-11a), 108.4 (O – CH_2 – O), 71.0 (C-7), 57.2 (C-6a), 43.1 (C-5), 29.2 (C-4).



(**12**)

Cleistopholine (**12**), yellow glassy solid; MS m/z : 281 (M^+); UV λ_{max} nm EtOH : 234, 272, 315; IR υ_{max} cm^{-1} : 1040, 945; ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm : 8.95 (1H, *d*,*J*= 4.8 Hz, H-2), 8.31 (1H, *dd*,*J*= 8.5;2.2 Hz, H-5), 8.21 (1H, *dd*,*J*= 8.5;2.2 Hz, H-8), 7.79 (1H, m, H-6), 7.79 (1H, m, H-7), 2.89 (1H, s, CH3); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ ppm : 184.7 (C-9), 181.9 (C-10), 153.4 (C-2), 151.6 (C-4), 150.1 (C-9a), 134.6 (C-7), 134.2 (C-6), 132.6 (C-10a), 131.2 (C-3), 129.1 (C-4a), 127.4 (C-5), 127.2 (C-8), 22.8 (CH3).

Table 2.

derivative in compound **2** gives increase to a bathochromic shift in the 235-250 nm bands on comparison with the corresponding compound **9.** The addition of acid will gives a substantial bathochromic shift of the longest-wavelength band. In oxoaporphine and aporphine, position 1 and 2 are constantly oxygenated. It is frequent to find further oxygen substituent at C-9, C-10 and C-11 and occasionally at C-8. Other than that, H-4 and H-5 will give a characteristic AB system with doublet of doublet at about 7.6 ppm and 8.7 ppm with a coupling constant about 5.4 Hz. The small *J* value is due to the adjacent of electronegative nitrogen atom. The methylenedioxyl group gives singlet peak at about 6.0 ppm due to the inductive effect cause by existence of the neighboring C-7 carbonyl. The C-11 proton usually the most deshielded and the C-3 protons always appeared at a higher field then the aromatic hydrogen (Cordell, 1981). Presented below are structures and spectroscopic data of the isolated compounds.

3. The family of Rubiaceae as source of anthraquinones

Rubiaceae is among the largest flowering plants family comprising of 450 genera and 13,000 species. In Malaysia, 70 genera and 555 species of Rubiaceous plants were reported (Wong, 1989). Most Rubiaceous plants are shrubs or small trees and infrequently herbs (Hutchinson, 1973). Rubiaceous plants are distributed worldwide but they are mainly tropical. They are easily recognized at family level by decussate, entire leaves, presence of stipules, actinomorph flowers and inferior ovary.

Rubiaceous plants are known to accumulate substantial amount of anthraquinones particularly in the roots (Han*, et al.*, 2001). Anthraquinones containing plants are used traditionally for various ailments and health complaints such as diarrhea, loss of appetite, fever, wounds and cancer. The plant extracts are used in form of poultice, lotion and decoction from various plant parts. *Morinda*, *Hedyotis*, *Primatomeris* and *Rennellia* are among anthraquinone containing-genera that are widely used in Malaysian traditional medicine (Ismail*, et al.*, 1997; Jasril*, et al.*, 2003; Ahmad*, et al.*, 2005; Lajis*, et al.*, 2006; Osman*, et al.*, 2010).

Morinda comprises of approximately 80 species, distributed worldwide in tropical areas. It is considered to be highly nutritious plant and is used as traditional medicine. In Malaysia *M. citrifolia* and *M. elliptica* are widely used. The roots of *M. elliptica* are used to treat jaundice and gastric complaints and the leaves are used to treat flatulence and fever. *Prismatomeris* and *Hedyotis* species on the other hand are recorded in various traditional medicine systems such as Traditional Chinese Medicine. Several well-known *Prismatomeris species* used in folk medicine in Malaysia are *P. glabra* and *P. malayana. P. glabra* is claimed to be aphrodisiac and widely used in the east coast of Malaysia. *P. Malayana* contained the anthraquinones, rubiadin and rubiadin-1-methyl ether (Lee, 1969). *Hedyotis* plants are generally consumed as tonic or febrifuge for treatment of diarrhea and dysentery (Lajis, et al., 2006). Several species of *Hedyotis* native to Malaysia are *H.capitellata*, *H. Herbaceae*, *H.dichtoma*, *H. diffusa* and *H. verticillata*. Besides anthraquinones, *Hedyotis* also contain β-cabolline alkaloids, flavonoids and triterpenes. *Rennellia* is another small genus of Rubiaceae family. Consists of shrubs and small trees, the plants may be found in lowland tropical rainforest of Peninsular Malaysia and Sumatra. *R. elliptica*, is used for general health improvements and dubbed as Malaysian Ginseng most likely due to the appearance of its yellow roots.

Anthraquinones of the Malaysian Rubiaceae are generally of the *Rubia* type. Rings A and B of the anthraquinone skeleton are biosynthetically derived from chorismic acid and *a*-ketoglutarate *via* o-succinylbenzoic acid, whereas ring C is formed from isopentenyl diphosphate *via* the terpenoid pathway (Han, *et al.*, 2001). Chorismate is first converted to isochorismate, and then to *o*-succinoylbenzoic acid (OSB) in the presence of α-ketoglutarate and thiamine diphosphate. OSB is activated at the aliphatic carboxyl group to produce an OSB-CoA ester. It is the ring closure of OSB-CoA which results in the formation of 1,4-dihydroxy-2-naphthoic acid (DHNA) leading to ring A and B. The prenylation of DHNA at C-3, leads to naphthoquinol or naphthoquinone. The ring C formation is a consequence of the cyclization *via* C-C bond between the aromatic ring of the naphthoquinone and an isoprene unit, isopentenyl diphosphate (IPP) or 3,3-dimethylallyl diphosphate (DMAPP).

Of the anthraquinone from Malaysian Rubiaceeae are substituted only on ring C while the remaining are substituted on both ring A and ring C. Anthraquinones from genus *Morinda* are typically substituted at C-1, C-2, and C-5, C-6 or C-7, C-8 and C-1, C-2 and C-3

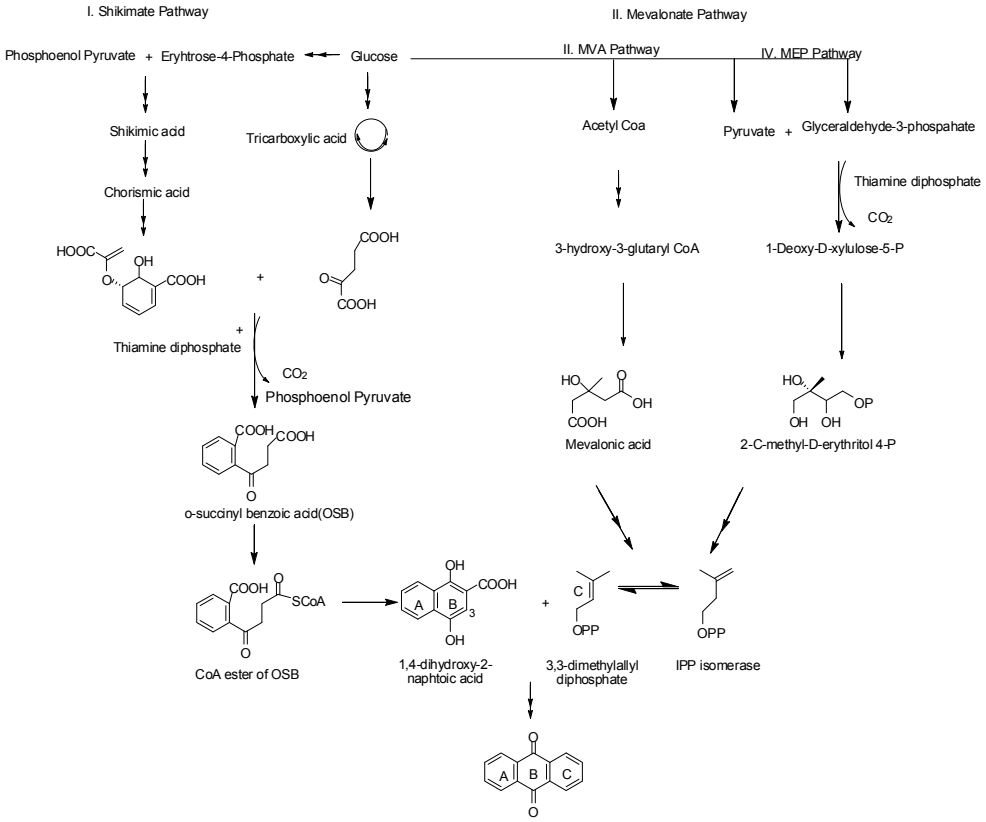


Fig. 2. Biosynthetic Pathway of Anthraquinones

meanwhile anthraquinones from *Hedyotis* are differed by rare substitution at C-1, C-2 and C-4. Anthraquinones from *Hedyotis* displayed wide structural variation. *H. capitellata* contains furanoanthraquinones (Ahmad, *et al.*, 2005) and *H. dichotoma* was reported to contain both 9,10- and 1,4-anthraquinone (Hamzah & Lajis, 1998). Genus *Rennellia* is closely related to *Morinda* and anthraquinones reported from *R. elliptica* are similar to those from genus *Morinda* (Osman *et al.*, 2010). One particular difference is the occurrence of anthraquinone with methyl substitution at C-6 which is characteristic to this plant.

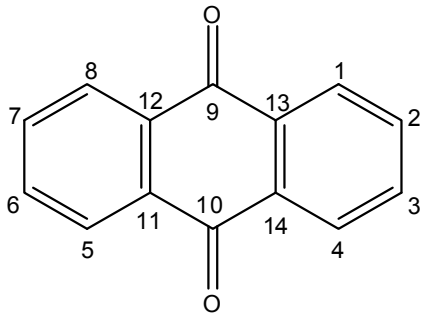


Fig. 3. Basic Skeleton of Anthraquinones

There are several characteristic spectroscopic data that distinguished anthraquinones from other types of compounds. In mass spectra, the major fragmentations are due to two consecutive loss of carbonyls, $[M\text{-}CO]^+$ and $[M\text{-}2CO]^+$. In the IR spectra, the unchelated carbonyl only viewed as one sharp stretching band at 1670 cm^{-1} due to symmetrical character of 9,10-anthraquinone (Derksen, *et al.*, 2002). Anthraquinones substituted with hydroxyl at *peri* position displayed two carbonyl absorption bands at about 1670 cm^{-1} and 1630 cm^{-1}. Anthraquinones give several chracteristic UV absorptions at 265-280 nm and 285-290 nm due to electron transfers bonds of benzoid chromophore and at 430-437 nm due local excitation of quinoid carbonyls. The location hydroxyl substituent can be distinguished by observing the absorption maxima in UV spectra. Addition of dilute sodium hydroxide solution caused bathchromic shift of absorption maxima. The shift is useful in distinguishing substitution pattern of polyhydroxyanthraquinones. Proton NMR spectra of 9,10-anthraquinones shows typical A_2B_2 substitution pattern of *ortho*-substituted aromatic ring. An unsubstituted anthraquinone ring can be easily distinguished by the presence of at least two sets of multiplets at ca. δ_H 8.10 and ca. δ_H 7.20 in the aromatic region. Anthraquinones substituted at both rings A and C will give several doublets in the aromatic region. The two carbonyl groups in the molecule can be easily distinguished if hydroxyl substituents present in *para* position. Hydroxyl groups adjacent to carbonyl can be seen as sharp singlets much downfield at δ_H 12-14 due to strong intramolecular hydrogen bonding to the adjacent carbonyl. The presence of hydroxyl adjacent to carbonyl cause significant shift of carbonyl carbon resonance to downfield region at 186-189 ppm.

3.1 Anthraquinones of *Rennellia elliptica* Korth.

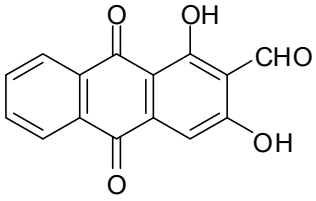
R. elliptica Korth. was also previously known as *R. elongata* (King & Gamble) Ridl. It is a shrub of about 2 m tall. This shrub can be found in lowland to hill forest to c. 500m above sea level. *R. elliptica* Korth. is widely distributed from Southern Myanmar to West Malaysia.

R. elliptica is used for general health improvements and dubbed as Malaysian Ginseng may be due to the appearance of its yellow roots. Its medicinal uses were documented as treatment of body aches, after-birth tonic and aphrodisiac (Mat Salleh & Latiff, 2002). The root extract of *R. elliptica* was reported to be antimalarial (Osman, *et al.*, 2010) and antioxidant (Ahmad, *et al.*, 2010). Further study is warranted to investigate the antimalarial potential of roots of *R. elliptca*.

Fig. 4. Rennellia elliptica Korth

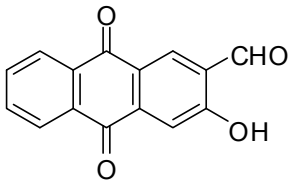
Phytochemical studies of the roots of *R. elliptica* Korth. resulted a new anthraquinone 1,2-dimethoxy-6-methyl-9,10-anthraquinone **18**, along with ten known ones. The known anthraquinones were nordamnacanthal **13**, 2-formyl-3-hydroxy-9,10-anthraquinone **14**, damnacanthal **15,** 1-hydroxy-2-methoxy-6-methyl-9,10-anthraquinone **16**, lucidin-ω-methyl ether **17**, 3-hydroxy-2-methoxy-6-methyl-9,10-anthraquinone **19**, rubiadin 2**0**, 3-hydroxy-2-methyl-9,10-anthraquinone **21**, rubiadin-1-methyl ether **22** and 3-hydroxy-2-hydroxymethyl-9,10-anthraquinone **23**.

Anthraquinone **18**, 1,2-dimethoxy-6-methyl-9,10-anthraquinone, isolated for the first time as bright yellow amorphous solid. The HREIMS of **18** displayed a $[M + H]^+$ peak at 283.0968 [calc 283.3067] suggesting a molecular formula of $C_{17}H_{14}O_4$. The absorption maxima in the UV spectrum were observed at 373, 341 and 257 nm, indicative of an anthraquinone moiety. The IR spectrum did not show presence of chelated carbonyl and hydroxyl groups. The sp^2 C-H stretch for the aromatic ring was observed at 3,081 cm^{-1}. With the exception of the sharp singlet in the downfield region for the hydrogen-bonded hydroxyl group, the ^1H NMR spectrum resembles that of compound **16**, suggesting a similar substitution pattern. Splitting pattern of the five aromatic proton signals suggested substitutions on both rings. Two overlapping doublets centered at δ_H 8.17 are due to H-8 (*d*, *J* = 7.8 Hz) and H-4 (*d*, *J* = 8.7 Hz), the *peri*-hydrogens. A doublet at δ_H 7.28 (*J* = 8.7 Hz) is due to H-3, meanwhile H-7 gave another doublet of doublet at δ_H 7.58 (J_o = 7.8 Hz, J_m = 1.7 Hz). These assignments were confirmed by their respective correlations in the COSY spectrum. H-5 resonated as a singlet at 8.06 ppm. In addition, two sharp singlets at δ_H 2.53 (3H, *s*) and 4.02 (6H, *s*) due to a methyl and two methoxy groups, respectively, were also observed. The location of the methoxy groups were established at C-1 and C-2 of ring C based on its NOE correlation with H-3. Thus, the only possible location for the methyl substituent is at C-6. This assignment was confirmed through NOE correlations of the methyl group with H-5 and H-7. The placement of methyl group at C-6 was further confirmed by HMBC experiment



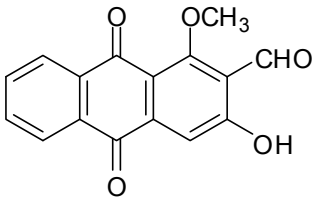
(13)

Nordamnacanthal. (**13**) Orange crystals. Mps 216-219 ° [lit. 220 ° C (Me_2CO) Chang (1984)]. UVλ_{max} EtOH nm: 421, 295, 259. UVλ_{max} EtOH/ -OH nm: 512, 357, 283. IR ν_{max} (KBr) cm^{-1} :3460, 1646, 1627, 1382. MS m/z 268 [M^+], 240, 212, 184, 138. ^{1}H NMR ($CDCl_3$, 300MHz): 14.05 (1H, s, 1-OH), 12.70 (1H, s, 3-OH), 10.52 (1H, s, 2-CHO), 8.30 (2H, m, H-5, H-8), 7.88 (2H, m, H-6, H-7), 7.36 (1H, s, H-4). ^{13}C NMR ($CDCl_3$, 75.5 MHz): 193.9 (2- CHO), 186.8 (C=O, C-9), 181.4 (C=O, C-10), 169.2 (C-OH,C-1), 168. 1 (C-OH, C-3), 139.1 (C-2), 134.8 (C-7), 134.7 (C-6), 133.3 (C-14), 133.2 (C-13), 127. 8 (C-8), 127.0 (H-5), 112.1 (C-14), 109.4 (C-4), 109.1 (C-13)



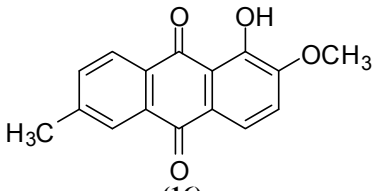
(14)

3-Formyl-2-hydroxy-9,10-anthraquinone (**14**). Bright orange needle crystals. Mps 212-214 °C [259-260 ° C, Rath et al.(1995)]. UVλ_{max} EtOH nm: 380, 277, 246. UVλ_{max} EtOH/ -OH nm: 466, 392, 310, 254. IR ν_{max} (KBr) cm^{-1}: 3467, 1655, 1657, 1564. MS m/z 252 [M^+], 229, 206, 167, 139. ^{1}H NMR ($CDCl_3$, 300MHz): 11.45 (1H, s, 3-OH), 10.17 (1H, s, 2-CHO), 8.68 (1H, s, H-4), 8.35 (2H, m, H-5, H-8), 7.88 (2H, m, H-6, H-7), 7.86(1H, s, H-1) .^{13}C NMR ($CDCl_3$, 75.5 MHz): 196.8 (2-CHO), 181.0 (C=O, C-9, C-10), 165.3 (C-OH, C-3), 139.1, 134.8 (C-4), 134.8, 133.3, 127.6, 127.5, 127.3, 126.1, 124.5, 123.4, 116.5 (C-1)



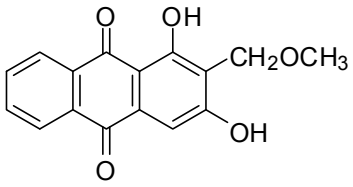
(15)

Damnacanthal (**15**). Yellow crystals. Mps 208-211°C [lit. 218-218.5 ° C (Me_2CO) Chang (1984)]. UVλ_{max} EtOH nm: 381, 284, 250, 213. UVλ_{max} EtOH/ -OH nm: 460, 379, 315, 262, 250. IR ν_{max} (KBr) cm^{-1}: 3437, 1644, 1561. MS m/z: 282 [M^+], 254, 225, 196. ^{1}H NMR ($CDCl_3$, 300MHz): 12.29 (1H, s, 3-OH), 10.49 (1H, s, 2-CHO), 8.25 (2H, m, H-5, H-8), 7.84 (2H, m, H-6, H-7), 7.68 (1H, s, H-4), 4.14 (3H, s, 1-OCH_3)



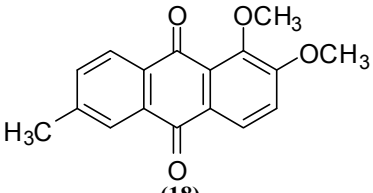
(16)

1-Hydroxy-2-methoxy-6-methyl-9,10-anthraquinone (**16**). Red needle crystals. Mps 220-221 °C. UVλ_{max} EtOH nm: 421, 278, 262, 231. UVλ_{max} EtOH/ -OH nm: 505, 314, 258. IR ν_{max} (KBr) cm^{-1}: 3467, 1653, 1637. MS m/z: 268 [M^+], 239, 197, 169,139, 115. ^{1}H NMR (CDCl3, 300MHz): 13.20 (1H, s, 1-OH), 8.23 (1H, d, *J*=8.1, H-8), 8.12 (1H, s, H-5), 7.89 (1H, d, *J*=8.4, H-4), 7.61 (1H, d, *J*=8.1, H-7), 7.19 (1H, d, *J*=8.4, H-3), 4.04 (3H, s, 2-OCH_3), 2.56 (3H, s, 6-CH_3). ^{13}C NMR (CDCl3, 75.5 MHz): 189.1(C=O, C-9), 181.8 (C=O, C-10), 154.0 (C-OH, C-1), 152.7 (C-OCH_3, C2), 146.2 (C-6), 134.6 (C-7), 134.0 (C-11), 131.10 (C-12), 127.8 (C-5), 127.1 (C-8), 125.5 (C-14), 121.0 (C-4), 116.1 (C-13), 115.6 (C-3), 56.4 (2-OCH_3), 22.0 (6-CH_3)



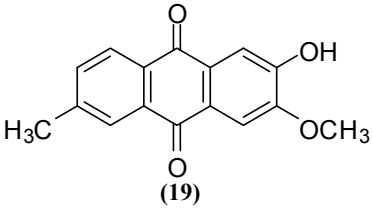
(17)

Lucidin-ω-methyl ether (**17**). Yellow crystals. Mps 175-179°C [lit 170 ° C, Dictionary of Natural Products (1995); 163-166 ° C, Leistner (1975)]. UVλ_{max} EtOH nm: 412, 280, 24. UVλ_{max} EtOH/ -OH nm: 491, 314, 242 IR ν_{max} (KBr) cm^{-1}: 3428, 2927, 1668, 162. MS m/z: 284 [M^+], 263, 241, 213, 185. ^{1}H NMR ($CDCl_3$, 300MHz): 13.29 (1H, s, 1-OH), 9.39 (1H, s br, 3-OH), 8.27 (2H, m, H-5, H-8), 7.80 (2H, m, H-6, H-7), 7.32 (1H, s, H-4), 4.90 (2H, s, 2-**CH_2**OCH_3), 3.59 (3H, s, 2-CH_2O**CH_3**). ^{13}C NMR ($CDCl_3$, 75.5 MHz): 186.9 (C=O, C-9), 182.2 (C=O, C-10), 164.1 (C-1), 161.7 (C-3), 134.1 (C-11), 134.1 (C-12), 133.6 (C-6), 133.5 (C-7), 126.8 (C-2), 114.4 (C-4), 109.7 (C-8), 109.7 (C-13), 109.6 (C-5), 109.6 (C-14), 68.9 (2-**CH_2**OCH_3), 59.3 (2-CH_2OCH_3)



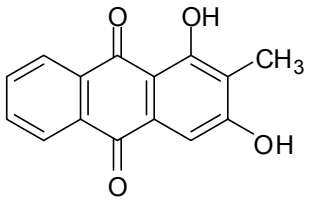
(18)

1,2-Dimethoxy-6-methyl-9,10-anthraquinone (**18**). Bright yellow crystals. Mps 193-196 °C. UVλ_{max} EtOH nm: 373, 341, 257, 222. UVλ_{max} EtOH/ -OH nm: 373, 342, 257, 222. IR ν_{max} (KBr) cm^{-1}:1666, 1601, 1327, 1267MS m/z: 282 [M^+], 253, 221, 194, 165, 139. ^{1}H NMR ($CDCl_3$, 300MHz): 8.17 (2H, dd, *J*=8.7, 7.8, H-4, H-8), 8.06 (1H, s, H-5), 7.58 (1H, d, *J*=7.8, H-7), 7.28 (1H, d, *J*=8.7, H-3), 4.02 (6H, s, 1-OCH_3, 2-OCH_3), 2.53 (3H, s, 6-CH_3).^{13}C NMR ($CDCl_3$, 75.5 MHz): 182.7 (C=O, C-9), 182.7 (C=O, C-10), 159.1 (C-1), 149.6 (C-2), 144.6 (C-6), 134.8 (C-7), 132. 9 (C-11), 132.9 (C-12), 127.5 (C-14), 127.4 (C-13), 127.1 (C-8), 126.9 (C-5), 125.2 (C-4), 115.9 (C-3), 61.3 (1-OCH_3), 56.3 (2-OCH_3), 21.8 (6-CH_3)



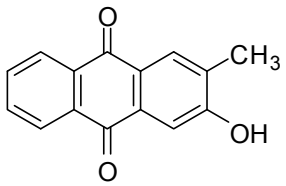
(19)

2-Hydroxy-3-methoxy-6-methyl-9,10-anthraquinone (**19**) Light yellow amorphous solid. Mp 210-215 °C. UVλ_{max} EtOH nm: 393, 286, 244. UVλ_{max} EtOH/ -OH nm: 509, 316, 250. IR ν_{max} (KBr) cm^{-1}: 3203, 2927, 2869, 1666, 1265. MS m/z: 268 [M^+], 239, 207, 169. ^{1}H NMR ($CDCl_3$, 300MHz): 8.18 (1H, d, *J*=8.1, H-8), 8.08 (1H, s, H-5), 7.79 (1H, s, H-1), 7.76 (1H, s, H-4),7.57 (1H, d, *J*=8.1, H-7), 6.23 (1H, s br, 2-OH), 4.11 (3H, s, 3-OCH_3), 2.54 (3H, s, 6-CH_3). ^{13}C NMR ($CDCl_3$, 75.5 MHz): 182.4 (C=O), 162.8(C-OH, C-2), 151.4 (C-OCH_3, C-3), 144.9, 134.5, 133.6, 127.4, 127.2, 112.6, 108.3, 56.6 (3-OCH_3), 21.9 (6-CH_3)



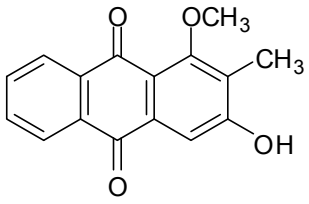
(20)

Rubiadin (**20**). Yellow crystals. Mps 250-258 °C [lit. 280-283 ° C, Leistner (1975)]. UVλ_{max} EtOH nm: 413, 279. UVλ_{max} EtOH/ -OH nm: 496, 314, 241 IR ν_{max} (KBr) cm^{-1}: 3436, 1653, 1626. MS m/z: 254 [M+], 226, 197, 152, 115. ^{1}H NMR (Acetone$_{-d6}$, 300MHz): 13.20 (1H, s, 1-OH), 8.31 (1H, m, H-8), 8.23 (1H, m, H-5), 7.92 (2H, m, H-6, H-7), 7.38 (1H, s, H), 2.20 (3H, s, 2-CH_3). ^{13}C NMR (Acetone$_{-d6}$, 75.5 MHz): 186.9 (C=O,C9), 181.8 (C=O, C-10), 163.2 (C-OH, C-1), 162.4 (C-OH, C-3), 134.3, 134.2, 133.5, 133.5, 132.4, 126.8, 126.5, 117.9, 107.17, 7.3



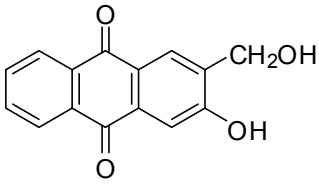
(21)

3-Hydroxy-2-methyl-9,10-anthraquinone (**21**). Yellow crystals. Mps 138- 142 ° C. UVλ_{max} EtOH nm: 379, 329, 274, 245, 239. UVλ_{max} EtOH/ -OH nm: 496, 314, 246 . IR ν_{max} (KBr) cm^{-1}: 3436, 1663, 651. MS m/z 238 [M^{+}], 238, 210, 181, 152, 105. ^{1}H NMR (Acetone$_{-d6}$, 300MHz): 8.23 (2H, m, H-5, H-8), 8.05 (1H, s, H-1), 7.89 (2H, m, H-6, H-7), 7.67 (1H, s, H-4), 2.39 (3H, s, 2-CH_3). ^{13}C NMR (Acetone$_{-d6}$, 75.5 MHz): 182.6 (C=O, C-10), 181.5 (C=O, C-9), 161.0 (C-OH, C-3), 134.1 (C-2), 133.8 (C-14), 133.7 (C-7), 133.6 (C-6), 132.2 (C-13), 130.1 (C-1),126.6 (C-5), 126.5 (C-8), 111.4 (C-4), 15.6 (2-CH_3)



(22)

Rubiadin-1-methyl ether (**22**). Light yellow crystal. Mps 302-304 ° C [282-284, Briggs (1976); 300 ° C, Roberts (1977)]. UVλ_{max} EtOH nm: 354, 332, 279. UVλ_{max} EtOH/ -OH nm: 440, 314, 246. IR ν_{max} (KBr) cm^{-1}: 3437, 2913, 2847, 1668, 1651. MS m/z : 268, 239, 207, 181. ^{1}H NMR (Acetone$_{-d6}$, 300MHz): 9.50 (1H, s, br, 3-OH), 8.20 (2H, m, H-5, H-8), 7.87 (2H, m, H-6, H-7), 7.63 (1H, s, H-4), 3.90 (3H, s, 1-OCH_3), 2.27 (3H, s, 2-CH_3). ^{13}C NMR (Acetone$_{-d6}$, 75.5 MHz): 182.7 (C=O), 180.4 (C=O), 161.2 (C-OH), 140.6, 134.4, 134.2, 133.0, 132.6, 126.8, 126.0, 60.4 (OCH_3), 8.4 (CH_3)



(23)

3-Hydroxy-2-hydroxymethyl-9,10-anthraquinone (**23**). Light yellow solid . UVλ_{max} EtOH nm: 374, 274, 238. UVλ_{max} EtOH/ -OH nm: 481, 311, 246 IR ν_{max} (KBr) cm^{-1}: 3468, 1628. ^{1}H NMR (Acetone$_{-d6}$, 300MHz): 8.38 (1H, s, H-4), 8.24 (2H, m, H-5, H-8), 7.89 (2H, m, H-6, H-7), 7.63 (1H, s, H-1), 4.803 (2H,s, 2-CH_2OH). ^{13}C NMR (Acetone$_{-d6}$, 75.5 MHz): 182.9 (C=O, C-9), 181.7 (C=O, C-10), 160.1 (C-OH, C-3), 136. 1 (C-2), 134.2 (C-14), 134.1 (C-11), 133.8 (C-6), 133.7 (C-7), 133.6 (C-12), 126.7 (C-4), 126.6 (C-5), 125.9 (C-13), 125.6 (C-8), 111.5 (C-1), 59.0 (2-**CH_2**OH)

Table 3.

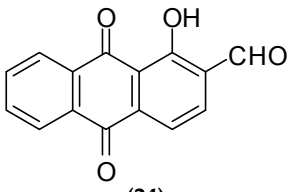
which showed a ^{3}J correlation with H-7. The methine carbons (C-3, C-4, C-5, C-7 and C-8) were assigned through HMQC correlations while the quaternary carbons (C-1, C-2, C-6, C-11, C-12, C-13 and C-14) were assigned based on careful analysis of HMBC spectrum. Both carbonyl carbons in this compound resonated very closely to each other with only 0.01 ppm difference at δ_C 182.70 and 182.71, which further confirmed the unchelated nature of the carbonyls. Presented below are structures and spectroscopic data of the isolated compounds.

3.2 Anthraquinones of *Morinda elliptica*

Morinda elliptica or locally known as 'mengkudu kecil' is a shrub or small tree and it is very common in wild state of Malay Peninsula and northwards Burma (Burkill, 1966). It can be seen growing wild in newly developed areas, bushes and lowland secondary forest throughout the peninsula. *M. elliptica* is very common and always available and mostly used by the Malays for medicinal purposes. Traditionally, different parts of the plant are used in various ways for a number of health problems and ailments. The leaves may be added to rice for loss of appetite and taken for headache, cholera, diarrhea and wounds. Sometimes a lotion is made and used for hemorrhoid and applied upon body after childbirth (Burkill, 1966). The extracts and anthraquinones isolated from *M. elliptica* were reported to possess wide spectrum of biological activities such as antioxidant (Ismail, *et al.*, 2002; Jasril, *et al.*, 2003), antimicrobial, anti-HIV and anticancer (Ali, *et al.*, 2000).

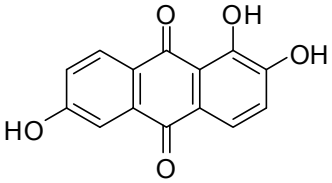
Fig. 5. *Morinda elliptica*

Five anthraquinones in roots of *M. elliptica* which are nordamnacanthal **13**, damnacanthal **15**, lucidin-ω-methyl ether **17,** rubiadin **20** and rubiadin-l-methyl ether **22** are the same constituents found in *R. elliptica*. The others are 1-hydroxy-2-methylanthraquinone **23**, soranjidiol **25**, morindone **26**, morindone-5- methyl ether **27** and alizarin-1-methyl ether **28.** In addition, 2-formyl-1-hydroxyanthraquinone **24** was reported as a new naturally occuring anthraquinone from roots of *M. elliptica*. HR-MS of **24** showed molecular ion peak at 252.0414 consistent with molecular formula of $C_{15}H_{14}O_4$. A bathchromic shift (407 to 531 nm) upon adding NaOH suggested the presence of OH at C-1 of the anthraquinone skeleton. The presence of hydroxyl group was evident from the broad stretching band observed at 3448 cm^{-1}. Two sharp stretching vibrations due to chelated and unchelated carbonyls were observed at 1638 and 1676 cm^{-1}, respectively. In the proton NMR, the signal for chelated hydroxyl group is at δ_H 13.26. The splitting pattern of ^{1}H NMR suggest substitution pattern



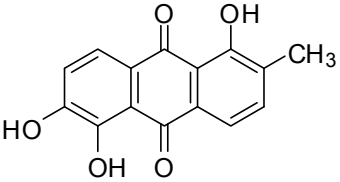
(24)

2-Formyl-1-hydroxy-9,10-anthraquinone (**24**). Mps 183-185 ° C [lit. 259-260 ° C, Rath et al. (1995)]. UVλmax EtOH nm: 229, 278, 331, 407. UVλmax EtOH/ -OH nm: 229, 280, 308, 531. IR νmax (KBr) cm-1: 3448 (OH), 1696 (aldehyde), 1676 (C=O unchelated), 1638 (C=O chelated), 1592 (C=C aromatic). MS m/z 252 (M+), 2224, 196, 168. ^{1}H NMR ($CDCl_3$, 500MHz): 13.26 (1H,s, 1-OH), 10.63 (1H, s, CHO), 8.35 (1H, m, H-8), 8.32 (1H, m, H-5), 8.23 (1H, d, J= 8.0 Hz, H-3), 7.89 (1H, d, J= 8.0 Hz, H-4), 7.88 (2H, m, H-6, H-7). 13C NMR(CDCl3, 125 MHz): 164.5 (C-1), 128.4 (C-2), 135.4 (C-3), 118.7 (C-4), 127.7 (C-5), 134.7 (C-6), 135.3 (C-7), 127.2 (C-8), 188.9 (C-9), 181.8 (C-10), 117.4 (C-11), 137.2 (C-12), 134.8 (C-13), 133.3 (C-14) and 188.0 (C-15)



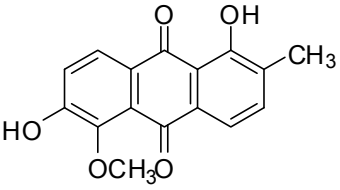
(25)

Soranjidiol. Yellow-orange neddles (**25**) Mps 276-273 ° C [lit. 271-272 ° C, Adesogan (1973)]. UVλ_{max} EtOH nm: 265, 409. UVλ_{max} EtOH/ -OH nm: 308, 489. IR ν_{max} (KBr) cm^{-1}: 3401 (OH), 1667 (C=O unchelated), 1635 (C=O chelated), 1593 (C=C aromatic). MS m/z 254 (M^+), 226, 197, 115. ^{1}H NMR ($DMSO_{-d6}$, 500 MHz): 13.10 (1H,s, 1-OH), 11.21 (1H, s, 6-OH), 7.63 (1H, d, J= 7.57 Hz, H-3), 7.57 (1H, d, J=7.57 Hz, H-4), 7.25 (1H, dd, $J_{7,8}$ = 8.55 Hz, $J_{7,5}$ = 2.69 Hz, H-7), 7.45 (1H, d, J= 2.69 Hz, H-5), 2.27 (3H, s, CH3). ^{13}C NMR ($DMSO_{-d6}$, 125 MHz): 187.6 (C=O), 181.8 (C=O), 163.8 (C-OH), 160.0 (C-OH), 136.9, 135.6, 134.2, 131.1, 129.8, 124.5, 121.4, 118.6, 114.7, 112.5, 15.8 (CH_3)



(26)

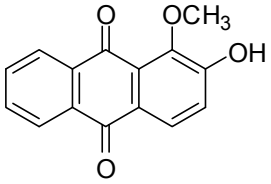
Morindone (**26**). Orange needles. Mps 240-241 ° C ($CHCl_3$) [lit. 248-249.5 ° C, Leistner (1975)]. UVλ_{max} EtOH nm: 260, 299, 448. UVλ_{max} EtOH/ -OH nm: 260, 302, 338, 558. IR ν_{max} (KBr) cm^{-1}: 3462 (OH), 1628 (C=O chelated). MS m/z 270 (M^+), 242, 135.^{1}H NMR ($CDCl_3$, 500MHz): 13.21 (1H, s, 1-OH), 12.95 (1H, s, 5-OH), 7.85 (1H, d- J=8.2 Hz, H-8), 7.75 (1H, d, J= 7.7 Hz, H-4), 7.52 (1H, d, J=7.6Hz, H-3), 7.26 (1H, d, J=8.2 Hz, H-7), 6.32 (1H, s, 6-OH), 2.39 (3H, s, CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz): 186.6 (C=O),179.9 (C=O), 170.4 (C-OH), 169.8 (C-OH), 161.4 (C-OH), 151.3, 149.4, 136.6, 130.9, 121.3, 119.8, 118.9, 115.3, 16.3 (CH_3)



(27)

Morindone-5-methyl ether (**27**). Orange cystals. Mp 232 ° C [lit. 223 ° C, Chang & Lee (1984)]. UVλ_{max} EtOH nm: 410, 497. UVλ_{max} EtOH/ -OH nm: 314, 388, 498. IR ν_{max} (KBr) cm^{-1}: 3389 (OH), 2926, 1672 (C=O unchelated), 1630 (C=O chelated), 1581 (C=C aromatic). MS m/z 284 (M^+), 266, 238, 197. ^{1}H NMR ($CDCl_3$, 500MHz): 13.02 (1H, s, 1-OH),8.14 (1H, d, J=8.55 Hz, H-8), 7.70 (1H, d, J= 8.06 Hz, H-4), 7.51 (1H, d, J=7.81 Hz, H-3), 7.35 (1H, d, J=8.54 Hz, H-7), 6.73

(1H, s, 6-OH), 4.03 (3H, s, OCH_3), 2.37 (3H, s, CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz): 187.8 (C=O), 182.0 (C=O), 160.6 (C-OH), 155.9, 146.8, 136.9, 134.5, 132.3, 127.1, 125.9, 125.5, 112.0, 118.9, 114.7, 62.3 (OCH_3), 16.1 (CH_3)



(28)

Alizarin-1-methyl ether. Yellow-orange crystals (**28**). Mp 164 [lit 178-179 °C, Chang & Lee (1984)]. UVλ_{max} EtOH nm: 313, 378, 485. UVλ_{max} EtOH/ -OH nm: 315, 333, 493. IR ν_{max} (KBr) cm^{-1}: 3443 (OH), 2926, 1671 (C=O unchelated), 1589 (C=C) aromatic. MS m/z 254 (M^+), 236, 208, 183. ^1H NMR ($DMSO_{-d6}$, 500MHz): 8.28 (2H, m, H-5, H-8), 8.15 (1H, d, J= 8.55 Hz, H-4), 7.78 (2H, m, H-7, H-6), 7.37 (1H, d, J = 8.54 Hz, H-3), 6.70 (1H, s, 2-OH), 4.04 (3H, s, OCH_3). ^{13}C NMR ($DMSO_{-d6}$ 125 MHz): 182.7 (C=O), 182.1 (C=O), 155.5, 146.6, 131.4, 133.9, 132.9, 127.5, 127.1, 126.8, 125.8, 125.6, 120.2, 62.3 (OCH_3)

on ring C only. H-3 and H-4 appeared as doublets at δ_H 8.23 and 7.89 respectively. A formyl group (δ_H 10.63) is attached to C-2. HMBC correlations of C-10 with H-3 and H-5 confirmed the assignment of the protons at their respective positions and supported by their respective COSY correlations. ^{13}C NMR showed fifteen carbons peaks as expected. One of the chelated carbonyl carbon was further downfield at δ_C 188.9 (C-9), confirming the chelated nature of this carbonyl. The assignment of carbons were accomplished using FGHMQC and FGHMBC experiment. Presented below are structures and spectroscopic data of the isolated compounds.

4. Conclusion

The phytochemical study on *Fissistigma latifolium* and *Meiogyne virgata* (Annonaceae) yielded twelve alkaloids; (-)-*N*-methylguattescidine **1**, liriodenine **2**, lanuginosine **3**, (-)-asimilobine **4**, dimethyltryptamine **5**, (-)-remerine **6**, (-)-anonaine **7**, columbamine **8**, lysicamine **9**, nornuciferine **10**, norushinsunine **11** and cleistopholine **12**. Tryptamine alkaloids have never been reported from *Fissistigma* species, whereas (-)-*N*-methylguattescidine **1** represents a rare finding of a naturally occurring 6a-methylated-7-oxo-aporphine alkaloid. Alkaloids **3**, **6**, **9** and **10** have never been reported from *Meiogyne* species.

Rennellia and *Morinda* are often confused with each other due to their similar traditional usage. Both plants are traditionally used for fever, postpartum and body ache treatment. Our phytochemical study on roots extract of *R. elliptica* showed significant similarities of major anthraquinones with those found in Morinda species. The major constituents of *R. elliptica*, nordamnacanthal, damnacanthal, rubiadian, rubiadin methyl ether and lucidin-ω-methyl ether are also present in *M. elliptica* and *M. citrifolia*.

5. Acknowledgment

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15

Phytochemistry of some Brazilian Plants with Aphrodisiac Activity

Cinara V. da Silva, Fernanda M. Borges and Eudes S. Velozo
Federal University of Bahia,
Brazil

1. Introduction

Since time immemorial man has used various parts of plants in the treatment and prevention of many ailments, including sexual impotence (Ayyanar & Ignacimuthu, 2009 as cited in Chah et al., 2006). Ancient people knew about herbal and animal aphrodisiacs, used in combinations like potions to mystical rites to infertility, to increase sexual performance, desire and pleasure (Malviya et al., 2011).

One of the first mentions of aphrodisiacs is in the Egyptian papyruses from 2300 to 1700 B.C. In the papyrus of Ebers, mandragora, garlic, onion and blue lotus were found as plants with aphrodisiac activity (Zanolari, 2003).

The tomb of Tutankhamon contain a gold plated shrine decorated with a bas-relief of a pharaoh holding a blue lotus and two mandragoras in his left hand, since the Egyptians believed in sexual life after death (Bertol et al., 2004).

Hindu poems dating from 2000 to 1000 B.C. and the Kama Sutra had already reported to the use of some products to enhance the sex (Zanolari, 2003). The traditional Chinese Medicine uses with aphrodisiac purpose, among others, ginseng, Chinese chive and parts of animals for example: dogs, rhino, bear and tiger penis and testicles (Still, 2003).

On this basis, the legendary love potions, such as Spanish fly, glandular products from musk deer and civet cats, varieties of natural oats (*Avena sativa*), ginseng, belladonna, and erotic foods like fish and oysters, are known aphrodisiacs (Drewes et al., 2003 as cited in Choudhary & Ur-Rahman, 1997).

The word aphrodisiac has its origin in Greek Mythology, most precisely from the goddess of love, Aphrodite. It has been used to define the products applied with proposal of increasing desire and drive associated with sexual instinct. Besides they have represented a passion of man, since historically, in all cultures, the sexual potency is considered as a significant part of the male ego and the anxiety and humiliation is frequently associated with a declining sexual ability (Malviya et al., 2011; Zanolari, 2003).

An aphrodisiac includes any food or drug that arouses the sexual instinct, induces venereal desire and increases pleasure and performance. There are two main types of aphrodisiacs: psychophysiological stimuli (visual, tactile, olfactory and aural) preparations and internal preparations (food, alcoholic drinks and love potion) (Malviya et al., 2011).

Currently, the increase in life expectancy of human beings has increased the demand for substances capable of improving quality of this longevity. Among these are products that enhance sexual performance, treat impotence or erectile dysfunction.

Brazil is the country with around 55,000 species of higher plants about a quarter of all known and greatest biodiversity in the world (Velozo et al., 2002). Many of these plants are used in folk medicine to aphrodisiac purposes in the form of teas, mixed with alcohol and other beverages. Some of them are belonging to the families like Anacardiaceae, Fabaceae, Sapindaceae, Amarantaceae, Amaryllidaceae, Aristolochiaceae, Bignoniaceae, Erythroxylaceae, Oleaceae, Asteraceae, Sapindaceae, Annonaceae and Dilleniaceae.

Several phytochemical studies, with species from these families above cited, have enabled the isolation of secondary metabolites possibly related to its pharmacological activity, such as alkaloids, flavonoids and saponins.

This chapter is a review on the chemical composition of Brazilian plants most used by the population for aphrodisiac purpose, searching rationalization between the chemical structure and biological activity (SAR).

2. Erectile dysfunction and aphrodisiac products

Erectile dysfunction (ED) is experienced at least some of the time by the most of men who have reached 45 years of age, and it is projected to affect 322 million men worldwide by 2025. This prevalence is high in men of all ages but increases greatly in the elderly (Seftel et al., 2002).

Sexual dysfunction, erectile dysfunction or male impotence is characterized by the inability to develop or maintain an erection of the penis and can be caused by psychological disorders like anxiety, stress and depression, physical disorders like chronic diseases: diabetes and hypertension; hormonal problems or sedentary life-style, alcohol and smoking abuses (Malviya et al., 2011; Sumalatha et al., 2010).

Drugs play a significant role in the pathogenesis of ED, altering hormonal or vascular mechanics needed for erection. Alterations in penile vessels can be observed in the elderly and in particular, lack of androgens may lead to a reduction of smooth muscle cells content in the penis and an increase in the caliber of vascular spaces (Vignera et al., 2011 as cited in Galiano et al., 2010).

An erection is a hemodynamic balance between inflow and outflow of blood within two chambers named corpus cavernosum and it starts with sensory and mental stimulation. There is a relaxation of the smooth muscles and arterioles which allows blood supply to flow in the sinusoidal space. The increased flow of blood, compress venules between sinusoids and the tunica albuginea of the corpus cavernosum. The lack of the distension of tunica albuginea results in venous occlusion, which increases the intracavernosal pressure, generating and sustaining a full erection (Zanolari, 2003).

The erection ends when the muscles of penis contract, opening outflow channels. The relaxation of cavernous smooth muscle is mediated by Nitric Oxide (NO) via cyclic guanosine monophosphate (cGMP). After sexual stimulation, nitric oxide is released by nerve endings and endothelial cells. Nitric oxide (NO) stimulates GMP cyclase to produce cGMP, which

leads to relaxation of smooth muscle. The erection ceases after a while because cGMP is hydrolysed by phosphodiesterase enzime into inactive GMP. Five types of phosphodiesterases are known to cause hydrolysis in cGMP. In the penis, phosphodiesterase is type V. Thus, a drug that inhibits the phosphodiesterase type V (cGMP-specific) should accelerate the action of nitric oxide and cGMP in erection (Drewes et al., 2003).

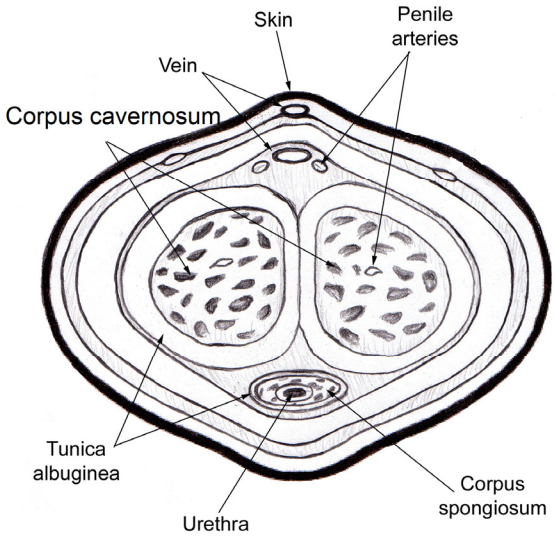


Fig. 1. Penis anatomy diagram

2.1 Male dysfunction therapies

The treatment with a psychotherapeutic approach is indicated to patients with psychological disorders. To patients with physical disorders, current treatments include oral medication, intracavernosal injection, vacuum pumps and penile prosthesis.

Some oral medications are available and well-established for ED treatment, among of them, two natural products: Cantharidin (Spanish fly) and Yohimbine, besides synthetic selective inhibitors, such as sildenafil (Viagra®), vardenafil (Levitra®), tadalafil (Cialis®), lodenafil (Helleva®) and udenafil (Zydena®) (see fig.2). The PDE-5 inhibitors have shown efficacy compared to placebo, in addition to present similar form of action and side effects like headache, flushing, dyspepsia and nasal congestion (Matheus et al., 2009; Wang et al., 2008).

The cantharidin is a lactone found in Spanish flies (also called Cantharides), beetles that have been cited in most of Asian and European Pharmacopoeias and have been used in dried form in internal preparations to impotence. Cantharides acts causing irritation of the urethra with vascular congestion, and inflammation of the erectile tissue. The Spanish flies are fallen into disuse due to their toxic effects (Zanolari, 2003).

Yohimbine is an indole alkaloid with a 2-adrenergic blocking activity. It comes from the bark of the African tree *Corynanthe yohimbe*, its first isolation was in the early 1930s and remained on the African market until 1973 like a drug marketed Aphrodex. Renewed

interest in yohimbine for ED has prompted several new investigative trials; however, there are indications of side-effects such as hypertension, anxiety, manic symptoms and interactions with used medications (Drewes et al., 2003).

Some natural products act like non-selective PDE inhibitors as the methylxanthines caffeine and theophylline, but others show similar effects to PDE-5 inhibitors, for example: flavonoids and derivatives (quercetin from *Allium cepa*, pyrano-isoflavones from *Eriosema kraussianum* - Kraussianone 1 and 2); alkaloids (Neferin from *Nelumbo nucifera*, Berberine from *Berberis aristata*, Papaverine from *Papaver somniferum* – used in association with Prostaglandin-E1 to injections intracavernosal), saponins (Steroidal saponins from *Allium tuberosum*), coumarins (Osthole from *Angelica pubescens*) and terpenes (Forskolin from *Coleus forskohlii*) (Drewes et al., 2003; Guohua et al., 2009; Rahimi et al., 2009; Sumalatha et al., 2010; Zanolari, 2003).

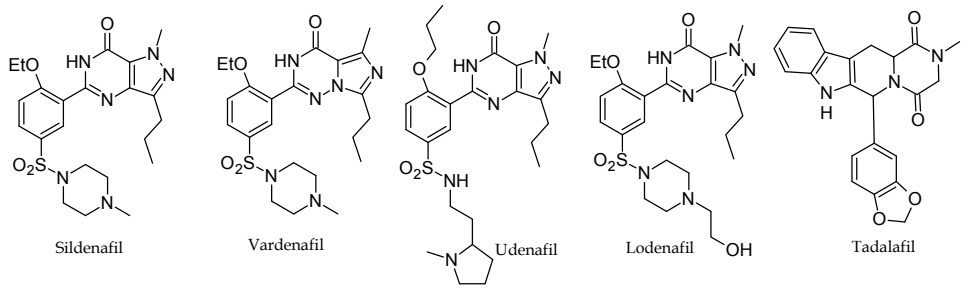


Fig. 2. Selective inhibitors of PDE-5

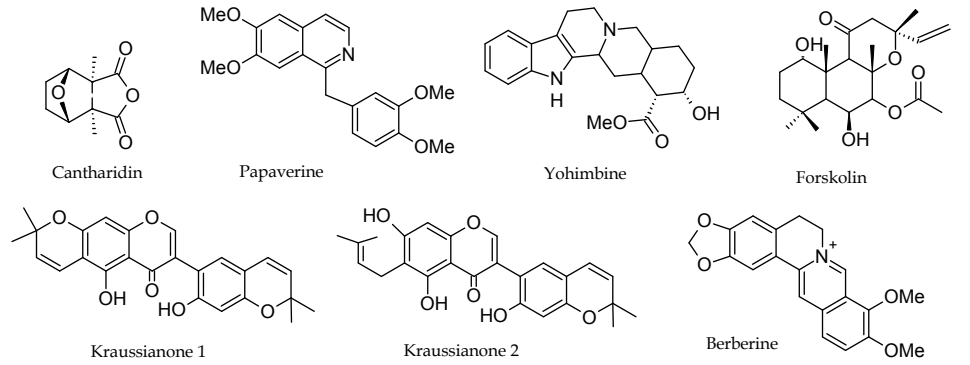


Fig. 3. Examples of natural products with aphrodisiac effect

2.2 Chemical of some Brazilian aphrodisiacs species and rationalization between structure and activity

The success of PDE-5 inhibitors, particularly of Viagra, the first inhibitor that has been marketed, the aging of the population and the quest for improved quality of life led to the search for new drugs with fewer side effects. As sources of research, plants used as aphrodisiacs have turned to folk medicine in whole world.

There are many herbal drugs that have been used by men with ED with varying degrees of success. Most potent aphrodisiacs herbal are available and have few side effects (Malviya et al., 2011).

Some of the genera and species listed in this work in *in vitro* tests showed satisfactory answers to such an aphrodisiac effect like *Turnera diffusa* (Estrada-Reyes et al., 2009), *Pfaffia paniculata* (Arletti et al., 1999), *Passiflora* (Patel et al. 2009), *Mucuna pruriens* (Suresh et al., 2009), *Mimosa pudica* (Pande & Pathak, 2009), *Mimosa tenuiflora* (Souza et al., 2008), *Achyrocline satureioides* (Hnatyszyn et al, 2004; Simões et al., 1986) and *Anemopaegma arvense* (Chieregatto, 2005).

The effects of the Brazilian herbal medicine Catuama® and each of its plant constituents (*Paullinia cupana, Trichilia catigua, Zingiber officinalis and Ptychopetalum olacoides*) were investigated on rabbit corpus cavernosum. Catuama® induced relaxations, but P. *cupana* was the most effective, increased the cAMP levels by 200% indicating that it is the main extract responsible for the relaxing effect (Antunes et al., 2001).

Specie (Family)	Part used	Popular Name
Achyrocline satureioides (Asteraceae)	Inflorescence	Macela do campo Macela
*Anacardium Ocidentale (*Anacardiaceae)	Nut Pseudo-fruit	Caju
Anemopaegma arvense (Bignoniaceae)	Stem bark Roots	Catuaba verdadeira Marapuama Alecrim do campo
Aristolochia cymbifera (Aristolochiaceae)	Stem	Cipó mil homens
Arrabidaea chica **(**Bignoniaceae)	Leaves	Cipó cruz Carajiru
Artocarpus integrifolia (Moraceae)	Seeds	Jaca
Davilla rugosa (Dilleniaceae)	Stem , Leaves	Cipó caboclo
Erythroxylum viceniifolium (Erythroxylaceae)	Stem bark	Catuaba
Hippeastrum psittacinum (Amaryllidaceae)	Bulbs	Alho-bravo Alho-do-mato Açucena-do-campo
Mimosa pudica (Fabaceae)	Stem bark	Dormideira
Mimosa tenuiflora (Fabaceae)	Stem bark	Jurema preta
Mucuna pruriensis (Fabaceae)	Seeds	Pó-de-mico Mucuna preta
Nymphaea ampla (Nymphaeaceae)	Whole plant	Ninfa branca
Passiflora sp. (*P. edulis, P. alata and P. caerulea)* (Passifloraceae)	Leaves	Maracujá
Paulinia cupana (Sapindaceae)	Seeds	Guaraná
Pfaffia paniculata (Amarantaceae)	Roots	Ginseng brasileiro
Ptychopetalum olacoides (Oleaceae)	Bark	Marapuama
Schinus terebinthifolius (Anarcadiaceae)	Bark	Aroeira vermelha
Trichilia catigua (Meliaceae)	Bark , Leaves	Catuaba
Turnera diffusa (Turneraceae)	Leaves	Damiana

Table 1. Main Brazilian species with aphrodisiac activity

2.2.1 Aphrodisiacs chemical classes

The classes of substances discussed were those with proven aphrodisiac activity or with this possible action. The compounds were separated in three main groups, according to structures similarities: flavonoids and others phenolics compounds; alkaloids, xanthins and others amines; and saponins.

2.2.1.1 Flavonoids and other phenolic compounds

Flavonoids are polyphenols with a diphenylpropane core. According to the chemical and biosynthetic routes, flavonoids are separated into different classes: chalcones, flavonols, flavones, dihydroflavonoids, anthocyanidins, isoflavones, aurones, pterocarpanes, neoflavonoids, bioflavonoids and are presents in all flowering plants.

The major classes are flavones, flavonols, anthocyanins, isoflavones and the flavan-3-ol derivatives (catechin and tannins) (Miean & Mohamed, 2001).

The flavonoids are widely distributed in gymnosperms and angiosperms with therapeutic potential because of their antioxidant, anti-inflammatory, hepatoprotective, cardio protective, antiulcer, anticancer, antimutagenic, antispasmodic, anti-allergic and antiviral activities, besides to show inhibit xanthine oxidase, protein kinase C and PDE (Rahimi et al., 2009; Ko et al., 2004).

Miean & Mohamed (2001) studied 62 tropical species to presence of flavonoids and observed that flavonol quercetin and derivatives, mainly quercetin glycosides, had major occurrence, however glycosides of kaempferol, luteolin and apigenin were also present. In fruits contained almost exclusively quercetin glycosides.

In plants surveyed, in addition to flavonoids, other phenols were found such as caffeic and chlorogenic acid in *Achyrocline satureioides* (Desmarchelier et al., 2000) and chlorogenic acid in *Trichilia catigua* (Lagos, 2006), besides anacardic acid in *Anacardium ocidentale* (Kubo et al., 1994).

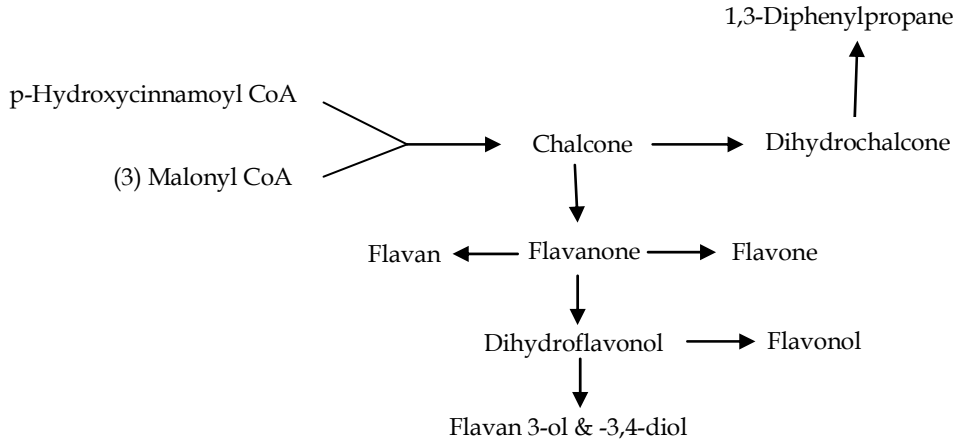


Fig. 4. Biosynthetic relationship among classes of flavonoids (Barron & Ibrahim, 1996)

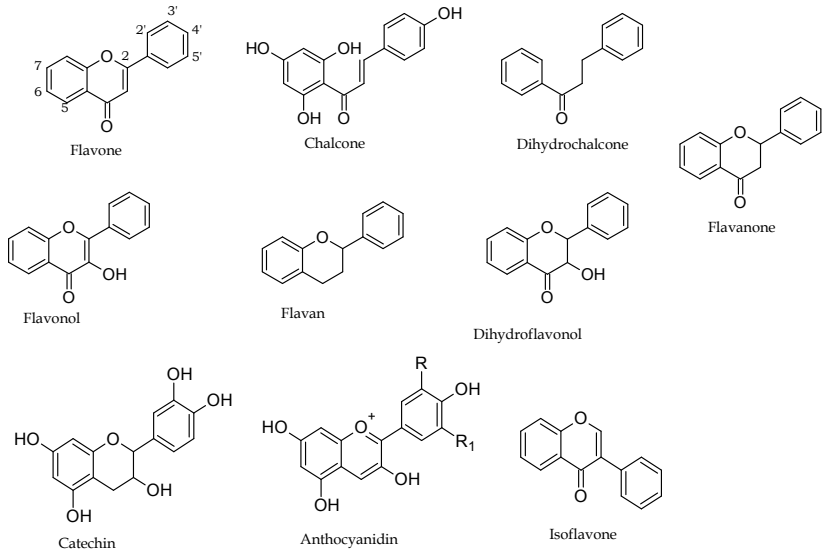


Fig. 5. Basic Structures of Flavonoids

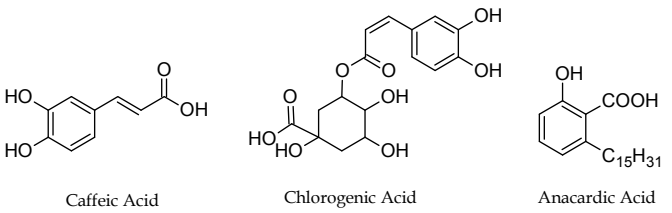
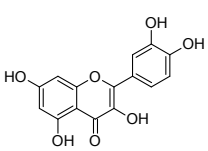
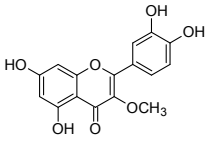
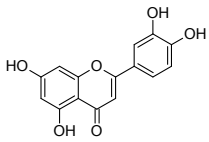
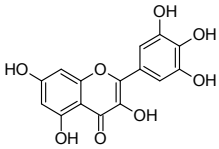
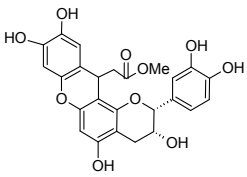
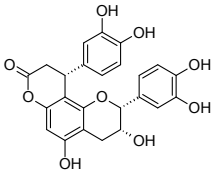
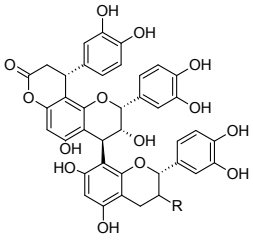
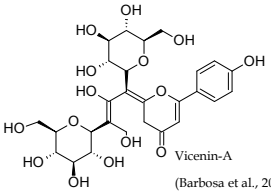
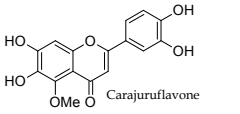
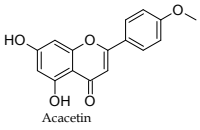
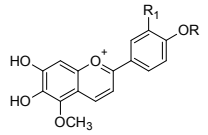
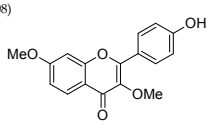
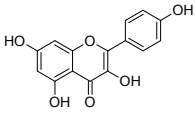
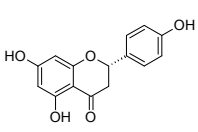
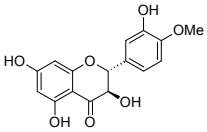
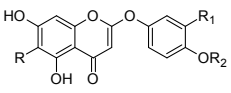
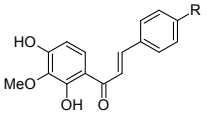
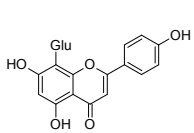
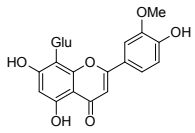
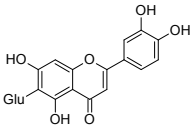
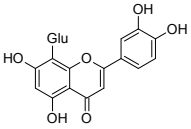
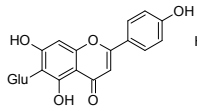
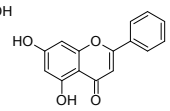


Fig. 6. Phenolic substances

Studies conducted by Ko and colleagues (2004) in flavonoids as inhibitors of PDE have suggested that C-4′ and C-5′ hydroxyl groups is not important for PDE-5 inhibition. The replacement of the hydroxyl by a methoxyl did not alter its inhibitory effect and it deletion resulted in no effect on PDE-5 inhibition. However, the C-7 hydroxyl group is very important for PDE-5 inhibition. C-7-glucoside showed no inhibition of the enzyme, being possible that the bulky glycosyl residues may hinder its binding to active site. Also, the C-3-hydroxyl group of flavonols seems difficult the binding with the PDE-5.

The luteolin showed more potent than other flavonoids, indicating that the presence of a double bond between C-2 and C-3 is important for PDE-5 inhibition. Between a flavon and an isoflavone, it may be easier for isoflavones than flavones to bind to the moiety of PDE-5. The removal of the C-5 hydroxyl group promoted the loss of inhibition of PDE, proposing that the hydroxyl group is vital for PDE-5 inhibition (Ko et al., 2004).

Specie (Family)	Flavonoids and phenols
Achyrocline satureioides (Asteraceae)	 Quercetin  3-O-Metil Quercetin  Luteolin And Caffeic, chlorogenic and isochlorogenic acids (Desmarchelier et al., 2000).
Anacardium Ocidentale (Anacardiaceae)	 Myricetin And Quercetin, Anacardic Acids and derivatives (Kubo et al., 1994; Miean & Mohamed, 2001).
Anemopaegma arvense (Bignoniaceae)	 Catuabine A  Cinchonain Ia  R = OH Cinchonain IIa R= OH KandelinAl (Tabanca et al.,2007)
Arrabidaea chica (Bignoniaceae)	 Vicenin-A (Barbosa et al., 2008)  Carajuruflavone (Takemura et al., 1995)  Acacetin (Zorn et al., 2001)  R_1= H; R = CH_3 - Carajurin  4'-hydroxi-3,7-dimethoxiflavone (Barbosa et al., 2008)  Kaempferol (Barbosa et al., 2008) R_1= OH; R= H - 6,7,3',4'-tetrahydroxy-5-methoxyflavylium R_1= H; R= H - 6,7,4'-trihydroxy-5- methoxyflavylium (Zorn et al., 2001)

Specie (Family)	Flavonoids and phenols
Davilla rugosa (Dilleniaceae)	 Narigenin  4'-O-methyltaxifolin Quercetin (David et al, 2006)
Mimosa tenuiflora (Fabaceae)	  R= O Me; R_1= OH; R_2= Me - Tenuiflorin A R= R_1= OMe; R_2= H - Tenuiflorin B R= H; R_1= OH; R_2= Me - Tenuiflorin C R= H; R_1= H; R_2= H - 6-Dimethoxycapilarisin R= H; R_1= H; R_2= Me - 6-Dimethoxy-4'-O-methylcapilarisin R= O Me - Kukulkan A R= OH - Kukulkan B (Souza et al., 2008)
Nymphaea ampla (Nymphaeaceae)	Quercetin derivatives (glycosides) (Marquina et al., 2005)
Passiflora sp. (Passifloraceae)	 Vitexin  Scoparin  Isoorientin  Orientin  Isovitexin  Chrysin (*P. Caeruleae*) Dhwan et al., 2002) Apigenin and luteolin derivatives (*P. edulis*) (Ferreres et al., 2007) Flavonoids above (*P. alata*)(Doyama et al, 2005)
Paulinia cupana (Sapindaceae)	Epicathechins, Cathechins (Ushirobira et al., 2007)
Schinus terebinthifolius (Anarcadiaceae)	Quercetin, myricetin, Kaempferol and derivatives (Ceruks et al., 2007; Johann et al., 2010)
Trichilia catigua (Meliaceae)	Chlorogenic acid, catechin and epicatechin (Lagos, 2006)

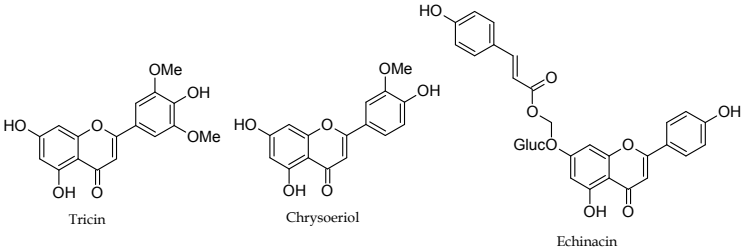
Specie (Family)	Flavonoids and phenols
Turnera diffusa (Turneraceae)	 Luteolin, apigenin, quercetin, orientin and vitexin derivatives (Zhao et al., 2007)

Table 2. Aphrodisiacs plants, their flavonoids and phenols

2.2.1.2 Alkaloids, xanthines and others amines

In broad sense, the alkaloids are natural nitrogen-containing secondary metabolites mostly derived from amino acids and found in about 20% of flowering plants. They are not limited to plants but also occur in marine organisms, insects, microorganisms and some animals (Rahimi et al., 2009).

Until 2005, 150,000 compounds were known and 14% these have been alkaloids. They are special interesting due to the heterogeneity of the group and the great bioactive potential particularly as inhibitors of PDE (Silva, 2006). Many of them have been used as a basis for design and development of new and more selective drugs with reduced side effects.

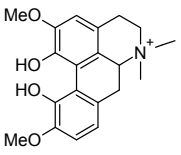
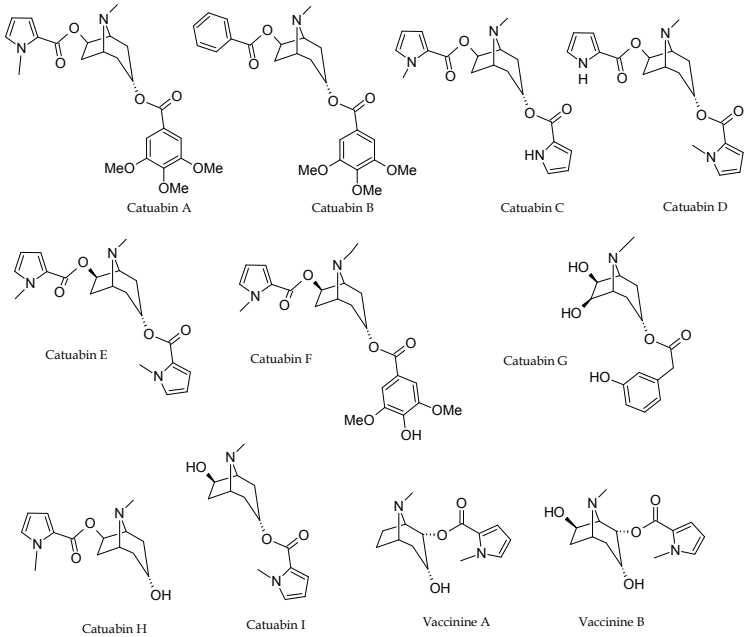
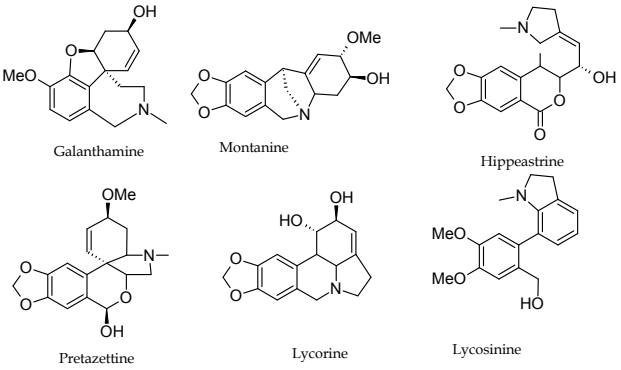
The methylxanthines are purine bases and have structural similarity with the cAMP and cGMP, therefore bind competitively to the sites of the various PDEs. They are considered non-selective inhibitors, such as caffeine found in *Paullinia cupana* seeds, theobromine and adenine from *Ptychopetalum olacoides*, which validate its aphrodisiac effect.

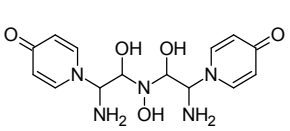
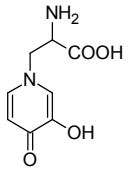
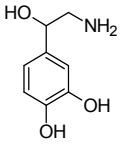
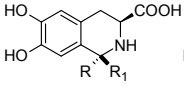
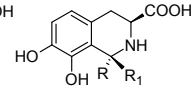
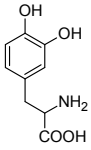
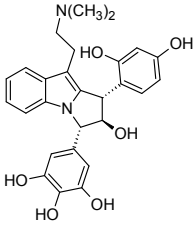
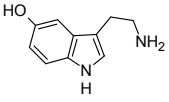
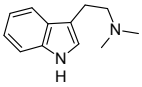
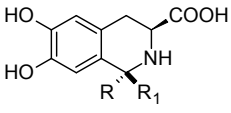
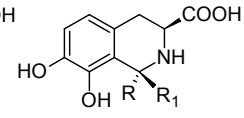
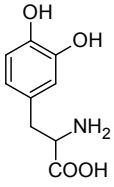
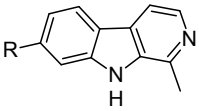
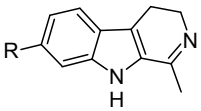
Introducing achiral cyclopenthyl and hexylamines moiety in xanthines analogues enhanced inhibitory activity. The ethyl group at the N-1 and N-3 positions showed the highest effect in PDE-5 (Wang et al., 2002).

Aporphines alkaloids act as dopamine agonists, due to their structural similarity. They improve central pro-erectile mechanisms by binding to receptors in the paraventricular nucleus of the hypothalamus. In clinical trials, apomorphine was found to be effective in patients with ED of various aetiologies and levels of severity, albeit with substantially less efficacy than any of the PDE-5 inhibitors (Seftel, 2002).

Other plants that seem to act this way are: *Mimosa tenuiflora, Mimosa pudica and Mucuna pruriens*, but they need more studies to investigation their aphrodisiac activities.

While many β-carbolines have effect as a selective inhibitor of PDE-5, the alkaloids of *Passiflora* seems to have effect as serotonin uptake inhibitors and therefore act with antidepressants. Recently, harmine and numerous related β-carboline derivatives were found as potent and specific inhibitors of cyclin-dependent kinases (CDKs), and the structure activity relationships (SARs) analysis demonstrated that the degree of aromaticity

Specie (Family)	Alkaloids, xanthines and others amines
Aristolochia cymbifera (Aristolochiaceae)	 Magnoflorine (Wu et al.,2005)
Erythroxylum viceniifolium (Erythroxylaceae)	 Catuabin A, Catuabin B, Catuabin C, Catuabin D, Catuabin E, Catuabin F, Catuabin G, Catuabin H, Catuabin I, Vaccinine A, Vaccinine B And derivatives (Zanolari, 2003)
Hippeastrum psittacinum (Amaryllidaceae)	 Galanthamine, Montanine, Hippeastrine, Pretazettine, Lycorine, Lycosinine (Jin, 2011; Pagliosa et al., 2010)

Specie (Family)	Alkaloids, xanthines and others amines
Mimosa pudica (Fabaceae)	 Mimopudine  Mimosiine  Pheniletylamine (Muthumani et al., 2010; Ueda & Yamamura, 1999a, 1999b)
Mimosa tenuiflora (Fabaceae)	 1) R=R$_1$= H 2) R=H; R$_1$=Me 3) R=R$_1$= Me  4) R=R$_1$= Me  L-dopa  Yuremamine  5 Hydroxytryptamine  N,N-dimethyltryptamine (Souza et al., 2008)
Mucuna pruriensis (Fabaceae)	 1) R=R$_1$= H 2) R=H; R$_1$=Me 3) R=R$_1$= Me  4) R=R$_1$= Me  L-dopa (Misra & Wagner, 2004; Siddhuraju &Becker, 2001)
Passiflora sp. (Passifloraceae)	 R=H- Harman R=OH - Harmol R=OCH$_3$ - Harmin  R=OH - Harmalol R=OCH$_3$ - Harmalin (Ingale & Hivrale, 2010)

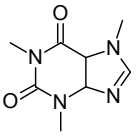
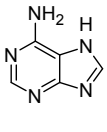
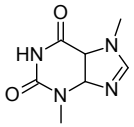
Specie (Family)	Alkaloids, xanthines and others amines
Paulinia cupana (Sapindaceae)	 Caffeine (Ushirobira et al., 2007)
Ptychopetalum olacoides (Oleaceae)	 Adenine  Theobromine And caffeine, muirapuamine (Montrucchio, 2005)

Table 3. Aphrodisiacs plants and their alkaloids, xanthines and others amines

of the tricyclic ring and the positioning of substituents were crucial for inhibitory activity. In addition, N-2-furoyl and N-2- pyrimidinyl β-carbolines were found to strongly inhibit activity against phosphodiesterases (PDEs) (Cao et al., 2007).

Tropane alkaloids present in *Erytroxylum* species have a structure similar to cocaine and seem to have the same action in the transport of dopamine (Singh, 2000).

2.2.1.3 Saponins

Saponins are a vast group of non-nitrogenous compounds, in general glycosides of steroids or polycyclic terpenes and widely distributed in higher plants. Their surfactant properties are what distinguish these compounds from others. They are soluble in water and form colloidal solutions that foam upon shaking (Schenkel et al., 2007; Sparg et al. 2004).

They have a diverse range of biological activities including hemolytic, hepatoprotective, antimutagenic, antiviral, antileishmanial and antiinflammatory (Rahimi et al., 2009).

Saponins are high molecular weight substances and occur in complex mixtures due to the concomitant presence of structures with varying number of sugars or because of the presence of various aglycones. As a result of structural complexity, isolation and structural elucidation of these compounds can be very difficult and has developed only recently (Schenkel et al., 2007).

Although some saponins inhibit PDE-5, like those present in *Allium tuberosum*, those found in plants studied did not have any reports for this activity (Guohua et al., 2009; Rahimi et al, 2009), except *Pfaffia paniculata* (Brazilian ginseng) presented saponins as the main active components due to its similarity with those saponins from *Panax ginseng,* known as ginsenosides (Rates & Gosmann, 2002). The ginsenosides are adaptogens substances or anti-stress agents, but their action mechanisms are not clear (Schenkel et al., 2007).

The term adaptogen, or resistogen, as it is called to classify a group of substances that can improve nonspecific resistance of body after being exposed to various stressing factors,

promoting a state of adaptation to the exceptional situation. Some plants like *Pfaffia paniculata, Paulinia cupana, Turnera diffusa, Anemopaegma arvense, Ptychopetalum olacoides* and *Trichilia catigua* are considered adaptogens (Mendes, 2011).

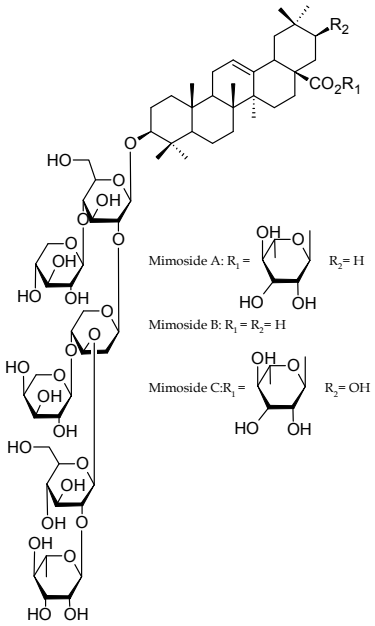
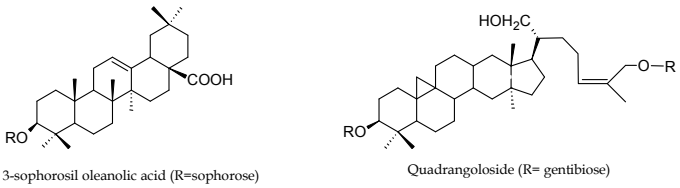
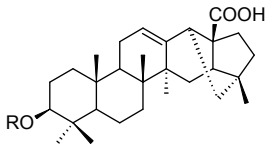
Specie (Family)	Saponins
Mimosa tenuiflora (Fabaceae)	 (Souza et al., 2008)
Passiflora sp. (Passifloraceae)	 3-sophorosil oleanolic acid (R=sophorose) Quadrangoloside (R= gentibiose) (*P. alata*) (Doyama et al., 2005)
Pfaffia paniculata (Amarantaceae)	 R= H - Pfaffic acid R= β-D-glucoronic acid(2-1)-β-D-xilose (Rates & Gosmann, 2002)

Table 4. Saponins and derivatives found in some Brazilian plants

Pharmacological studies of *P. paniculata* extracts indicate that they might act mainly by increasing central noradrenergic and dopaminergic tone, and possibly (indirectly) oxytocinergic transmission (Arletti et al., 1999).

It is possible to speculate that the activity is related to the distance between the groups at C-3 and groups at C-17 and the architecture of the molecule must be important.

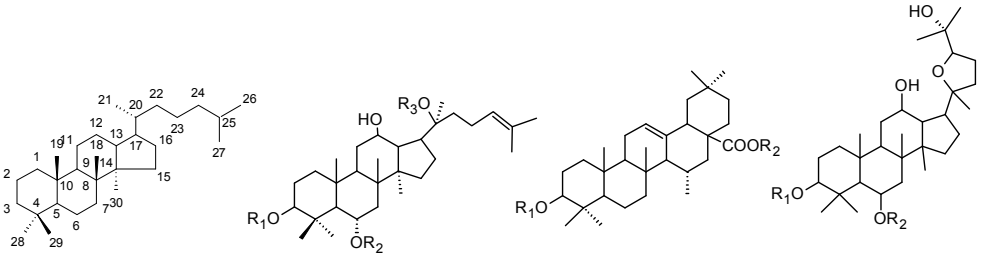


Fig. 7. Saponins basic strutures from *Panax Ginseng* (Jia & Zhao, 2009)

3. Conclusion

Despite the search promoted by pharmaceutical companies for analogues of sildenafil, the use and interest in herbal products based on folk and traditional medicine is growing globally, aiming to increase access to treatment for erectile dysfunction and to reduce the adverse effects and costs, improving the quality of life.

The investigation of classes of metabolites present in plants can indicate a possible rationalization of relations between the structure - aphrodisiac activity of substances, contributing to the development and generation of new drugs more effective and secure derivatives from regional floras.

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16

A Phytochemical and Ethnopharmacological Review of the Genus *Erythrina*

João X. de Araújo-Júnior, Mariana S.G. de Oliveira, Pedro G.V. Aquino, Magna S. Alexandre-Moreira and Antônio E.G. Sant'Ana
Universidade Federal de Alagoas
Brazil

1. Introduction

Considered in acient times as a connection to the divine, the use of this medicinal plant is as old as human civilization itself. Whole nations dominated its secrets, often associated with magic and religious rites, searching in nature's resources to improve life conditions, and increase chances of survival (Herbarium, 2008).

In 1978, the World Health Organization (WHO) recognized folk medicine and its beneficial effects to health, during the *Alma Ata* conference, which published in 1985 that approximatly 80% of the global population, resorted to traditional medicine as their primary health treatment (Herbarium, 2008). Medicinal plants have been used as a means of curing or preventing diseases, now called phytotherapy, in all regions of the world, with regional variations due to the influence of cultural characteristics of the population, as well as its flora, soil and climate (Lewinsohn, 2003).

Since the nineteenth century, humanity discovered the endless and diverse therapeutic arsenal present in medicinal plants, due to the discovery of active substances that in their natural state or after chemical transformation showed biological activity, and often already confirmed by popular use and/or proven scientifically (Miguel & Miguel, 2004).

According to Yamada (1998) it is necessary to carry out more studies and to propagate medicinal plant utilization as a way to diminish the costs of public health programs since the utilization of these plants may constitute a very useful therapeutic value due their efficacy coupled with low operating costs and the relative ease of obtaining the plants (Matos, 1994).

According to Brazilian legislation, a new herbal medicine can be introduced to the market in two forms: as a finished product – industrially produced, or as an official product – manufactured in pharmacies. Both forms should ensure quality, safety and efficacy of the herbal medicines supplied to the consumer. On the other hand, medicinal plants sold at popular markets or obtained directly from farmers at an informal market, have no guarantee provided by law, especially with regards to safety and efficacy (Herbarium, 2008). However we cannot rule out the cultural importance that popular knowledge inputs, being transmitted from generation to generation.

The WHO strategy on traditional medicine for the period of 2002-2005 has brought as one of its objectives, the strengthening of traditional remedies by placing them in the National Health Systems through policies and programs determined by their respective governments. The National Policy on Integrative and Complementary Practices of the Brazilian Unified Health System (SUS, *Sistema Único de Saúde*) (2006), for example, fulfills these requests by proposing the inclusion of medicinal plants, phytotherapy, homeopathy, traditional Chinese medicine, acupuncture, hydrotherapy and crenotherapy as therapeutic options for the SUS. Another example is the Brazilian National Policy on Medicinal Plants and Herbal Medicines, which includes as one of its guidelines the promotion and recognition of popular practices in the use of herbal and home remedies. Therefore, a strategy that can be used to meet this demand proposed by the federal government is to conduct a survey of plants used by communities in order to strengthen with the establishment a list of Medicinal Plants of Interest to SUS (RENISUS), which aims to give priority to the naturally occurring species of regions or to those easily cultivated. In this context, the Brazilian Ministry of Health released the RENISUS list, containing 71 species of medicinal plants for therapeutic use (http://portal.saude.gov.br/portal/ arquivos/pdf/RENISUS.pdf).

2. The Fabaceae family

Also known as a sub-family of Leguminosae, the Fabaceae family is one of the largest botanical families and widely distributed around the world, spread out over temperate, tropical and cold regions. Thus family is composed of 32 tribes, whose genera are chemically represented by a variety of flavonoid skeletons, notably pterocarpans and isoflavones. There are about 650 genera comprising about 18,000 species (Polhil & Raven, 1981). The genus *Erythrina* is represented by about 290 species (Cronquist, 1981; http://www.tropicos.org/Name/40005932). The Fabaceae family produces valuable medicinal drugs, ornamental species, fodders plants, oil producing plants, inseticides and species with various other functions (Salinas, 1992).

3. The *Erythrina* Genus

The genus *Erythrina* is one among several genera from the Fabaceae family. The origin of the name *Erythrina* comes from the Greek word "erythros" which means red, alluding to the bright red flowers of the trees of the genus (Krukoff & Barneby, 1974). Over 130 species of "coral tree" belong to the genus *Erythrina*, which has been widely studied and are distributed in tropical and subtropical regions of the world. In South America, these species are present in Argentina, Bolivia, Paraguay, French Guiana, Colombia and Peru (Hickey & King, 1981). In Brazil the genus is spread throughout all of the Brazilian biomes, like the Atlantic forest, *cerrado*, Amazon rainforest and Brazilian northeast *caatinga* (Corrêa, 1984). In Brazil, there are eight species found: *E. mulungu, E. velutina, E. cista-galli, E. poeppigiana, E. fusca, E. falcata, E. speciosa* and *E. verna* (Lourenzi, 1992).

Phytochemical analysis has demonstrated the presence of terpenes in plants from the *Erythrina* genus (Serragiotto et al., 1981; Nkengfack et al., 1997), that are also recognized as bioactive alkaloid-rich plants (Ghosal et al., 1971; Barakat et al., 1977) and flavonoids, especially, isoflavones, pterocarpanes, flavanones and isoflavanones (Chacha et al., 2005). Some of these flavonoids have demonstrated a wide variety of biological activities (Table 2).

Studies have demonstrated the presence of analgesic and anti-inflammatory effects in extracts obtained from *E. senegalensis*, *E. velutina* and *E. mulungu* (Vasconcelos et al., 2003). In folk medicine, various species are utilized as a tranquilizer, against insomnia and to treat inflammation (Garcia-Mateos et al., 2001).

3.1 Bibliographic review

We conducted a literature review using the database SciFinder Scholar®, and from the results obtained, we prepared two tables of data showing the correlation between popular use and the plant part utilized, as well as the form of utilization (Table 1), and the biological activities of the extracts obtained from *Erythrina* species (Table 2). Due to the large amount of data for phytochemicals isolated from the *Erythrina* species, we organized them in a simplified table (Table 3).

Uses	**Part Utilized**	**Kind of Extract/ Way of Use and Administration**	**Species**	**Locality**	**Reference**
Trachoma Malaria Syphilis Elephantiasis	Bark Roots Roots Bark	Unspecified, oral Unspecified,oral Unspecified Unspecified, external	*Erythrina abyssinica*	Kenya	Ichimaru et al. (1996) Kamat et al. (1981) Moriyasu et al. (1998)
Colic	Roots	Decoction, oral	*Erythrina abyssinica*	Tanzania	Chhabra et al. (1984)
Syphilis	Flowers	Infusion, oral	*Erythrina abyssinica*	Uganda	Kamusiime et al. (1996)
Fever Leprosy Dysentery Gonorrhea Hepatitis	Leaves Stalk Stalk	Unspecified, oral	*Erythrina abyssinica*	Rwanda	Chagnon (1984) Boily & Van Puyvelde (1986) Maikere-Faniyo et al. (1989) Vlietinck et al. (1995)
Schistosomiasis of the urinary tract	Unspecified	Decoction, oral	*Erythrina abyssinica*	Zimbabwe	Ndamba et al. (1994)
Poison antidote	Roots	Unspecified	*Erythrina abyssinica*	India	Selvanayahgam et al. (1994)
Anthelmintic	Green bark stem	Unspecified, oral	*Erythrina abyssinica*	East Africa	Kokwaro (1976)
Contraception Parturition Malaria Insomnia	Bark Bark Whole plant Flowers	Unspecified, oral Unspecified, oral Unspecified, oral Unspecified, oral	*Erythrina americana*	Mexico	Hastings (1990) Dominguez & Alcorn (1985)

Uses	Part Utilized	Kind of Extract/ Way of Use and Administration	Species	Locality	Reference
Hypnotic Inflammation of the arms, legs, hair and eyes. Abscesses Insect bites Ulcers Curare-like effect	Flowers Fruits Leaves Leaves Leaves Seeds	Unspecified, oral Unspecified, External Unspecified, external Unspecified, oral Unspecified, external Unspecified, external			
Anthelmintic Earache	Bark Leaves	Decoction, oral Juice of leaves, aural	*Erythrina arborescens*	Nepal	Manandhar (1995) Bhattarai (1991)
Pork skin disease	Leaves	Unspecified, external	*Erythrina arborescens*	India	Rao (1981)
Snakebite Abscesses Boils Infections of skin and mucous Dermatitis and inflammation	Bark Leaves Leaves Leaves Leaves	Infusion, oral Unspecified, external Unspecified, external Unspecified, external Unspecified, external	*Erythrina berteroana*	Guatemala	Giron et al. (1991) Caceres et al. (1987)
Poison antidote	Bark	Infusion, oral	*Erythrina berteroana*	India	Selvanayahgam et al. (1994)
Fish poison Female diseases Sedative Bleeding Dysentery Poison Narcotic	Branches Whole plant Flowers Flowers Flowers Seeds Unspecified	Unspecified Unspecified, oral Decoction, oral Decoction, oral Decoction, oral Unspecified, oral Decoction, Unspecified	*Erythrina berteroana*	Mexico	Hastings (1990)
Sedative Bleeding Dysentery	Levaes and flowers Flowers Flowers	Infusion, oral Unspecified, oral Unspecified, oral	*Erythrina berteroana*	Central America	Morton (1994)
Female diseases	Unspecified	Unspecified, oral	*Erythrina berteroana*	Panama	Duke & Ayensu (1994)
Antiasthmatic Expel placenta	Bark Leaves	Unspecified Unspecified, oral	*Erythrina corallodendron*	Antilles	Ayensu (1978)
Measles	Seeds	Unspecified, external	*Erythrina coralloides*	Mexico	Hastings (1990)

Uses	Part Utilized	Kind of Extract/ Way of Use and Administration	Species	Locality	Reference
Urinary Tract Infection Respiratory Tract Infection Diarrhea Anti-hemorrhoids Narcotic Antiseptic	Bark Bark Bark Leaves Stalk Stalk	Decoction, oral Decoction, oral Decoction, oral Unspecified, external Unspecified, oral Unspecified, external	*Erythrina crista-galli*	Argentina	Perez & Anesini (1994) Bandoni et al. (1976)
Antimicrobial Throat infections Astringent in wound healing	Stalk+leaves Stalk+leaves Stalk+leaves	Unspecified, external Unspecified, oral Unspecified, external	*Erythrina crista-galli*	Brazil	Simões et al. (1999)
Swelling Healing	Bark Bark	Suspension in water, oral	*Erythrina dominguezii*	Argentina	Filipoy (1994)
Diarrhea Toothache Erotic dreams Toxic Purgative Contraceptive	Leaves Seeds Seeds Seeds Seeds Seeds	Infusion, oral Unspecified, oral Unspecified, oral Unspecified, oral Unspecified, oral Unspecified, oral	*Erythrina flabelliformis*	Mexico	Hastings (1990) Diaz (1977) Pennington (1973) Bye (1986)
Inflammation of uterus Appendicitis Diuretic	Bark Whole plant Seeds	Decocction, oral Unspecified, oral Unspecified	*Erythrina folkersii*	Mexico	Zamora-Martinez & Pola (1992) Hastings (1990)
Migraine Infected wounds Fungal dermatosis Antitussive	Bark Bark Bark Flowers	Infusion, external Decoction, external Decoction, external Decoction, oral	*Erythrina fusca*	Peru	Duke (1994)
Anti-inflammatory	Bark and leaves	Unspecified, oral	*Erythrina fusca*	Thailand	Wasuwat (1967)
Skin infections Itch	Seeds Seeds	Unspecified Unspecified	*Erythrina fusca*	Indonesia	Widianto (1980)
Headache Narcotic Kidney	Bark and leaves Bark and	Infusion, oral Infusion, oral Infusion, oral	*Erythrina glauca*	Peru	Jovel et al.(1996) Duke (1994)

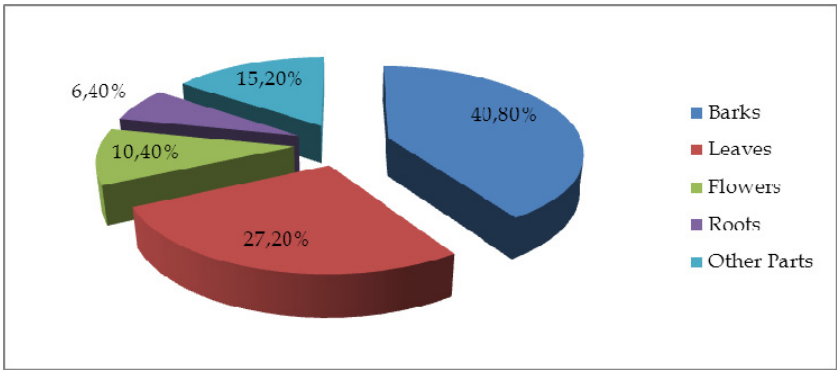
Uses	Part Utilized	Kind of Extract/ Way of Use and Administration	Species	Locality	Reference
inflammation Purgative Antimalarial	leaves Bark and leaves Bark and leaves Unspecified	Infusion, oral Decocction, oral			
Rats and dogs poison	Seeds	Unspecified, oral	*Erythrina herbacea*	Mexico	Hastings (1990)
Tuberculosis	Bark	Infusion, oral	*Erythrina humeana*	South Africa	Pillay et al. (2001)
Antipyretic Anthelmintic Astringent Expectorant Eye drops Antibilious Stomach upset Menstrual regulator Aphrodisiac Laxative Diuretic Stimulation of milk production	Bark Bark and leaves Bark Bark Bark Bark Bark Bark+roots Leaves Leaves Leaves Leaves	Unspecified, oral Unspecified, oral Unspecified, external Unspecified, oral Unspecified, ophthalmic Unspecified, oral Juice, oral With milk, oral Unspecified, oral Unspecified, oral Unspecified, oral Unspecified, oral	*Erythrina indica*	India	Khan et al. (1994) John (1984) Chopra & Ghosh (1935) Pushpangadan & Atal (1984)
Poison	Whole plant	Unspecified	*Erythrina lanata*	Mexico	Hastings (1990)
Aphrodisiac	Bark	Unspecified, oral	*Erythrina mildbraedii*	Guinea	Vasileva (1969)
Antipyretic	Bark	Decoction, oral	*Erythrina mulungu*	Brazil	Brandão (1985)
Antimalarial	Leaves an roots	Decoction/infusion, oral	*Erythrina sacleuxii*	Tanzania	Gessler et al.(1995)
Postpartum (women) Treatment of female sterility	Bark Bark+leaves	Unspecified, oral Unspecified, oral	*Erythrina senegalensis*	Guinea	Vasileva (1969)
Serious injury Yellow fever Bronchial diseases Eye disorders Injuries	Bark Bark Bark Bark Twigs and leaves	Unspecified, oral, external Unspecified, oral Unspecified, oral Unspecified, oral Unspecified, external	*Erythrina senegalensis*	Senegal	Le Grand & Wondergem (1987) Le Grand (1989)

Uses	Part Utilized	Kind of Extract/ Way of Use and Administration	Species	Locality	Reference
Ulcers Venereal diseases	Twigs and leaves Twigs and leaves	Unspecified, oral Unspecified, oral			
Antimalarial	Roots	Unspecified	*Erythrina senegalensis*	Nigeria	Etkin (1997)
Broken bones Antipyretic	Bark Leaves	Decoction, external Unspecified, oral	*Erythrina species*	Thailand	Anderson (1986) Mokkhasmit et al. (1971)
Analgesic	Leaves	Unspecified, oral	*Erythrina species*	Solomon Islands	Blackwood (1935)
Parturition induction Toothache Nosebleed	Bark and leaves Roots Roots	Infusion, oral Unspecified, oral Unspecified, external	*Erythrina standleyana*	Mexico	Hastings (1990) Dominguez & Alcorn (1985)
Epilepsy Leprosy	Bark Bark	Unspecified, oral Unspecified	*Erythrina stricta*	India	Chopra (1933)
Menorrhagia	Leaves	Unspecified, oral	*Erythrina subumbrans*	East Indias	Burkill (1966)
Antiseptic	Bark	Unspecified, external	*Erythrina ulei*	Peru	Desmarcheilier et al. (1997) Desmarcheilier et al. (1996)
Antiseptic	Stem Bark	Unspecified, external	*Erythrina ulei*	Argentina	Desmarcheilier et al. (1996)
Antipyretic	Bark	Decoction, oral	*Erythrina variegata*	Adaman Islands	Awasth (1991)
Epilepsy Stomach ache	Bark Bark	Unspecified, oral Juice, oral	*Erythrina variegata*	India	Pushpangadan & Atal (1984) John (1984)
Swelling	Bark	Unspecified, external	*Erythrina variegata*	New Guinea	Holdsworth (1984)
Amenorrhea Conception Dysmenorrhea	Bark Bark Bark	Infusion, oral Infusion, oral Infusion, oral	*Erythrina variegata*	Rotuma	Mc Clatchey (1996)
Antipyretic Sedative Antiasthmatic	Flowers Flowers Flowers	Unspecified, oral Unspecified, oral Unspecified, oral	*Erythrina variegata*	Brazil	Sarragiotto et al. (1981)
Induce menstruation	Leaves	Juice, oral	*Erythrina variegata*	India	Das (1955)

Table 1. Popular uses of *Erythrina* species

3.1.1 Ethnopharmacological data

Plants of the *Eryhtrina* genus are utilized for a wide array of human diseases (Table 1). With regards the parts of the plants that are utilized, the most used is the bark, being 40.8% of the total of citations, as shown in Graphic 1.



Graphic 1. Parts of the plants utilized in folk medicine.

3.1.2 Biological activity data

Analysis of the biological activity data (Table 2) shows the wide variety of biological activity of plants from the *Erythrina* genus, and shows too that most of this corroborates with popular knowledge and uses.

It is noteworthy to point out that most of these activities, mainly the antibacterial and analgesic properties, confirm the different popular applications of extracts obtained from plants of this genus. We would like also to draw attention to the fact that in the Brazilian market there is the availability of a phytotherapeutic product from *Erythrina mulungu* widely used for anxiolytic purposes and as a sedative, activities confirmed by popular knowledge, but that, to our knowledge, have not yet been confirmed in pharmacological tests, showing that, despite the wide array of available data related to plants of this genus, there is still a need for more research about some of them.

It is important to note that some of the activities shown in the biological tests were not cited in the ethnopharmacological studies, which indicates yet another importance for plants of the *Erythrina* genus, which have the potential to provide new compounds for the development of drugs for the treatment of diseases such as cancer, diabetes and hypertension.

3.1.3 Phytochemical data

The phytochemical data (Table 3) analysis allowed for the verificationof a predominance of alkaloids and flavonoids in the *Erythrina* genus. It is important to note that alkaloids are recognized as markers for plants of this genus in addition to showing a wide array of biological activities, and being important candidates in the development of new drugs.

Species	Part of the Plant	Biological Activities	Location	Reference
Erythrina abyssinica	Bark Leaves Roots Roots Root Bark Root Bark	Mitogenic activity Cell Culture Molluscicidal (*Biomphalaria pfeifferi*) Anti-bacterial Anti-bacterial (Gram-positive species, *Escherichia coli*) Anti-yeast (*Saccharomyces cerevisiae*) Antimalárico	Kenya	Tachibana et al (1993) Kloos et al (1987) Kamat et al. (1981) Taniguchi et al. (1978) Yenesew et al. (2003a)
Erythrina abyssinica	Bark	Anti-bacterial (*Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* e *Staphylococcus aureus*)	Sudan	Omer et al. (1998)
Erythrina abyssinica	Leaves Trunk	Uterine relaxing and stimulant Muscle Relaxing and stimulant Periferic muscle relaxing and stimulant Toxic effect in rats Antidiarrheal Anti-bacterial (*Salmonella typhi*, *Shigella flexnerishigella dysenteriae*, *Shigella boyd*, *Shigella sonnei*) Antiviral	Rwanda	Chagnon (1984) Maikere-faniyo et al. (1989) Vlietinck et al. (1995)
Erythrina abyssinica	Root bark	Anti-bacterial Anti-fungal	East Africa	Taniguchi & Kubo (1993)
Erythrina addisoniae	Stem Bark	Anti-inflammatory	Cameroon	Talla et al. (2003)
Erythrina americana	Bark	Plant germination inhibition Molluscicidal	Unspecified	Dominguez & Alcorn (1985)
Erythrina americana	Seeds	Central Nervous System depressor	Mexico	Garin-Aguilar et al. (2000)
Erythrina arborescens	Leaves Leaves, stem Stem Stem	Hypotensive Cytotoxic Antispasmodic Uterine stimulant	India	Dhar et al. (1968)
Erythrina berteroana	Leaves	Anti-yeast Anti-bacterial	Guatemala	Caceres et al. (1987)
Erythrina	Leaves+Twigs	Cytotoxic	Panamá	Chapuis et al.

Species	Part of the Plant	Biological Activities	Location	Reference
berteroana	Root bark	Anti-fungal		(1988) Maillard et al. (1987)
Erythrina berteroana	Stem	Pherormone	Puerto Rico	Keiser et al. (1975)
Erythrina bidwillii hybrid	Root bark	Anti-fungal Anti-bacterial Anticoagulant	Okinawa	Iinuma & Tanaka (1994) Iinuma et al. (1994)
Erythrina breviflora	Leaves+stem	Cancer induction	USA	Caldwell & Brewer (1983)
Erythrina caffra	Bark, leaves	COX1 inhibitor Anti-bacterial	South Africa	Pillay et al. (2001)
Erythrina corallodendron	Dry fruit + leaves + stem	Antiphagocytic	Greece	Yannitsaros (1996)
Erythrina corallodendron	Seeds	Trypsin inhibition	Israel	Joubert & Sharon (1985)
Erythrina coromandelianum	Whole plant	Molluscicidal	Puerto Rico	Medina& Woodbury (1979)
Erythrina crista-galli	Aerial parts Bark	Analgesic Anti-inflammatory Anti-bacterial Anti-fungal	Argentina	Mino et al. (2002) Perez & Anesini (1994)
Erythrina crista-galli	Flowers	Anti-mutagenic	Unspecified	Ishii et al. (1984)
Erythrina crista-galli	Fresh fruit + leaves + stem	Anti-phagocytic	Greece	Yannitsaros (1996)
Erythrina crista-galli	Leaves	Anti-fungal Anti-bacterial	Egypti	Ross et al. (1980)
Erythrina crista-galli	Leaves + stem	Cytotoxic Antiviral	Brazil	Simoes et al. (1999)
Erythrina crista-galli	Leaves + stem	Animal repellent	Germany	Wink (1984)
Erythrina crista-galli	Root and stem bark	Anti-bacterial Anti-mycobacterial	Bolivia	Mitscher et al. (1984) Mitscher et al. (1988)
Erythrina crista-galli	Seeds	Trypsin inhibition	Uruguay	Joubert & Sharon (1985)
Erythrina eriotricha	Root bark	Anti-bacterial	Cameroon	Nkengfack et al. (1995)
Erythrina excelsa	Root bark	Anti-bacterial/anti-fungal	East Africa	Taniguchi et al. (1993)
Erythrina	Seeds	Larvicidal	Unspecified	Janzen et al.

Species	Part of the Plant	Biological Activities	Location	Reference
flabelliformis				(1977)
Erythrina fusca	Leaves	Hypotensive Uterine stimulant Diuretic	Thailand	Unakul (1950)
Erythrina fusca	Seeds	Central Nervous System depressor	Indonesia	Widianto et al. (1980)
Erythrina glauca	Bark	Antiviral	Guatemala	Mc Kee et al. (1997)
Erythrina humeana	Bark, leaves	Anti-bacterial COX1 inhibitor	South Africa	Pillay et al. (2001)
Erythrina indica	Leaves	Anti-fungal Anti-bacterial	Egypt	Ross et al. (1980)
Erythrina indica	Leaves	Central Nervous System depressor	Sri Lanka	Ratnasooriya & Dharmasiri (1999)
Erythrina indica	Unspecified	Stimulant and inhibitor of lymphocyte blastogenesis	India	Singh & Chatterjee (1979)
Erythrina indica	Root bark Stem bark	Anti-mycobacterial Anti-bacterial Cytotoxic	Nigeria	Waffo et al. (2000) Nkengfack et al. (2001)
Erythrina indica	Seeds	Anti-fungal Anti-bacterial	Egypt	Ross et al. (1980)
Erythrina indica	Seeds	Immunosuppressor	India	Singh (1979)
Erythrina latissima	Bark, leaves	COX1 inhibitor Anti-bacterial	South Africa	Pillay et al. (2001)
Erythrina lysistemon	Bark, leaves	COX1 inhibitor Anti-bacterial Anti-yeast	South Africa	Pillay et al. (2001) Rabe & Van Staden (1997) Motsei et al. (2003)
Erythrina lysistemon	Root	Antiviral	Tanzania	Mc Kee et al. (1997)
Erythrina lysistemon	Stem bark	Estrogenic Bone formation stimulant Antidiabetic Rises seric LDL	Cameroon	Njamen et al. (2007)
Erythrina mildbraedii	Whole plant	Anti-tumoral Toxic effect Cytotoxic	Unspecified	Suffness et al (1988)
Erythrina mildbraedii	Root	Anti-mycobacterial Anti-bacterial	Nigeria	Mitscher et al. (1988)

Species	Part of the Plant	Biological Activities	Location	Reference
Erythrina poeppigiana	Unspecified	Cytotoxic	Colombia	De Cerain et al. (1996)
Erythrina resupinata	Roots	Fetal anti-implantation Anti-tumoral Uterine stimulant Abortive Toxicity evaluation	India	Aswal et al. (1984)
Erythrina rubrinervia	Twigs	"DNA linker" Cytotoxic	Unspecified	Pezzuto et al. (1991)
Erythrina sacleuxii	Leavess, root bark	Antimalarial Cytotoxic	Tanzania	Gessler et al. (1994) Gessler et al. (1995)
Erythrina senegalensis	Bark, root, stem bark Flowers	Antimalarial Analgesic Anti-inflammatory Anti-bacterial Molluscicidal	Nigeria	Saidu et al. (2000) Etkin (1997) Ajaiyeoba et al. (2004) Hussain & Deeni (1991) Okunji & Iwu (1988)
Erythrina senegalensis	Bark	Anti-bacterial Anti-fungal	Senegal	Le Grand & Wondergem (1988)
Erythrina senegalensis	Raiz	Antiviral	Guinea-Bissau	Silva et al. (1997)
Erythrina sigmoidea	Bark Bark, root bark Stem bark	Anti-yeast Anti-bacterial Anti-fungal Skeletal muscle relaxing Antispasmodic Spasmolytic	Cameroon	Biyiti et al. (1988) Nkengfack et al. (1994) Benedicta et al. (1993) Nkeh et al. (1996)
Erythrina species	Bark	Anti-bacterial	China	Gaw & Wang (1949)
Erythrina species	Leaves	Pherormone	Puerto Rico	Keiser et al. (1975)
Erythrina species	Leaves	Anti tumoral	Indonesia	Itokawa et al. (1990)
Erythrina species	Leaves	Antipyretic	Thailand	Mokkhasmit et al. (1971)
Erythrina species	Stem bark	Anti-leishmaniasis Anti-trypanossomiasis	Bolivia	Fournet et al. (1994)

Species	Part of the Plant	Biological Activities	Location	Reference
Erythrina standleyana	Bark	Molluscicidal Inhibition of plant germination	Unspecified	Dominguez & Alcorn (1985)
Erythrina stricta	Stem Leaves	Spasmolytic Hypotermic Diuretic Anticonvulsant Analgesic Antiviral Anti-fungal Anti-yeast Anti-protozoan Toxicity evaluation Cytotoxic	India	Bhakuni et al. (1988) Dhar et al. (1968)
Erythrina suberosa	Leaves Stem bark	Hypotensive Anti-spermatogenic Anti-androgen Anti-gonadotropin Anti tumoral Toxicity evaluation Hypoglicemic Cytotoxic Antispasmodic	India	Dhar et al. (1968)
Erythrina suberosa	Leaves, seed oil	Anti-bacterial Anti-fungal	Thailand	Silpasuwon (1979) Joshi et al. (1981)
Erythrina subumbrans	Aerial parts	Fetal anti-implantation Uterine stimulant Anti tumoral Abortive effect Toxicity evaluation	India	Aswal et al. (1984)
Erythrina ulei	Bark	Anti-crustacean "DNA linker" Antioxidant	Peru	Desmarcheilier et al. (1996) Desmarcheilier et al. (1997)
Erythrina variegata	Bark	Anti gastric ulcer	Japan	Muto et al. (1994)
Erythrina variegata	Bark, leaves Seeds oil Stem	Inhibition of plant germination and growing Anti-bacterial Anti-fungal Juvenile hormone activity	India	Chauhan et al. (1989) Bhale et al. (1979) Tripathi & Rizvi (1984) Prabhu & John (1975)

Species	Part of the Plant	Biological Activities	Location	Reference
Erythrina variegata	Bark Stem bark	Phospholipase A2 inhibitor Prostaglandin synthesis inhibidor Central Nervous System effects Spasmolytic	Samoa	Hegde et al. (1997) Dunstan et al. (1997) Cox et al. (1989)
Erythrina variegata	Flowers	Anti-yeast Anti-bacterial	Thailand	Avirutnant & Pongpan (1983)
Erythrina variegata	Fresh flowers	Anxiolytic	Brazil	Flausino et al. (2007)
Erythrina variegata	Leaves Unspecified	Anti-inflammatory Skeletal muscle relaxing Barbiturates potentiator	Vietnam	Nguyen et al. (1991) Nguyen et al. (1992)
Erythrina variegata	Roots	Inhibitor of glutamate-pyruvate-transaminase	Taiwan	Yanfg et al. (1987)
Erythrina variegate var. orientalis	Leaves Roots Stem bark	Antispasmodic Cytotoxic Toxicity evaluation Anti-yeast Anti-bacterial Anti-mycobacterial Cytotoxic Antispasmodic	India	Dhar et al. (1968) Telikepalli et al. (1990) Dhar et al. (1968)
Erythrina variegate var. orientalis	Leaves	Anti tumoral	Philippines	Masilungan et al. (1971)
Erythrina velutina	Leaves Stem bark Trunk bark	Analgesic Anti-inflammatory Uterine stimulant Molluscicidal	Brazil	Marchioro et al. (2005) Barros et al. (1970) Pinheiro de Sousa & Rouquayrol (1974)
Erythrina vespertilio	Bark	Inhibition of platelet aggregation Serotonin release inhibition	Australia	Rogers et al. (2001)
Erythrina vogelii	Root bark	Anti-fungal	Ivory Coast	Queiroz et al. (2002)
Erythrina zeyheri	Leaves	Anti-bacterial COX1 Inhibitor	South Africa	Pillay et al. (2001)

Table 2. Biological activity of *Erythrina* extracts.

Classes of Compounds	Occurrence	Percentage
Alkaloids	461	41.57
Coumarins	1	0.09
Steroids	29	2.62
Flavonoids	330	29.76
Lipids	32	2.88
Proteins	112	10.10
Triterpenes	31	2.80
Other compounds	113	10.19
Total	1109	100

Table 3. Occurrence of the different classes of compounds in the *Erythrina* genus

Some important alkaloids that are distributed within plants from the *Erythrina* genus are erytharbine, erythartine, erysotramidine and erysotrine, shown in figure 1. It is noteworthy that a characteristic feature of these alkaloids is the spiro structure in the rings bearing the nitrogen atom.

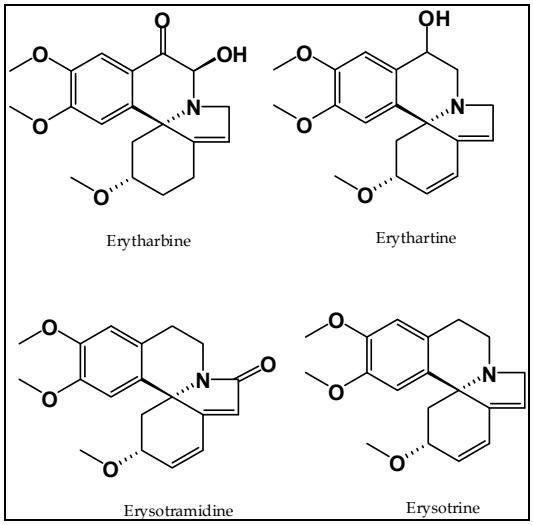


Fig. 1. Common alkaloids found in the *Erythrina* genus.

4. Conclusion

This review showed that *Erythrina* species are commonly utilized for numerous diseases and that many ethnopharmacological studies have been performed in order to confirm the activities attributed to these species. Moreover, several classes of substances have been isolated from the *Erythrina* genus, mainly alkaloids (41.57%) and flavonoids (29.76%).

Despite the large amount of available data, some of the plants of this genus remain to be studied. An example is *Erythrina mulungu*, largely used in Brazil, yet a significant number of studies regarding its pharmacological properties and chemical composition were unable to

be found. A recent contribution to the knowledge about this plant is given by our group, regarding the anti-inflammatory and antinociceptive activities of a hydroalcoholic extract obtained from *E. mulungu* (Oliveira et al., *in press*).

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6. References

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Phytochemistry, Pharmacology and Agronomy of Medicinal Plants: *Amburana cearensis*, an Interdisciplinary Study

Kirley M. Canuto, Edilberto R. Silveira, Antonio Marcos E. Bezerra, Luzia Kalyne A. M. Leal and Glauce Socorro B. Viana
Empresa Brasileira de Pesquisa Agropecuária, Universidade Federal do Ceará, Brazil

1. Introduction

Plants are an important source of biologically active substances, therefore they have been used for medicinal purposes, since ancient times. Plant materials are used as home remedies, in over-the-counter drug products, dietary supplements and as raw material for obtention of phytochemicals. The use of medicinal plants is usually based on traditional knowledge, from which their therapeutic properties are oftenly ratified in pharmacological studies.

Nowadays, a considerable amount of prescribed drug is still originated from botanical sources and they are associated with several pharmacological activities, such as morphine (**I**) (analgesic), scopolamine (**II**) atropine (**III**) (anticholinergics), galantamine (**IV**) (Alzheimer's disease), quinine (**V**) (antimalarial), paclitaxel (**VI**), vincristine (**VII**) and vinblastine (**VIII**) (anticancer drugs), as well as with digitalis glycosides (**IX**) (heart failure) (Fig. 1). The versatility of biological actions can be attributed to the huge amount and wide variety of secondary metabolites in plant organisms, belonging to several chemical classes as alkaloids, coumarins, flavonoids, tannins, terpenoids, xanthones, etc.

The large consumption of herbal drugs, in spite of the efficiency of synthetic drugs, is due to the belief that natural products are not toxic and/or have fewer side effects, the preference/need for alternative therapies, and their associated lower costs. In developing countries, herbal medicine is the main form of health care. In Brazil, where there is one of greatest biodiversity of plants in the world, pharmaceutical assistance programs, such as "Living Pharmacies", have a prominent role in spreading the rational use of medicinal plants mainly for poor people, under recognition by World Health Organization (WHO). Furthermore, herbal medicines also represent a significant pharmaceutical market share in some industrialized countries like Germany.

On the flip side, herbal drugs are discredited by most of the health related professionals, owing to a lack of scientific research supporting its efficacy and safety. In general, physicians feel insecure in prescribing herbal medicines, as most of them do not undergo through clinical trials, phytochemical analysis, and their active principles not being

determined. Therefore, herbal medicines do not have a defined dosage, information on the chemical composition and warnings about possible risks. Additionally, the poor quality control of herbal drugs, which are subject to adulteration and intrinsic factors related to used raw material, do produce variables and inconsistent effects. Furthermore, most herbal drugs are produced from wild source, limiting the production at industrial level and putting the species used under threat of extinction.

Due to these aforesaid limitations, disadvantages and drawbacks of herbal medicine, we would like to present an updated review of chemical, pharmacological and agronomic studies of *Amburana cearensis* as a well succeeded example of a scientific research on wild plants and a model of a sustainable economic utilization of medicinal plants.

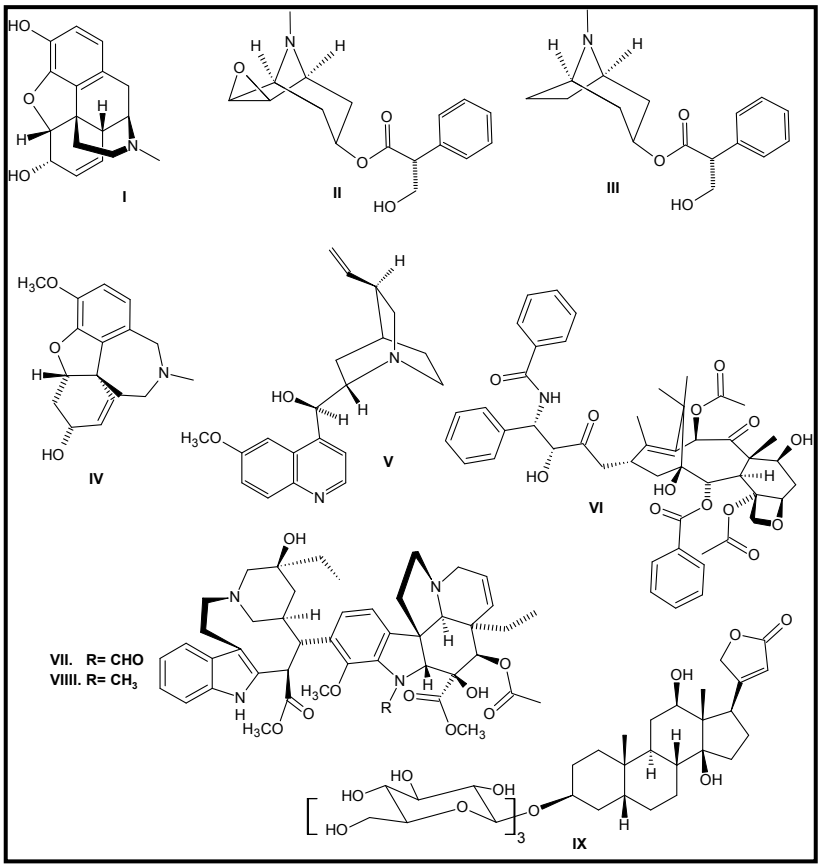

Fig. 1. Chemical structures of plant-derived drugs

2. Herbal drugs and phytopharmaceuticals

According to the WHO definition, herbal drugs are preparations containing plant parts (leaves, roots, seeds, stem bark, etc.) or whole plant materials in the crude or processed form, as active ingredients, besides some excipients. Herbal preparations can be found

under different forms: oral tablets, capsules, gel caps, syrup, extracts and infusions. In general, combinations with chemically defined active substances or isolated constituents, are not considered to be herbal medicines. (Calixto, 2000).

The information about the therapeutical properties and usage of medicinal plants are commonly based on the empirical knowledge of ancient people, which was passed over several generations and originated the traditional medicine systems, utilized all over the world (Traditional Chinese Medicine, Ayurvedic system, Western and African Herbalisms). An estimated quantity of 50 000 plant species are used for medicinal purposes, from which the stand out species of the following families, such as Apocynaceae, Araliaceae, Apiaceae, Asclepiadaceae, Canellaceae, Clusiaceae and Menispermaceae (Schippmann et al., 2002). From the total of 252 drugs in the WHO's essential medicine list, 11% are exclusively derived from plant origins (Sahoo et al., 2010)

Herbal drugs are consumed by three-quarters of the world's population in the treatment of mainly chronic diseases, particularly headache, rheumatological disorders and asthma (Inamdar et al., 2010). In the developing countries, the population relies basically on medicinal plants for primary health care, since modern medicine is expensive and not easily accessible. However, the consumption of herbal drugs is also large in developed countries. Phytotherapy is popular in many countries of Western Europe (Germany, France, Italy, etc.), since people believe that either herbal drugs are devoid of side-effects or seek a healthier life style. Americans usually buy herbal products as a dietary supplement in the United States, aiming at preventing aging and diseases like cancer, as well as diabetes (Calixto, 2000).

Herbal drugs have some features which distinguish themselves considerably from synthetic drugs. Herbal medicines are always formed from a complex mixture of chemical compounds (eg. *Scutellaria baicalensis* has over 2000 components), and they may be constituted by many plants, therefore herbal drugs show an ample therapeutic usage. It is quite common to find a medicinal plant with several therapeutic properties (Sahoo et al., 2010; Calixto, 2000). The combination of either many plants, containing diverse bioactive substances or a pool of structural analogs, can produce a synergistic action that results in a stronger effect, therefore, permitting a reduction of dosage, which implies in lower risks of intoxication and undesirable side effects. As some diseases (e.g. AIDS or various types of cancer) possess a multi-causal etiology and a complex pathophysiology, a medical treatment may be more effective through well-chosen drug combinations than a single drug. Ginkgolides A and B, isolated from *Ginkgo biloba*, duly demonstrated a greater effect on the thrombocyte aggregation inhibition, when used as a mixture as opposed to what would be expected from the sum of the two compounds separately (Wagner, 2011).

On the other hand, plant-based products do not possess a well-defined chemical composition, due partially to chemical complexity stated above. Hence, the active principles of herbal drugs are frequently unknown, in addition to their standardization and quality control, being hardly achieved (Calixto, 2000) owing to mainly chemical variability in raw material. Secondary metabolites are the bioactive components from herbal drugs and their contents are strongly influenced by several factors: genetic (genotypes, chemotypes), physiologic (circadian rhythm, phenology, age), environmental (climate, sunlight exposure, water availability, soil, agronomic conditions) and manufacturing conditions (harvesting, storage and processing) (Tab. 1), (Sahoo et al., 2010; Gobbo-Beto & Lopes, 2007).

Evaluated Effect	Plant	Compound (s)	Result
Seasonality	*Hypericum perforatum*	Hypericin	300x content larger in summer than winter. (Southwell & Bourke, 2001)
Harvesting time	*Ocimum gratissimum*	Eugenol	98 % at 17h, but it is 11 % at midday (Silva et al. 1999)
Age	*Papaver somniferum*	Morphine	6x content larger on the 75th day after germination than on the 50th day (Williams & Ellis, 1989)
Phenology	*Gentiana lutea*	Mangiferin/ isoorientin	Before flowering-↑ [Mangiferin] ; during flowering- ↑ [Isoorientin] (Menković et al., 2000)
Temperature	*Nicotiana tabacum*	Scopolamine	[Scopolamine] 4x larger after freezing (Koeppe et al, 1970)
Water availability	*H. perforatum*	Hyperforin/ Hypericin	Under water stress: ↑ 2x [Hyperforin] and ↓ [Hypericin] (Zobayed et al, 2007)

Table 1. Effects on the production of secondary metabolites

Furthermore, most herbal drugs are utilized and commercialized without having a proven efficacy and safety through well-controlled double-blind clinical and toxicological trials, as pharmaceuticals are usually tested prior to being marketed. The safety and efficacy of herbal drugs are supported by their long historical use. Nevertheless, it is known that various herbal drugs fail, after testing in clinical trials and there are numerous reports on intoxication cases associated with their consumption. The WHO database has over sixteen thousand suspected case reports, related to intoxication by herbal drugs. The most frequent adverse reactions are hypertension, hepatitis, convulsions, thrombocytopenia and allergic reactions. Cardiovascular problems with the use of ephedra, hepatotoxicity caused by the consumption of kava-kava and comfrey, as well as licorice-related water retention, are some side effects claimed by the pharmacovigilance authorities. In addition to intrinsic factors mentioned above, the herbal drugs efficacy and safety, may also be seriously affected due to botanical misidentification or intentional usage of fake plants, contamination with pesticide residue, toxic heavy metals, pathogens and mycotoxins, as well as adulterants added to increase potency (synthetic substances) or the weight of herbal products in order to reduce costs (Sahoo et al., 2010; Calixto, 2000).

For the purpose of overcoming or mitigating the aforesaid inconvenient issues, WHO has developed a series of technical guidelines and documents in relation to the safety and the quality assurance of medicinal plants and herbal drugs preparations, such as "Quality Control Methods for Medicinal Plant Materials" (a collection of recommended test procedures for assessing the identity, purity and content of medicinal plant materials), "Guidelines on good agricultural and collection practices for medicinal plants", as well as "WHO guidelines for assessing quality of herbal medicines with reference to contaminants and residues". In turn, pharmaceutical laboratories have been investing in the enhancement of the quality for herbal products, aiming at the approval by governmental regulatory agencies, as a strategy to offer more reliable products, therefore conquering the confidence of health care professionals and consumers (Sahoo et al., 2010).

The interest in herbal drugs is continuously growing and they account for a significant share in the pharmaceutical market. The global herbal pharmaceutical industry (including drugs from herbal precursors and registered herbal medicines) invoices approximately US$ 50 billion/year (2008). In 2006, the best selling herbal products were: Ginseng (>U$ 1 billion global sales), Ginkgo (U$ 1 billion), Noni (U$ 1 billion), Saw Palmetto (U$ 600 millions) Echinacea (U$ 500 millions), Valerian (U$ 450 millions), and Green Tea (U $ 450 millions) (Gruenwald, 2008- Entrepreneur). The United States, China, Japan, Germany, South Korea and India, are the largest market. Medicinal plants have also been utilized as source of phytochemicals for the pharmaceutical, cosmetic and agrochemical industries. The most successful examples are paclitaxel (an anticancer drug from *Taxus baccata*), artemisinin (an antimalarial agent from *Artemisia annua*), vincristine/vinblastine (anticancer substances from *Catharanthus roseus*). The Pharmaceutical industry is interested in phytochemicals, however, the availability of quantities of pure chemical substances is normally a limiting factor, since the market demand for phytochemicals, usually reaches a scale from hundreds to thousands of kilograms per annum. (McChesney, 2007)

3. Medicinal plants threatened by extinction

The increasing demand for medicinal plants has endangered several species, since the main source of herbal drugs is the wild plant and the amount required from plant materials invariably exceeds the supply available from its natural source. Although the Convention on Biological Diversity (CBD), held in 1992, has established as goals, the conservation of biological diversity, the sustainable use of its components as well as the fair and equitable sharing of the benefits from the usage of genetic resources, it is still estimated that slightly more than 4000 medicinal plant species are under threat of extinction. The Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES), being the principal tool for monitoring or restricting the international trade of species threatened by over-exploitation, has published a biannual list of medicinal species like: *Taxus wallichiana*, *Panax quinquefolius*, *Dioscorea deltoidea*, *Hydrastis canadensis*, *Prunus africana* , *Rauvolfia serpentina* and *Pterocarpus santalinus* (Schippmann et al., 2002).

The overexploitation of a certain wild medicinal plant and consequent depletion of its raw material affect inevitably the economic feasibility of any phytopharmaceutical business in medium or long-term, since the production cost tends to be higher and the product supply become discontinuous. Furthermore, the extractivism provokes loss of genetic diversity, becoming the remaining plant population more vulnerable to diseases/pests and diminishing the variability of genotypes with features of interest such as yielding, bioactive substance content and resistance to biotic and abiotic factors (Rao et al., 2004). Hence, agencies concerned with conservation policies are recommending that wild species be brought into cultivation systems in order to assure the economic and environmental sustainability of herbal medicines trade (Schippmann et al., 2002). *Ginkgo biloba* and *Hypericum perforatum* are some of the top-selling medicinal plants, however they are not endangered, because their plant materials have been obtained by cultivation for a long time (Canter et al., 2005).

4. Cultivation of medicinal plants

The cultivation of medicinal plants is advocated as a means for meeting current and future demands for large quantities of herbal drugs, but also as a way to relieve the pressure of

harvesting on wild populations (Schippmann et al., 2002). In China, one of the largest markets of herbal medicine, 380,000 ha of lands are utilized for farming of medicinal plants.

Medicinal plants are also cultivated for supplying phytochemicals. Bristol-Myers Squibb developed a system of production based upon isolation of a precursor of Taxol from the leaves or needles of cultivated *Taxus baccata* or *T. wallichiana* that provide the hundreds of kilograms of Taxol required per year for the treatment of cancer patients. (McChesney, 2007)

From the perspective of the market, domestication and cultivation provide a number of advantages over wild harvest for production of herbal drugs: (1) reliable botanical identification; (2) uniform and high quality raw material. As wild plants are dependent on many factors that cannot be controlled and the irregularity of supply is a common feature, the cultivation assures a steady source of raw material; (3) price and volume between farmer and pharmaceutical companies can be more easily negotiable, since the production forecast is more precise; (4) genetic breeding and biotechnology tools can lead to the development of plant materials with agronomically and commercially desirable features, permitting to optimize yield and to meet regulations as well as consumer preferences, respectively; (5) cultivated material can be easily certified as "organic product" (Schippmann et al., 2002; Canter et al, 2005).

Cultivated plants account for 60-90 % in terms of amount of plant material employed by Herbal medicines companies, but the number of wild species still is larger. Although the cultivation is apparently more advantageous than wild harvesting, only 130-140 species are cultivated in Europe, while just 20 out of 400 medicinal plants marketed in India are grown in field. Likewise, amongst 1000 plants more commonly used with medicinal purposes in China, only 100-250 species are sourced from cultivation. There are some reasons that can explain this low utilization of cultivated plants:

(1) Belief of that wild specimens are more potent than cultivated plants. Chinese believe that the physical appearance of wild roots to the human body symbolizes vitality and this feature is crucial for the potency of the ginseng roots, nevertheless cultivated roots do not exhibit this characteristic shape. Furthermore, some scientific studies support partly this hypothesis saying that secondary metabolites, the main responsible for therapeutic properties of herbal medicines, are biosynthesized by plants under particular conditions of stress and competition in their natural environments. Hence, perhaps the secondary metabolites would not be so expressed in monoculture conditions, therefore the active ingredient levels can be much lower in cultivated plant.

(2) Domestication of wild plant is not always technically possible. Many species are difficult to cultivate because of certain biological features or ecological requirements (slow growth rate, special soil requirements, low germination rates, susceptibility to pests, etc.).

(3) Economical feasibility. Domestication requires a long time of agronomical studies and high financial investment for the plantation. Generally, production costs through cultivation are higher than wild harvesting, thus few species can be marketed at a high sufficient price to make cultivation profitable, for instance *Garcinia afzelii, Panax quinquefolius, Saussurea costus* and *Warburgia salutaris*. Hence, many endangered medicinal plants only will bring into cultivation, if exists governmental incentive (Schippmann et al., 2002).

However, the cultivation of medicinal plant in agroforestry system can be a good alternative for more viable and environmentally sustainable farming. In China, ginseng (*Panax ginseng*) and other medicinal plants are grown in pine (*Pinus* spp.), *Paulownia tomentosa* and spruce (*Picea* spp.) forests; besides some medicinal herbs are often planted with bamboo (*Bambusa* spp.). In New Zealand, American ginseng showed better growth under *Pinus radiate.* The shade offered by forest species seems to favor the growth of medicinal plants. Likewise, quinine yields of *Cinchona ledgeriana* increase when it is protected by shade of other species, such as *Crotalaria anagyroides* and *Tephrosia candida*. In India, some medicinal plants that have also been successfully intercropped with fuel wood trees (e.g., *Acacia auriculiformis* and *Eucalyptus tereticornis*) and coconut. Intercropping gives some income to farmers during the period when the main trees have not started production. (Rao et al., 2004).

Application of traditional and biotechnological plant-breeding techniques can become the cultivation of medicinal plants a trade more attractive (eg. increasing the yielding) as well as it can improve features of the plant that affect the efficacy and safety of a herbal drug (eg. levels of bioactive compounds or presence of potentially toxic substances). *Mentha* spp (mints) have been engineered to modify essential oil production and to enhance the resistance of the plant to fungal infection and abiotic stresses. Genetic engineering allowed the enhancement of scopolamine and artemisinin in *Atropa belladonna* and *Artemisia annua*, respectively. (Canter et al., 2005)

5. Amburana cearensis

A. cearensis (Fabaceae) is a native tree from "Caatinga" (a kind of vegetation found in the Brazilian semi-arid region), where it is popularly known as "cumaru" or "imburana-de-cheiro" (Fig. 2). Because of these said popular names, *A. cearensis* is usually misidentified as *Dipteryx odorata* (Fabaceae) and *Commiphora leptophloeos* (Burseraceae). *A. cearensis* occurs widely in South America (from Peru to Argentina), along with another species of this taxon, *Amburana acreana*, which is found chiefly in the southwestern region of the Amazon Forest. *A. cearensis* can reach 15 m of height and 50 cm of diameter, but it is characterized by white flowers and dark pods containing only one seed each, besides its stem bark possessing reddish stains and a vanilla-like aroma of coumarin (**1**). At the early stage of development (seedlings), *A. cearensis* displays a hypertrophied and subterraneous tube-like structure, called xylopodium, which acts as a storage of water and nutrients, therefore it is considered an adaptive strategy for arid habitats (Lima, 1989; Cunha & Ferreira, 2003).

Given the various applications, *A. cearensis* has a great commercial importance in Northeastern region of Brazil. Its wood is used in the carpentry for the manufacturing of furniture, doors and crates, owing to its recognized durability, whereas the seeds are used as flavoring and insect repellents. The wood powder from it can be added to alcoholic beverage barrels for accelerating the aging process of sugar cane distilled spirits (cachaça) (Aquino et al, 2005). The seeds and stem bark are traditionally utilized for treating respiratory diseases, such as influenza, asthma and bronchitis due to anti-inflammatory, analgesic and bronchodilator properties. As far as folk medicine is concerned, *A. cearensis* is consumed as a homemade medication called "lambedô (a sugary drink), however in an industrial scale, the syrup is a pharmaceutical form, which is produced by the government and private laboratories (Fig. 3).

Fig. 2. A wild specimen of *Amburana cearensis* in its natural habitat.

The medicinal use of *A. cearensis* is based on scientific studies, which demonstrated that this plant possesses therapeutic properties that justify its recommendation for the treatment of respiratory illnesses. Preclinical tests demonstrated bronchodilator, analgesic and anti-inflammatory activities for the hydro-alcohol extract from the stem bark of the *A. cearensis*, which also showed to be free of toxicity in therapeutic doses. The chemical composition of the stem bark and seeds from it, consists basically of coumarin, flavonoids, phenol acids and phenol glucosides. Some of them were tested individually and showed pharmacological activities similar to the extract, hence they were considered the active principles of the *A. cearensis*.

However, the intense commercial use of *A. cearensis* has led to the threat of extinction for this specie. In order to ensure the conservation and the economic utilization of *A. cearensis*, we proposed the replacement of its stem bark of a wild adult plant for a young specimen, cultivated under controlled agronomic parameters. In an interdisciplinary study, ethanol extracts of cultivated plants were compared to the extracts of this wild plant through preclinical trials and phytochemical analysis.

Fig. 3. Syrups made from the trunk bark of *Amburana cearensis*.

5.1 Agronomical study of *A. cearensis*

The agronomical study of *A. cearensis* was carried out with seedlings obtained by seed germination. Each plot consisted of six regularly spaced rows of 20 cm, whose sowing density was 50 seeds/row. The seedlings were transplanted to four garden beds (1.2m×10 m), fertilized prior to an organic fertilizer (2.8 kg m^{-2}), containing each of them 20 young plants.

Fig. 4. Seedlings of *A. cearensis* harvested in 8th month of growth

Eight harvestings were performed monthly, starting on the 2nd month until the 9th month after the sowing. The plants harvested were evaluated with the following parameters: fresh plant weight, plant height, xylopodium diameter, root size, ethanol extract yield from the aerial part and xylopodium (Fig. 4). The fresh biomass production of *A. cearensis* seedlings increased almost eight-fold, during the 2nd through 9th month after the sowing. With reference to ethanol extract yield, there was a tendency of decrease for the extract weigh/xylopodium weight ratio over a period of time, while an oscillatory behavior was observed for yield of the ethanol extract from its aerial part, achieving a plateau on the 3rd and 7th month (Leal et al. 2011)

5.2 Phytochemistry of *A. cearensis*

5.2.1 Wild plant

While the pharmacological research about *A. cearensis* advanced, it was necessary to have a better understanding of the chemistry in this specie, previously limited to coumarin and a few phenol compounds, in order to discover its active principles. Bastos (1983), in her master thesis, found coumarin (**1**) as the most abundant component and described the isolation of isokaempferide (**2**), methyl 3,4-dimethoxy-cinnamate (**3**), afrormosin (**4**), 7-hidroxy-8,4'-dimethoxy-isoflavone (**5**), 24-methylenecycloartanol (**6**), β-sitosterol (**7**) and 6-hidroxy-coumarin (**8**). Additionally, the seeds presented a high oil content (23 %), which is composed of triglycerides of the following fatty acids: oleic acid (53 %), palmitic acid (19 %), stearic acid (8 %) and linoleic acid (7 %). Bravo et al. (1999) isolated amburosides A [(4-*O*-β-D-glucopyranosylbenzyl) protocatechuate] (**9**) and B [(4-*O*-β-D-glucopyranosylbenzyl) vanillate] (**10**) from the ethyl acetate extract of the trunk bark, utilizing just silica gel preparative Thin-Layer Chromatography (TLC) (Fig. 5)

Canuto & Silveira (2006) carried out a phytochemical investigation of the ethanol extract from the stem bark. The ethanol extract was partitioned with water and ethyl acetate. The aqueous phase showed to be very rich in sucrose (**11**), whereas the organic phase was dried and submitted to successive chromatographic columns on silica gel and dextran gels. These chromatographic separations led to the isolation of coumarin (**1**), two phenol acids [vanillic acid (**12**) and protocatechuic acid (**13**)], five flavonoids [isokaempferide (**2**), afrormosin (**4**), kaempferol (**14**), quercetin (**15**) and 4'-methoxy-fisetin (**16**)], amburoside A (**9**) and a mixture of β-sitosterol and stigmasterol glycosides (**17-18**). Later on, this same methodology was applied to isolate an isoflavone formononetin (**19**) and a novel coumarin 6-coumaryl protocatechuate (**20**) (Canuto et al., 2010) (Fig. 5). Continuing with the phytochemical study of *A. cearensis*, the seeds revealed high presence of phenol glucosides. Liquid-liquid partitioning from the ethanol extracts followed by chromatography on Sephadex LH-20 and a reversed-phase HPLC chromatography of the ethyl acetate fraction, resulted in the isolation of six new amburosides (C-H). 4-*O*-β-D-(6´´-*O*-galloylglucopyranosyl)-benzyl protocatechuate (**21**), 4-*O*-β-D-(6´´-*O*-acetylglucopyranosyl)-benzyl protocatechuate (**22**), 4-*O*-β-D-(6´´-*O*-protocatechuoylglucopyranosyl)-benzyl protocatechuate (**23**), 4-*O*-β-D-(6´´-*O*-feruloylglucopyranosyl)-benzyl protocatechuate (**24**), 4-*O*-β-D-(6´´-*O*-vanilloylglucopyranosyl)-benzyl protocatechuate (**25**) and 4-*O*-β-D-(6´´-*O*-sinapoylglucopyranosyl)-benzyl protocatechuate (**26**). Additionally, amburoside A (**9**), isokaempferide (**2**), vanillic acid (**12**), 6-hydroxycoumarin (**8**) and (*E*)-*o*-coumaric acid (**27**) were isolated from this same extract. (Canuto et al., 2010) (Fig. 5).

The isolation of 6-coumaryl protocatechuate (**20**) (trunk bark) and 6-hydroxycoumarin (**8**) (seeds) from *A. cearensis* presents an intriguing finding for biosynthesis of coumarins, since monoxygenated-coumarins are preferentially substituted at C-7 position (umbelliferone and its derivatives), according to biogenetic rules. This substitution pattern is due to the usual precursor of coumarins, *p*-coumaric acid, which is biosynthentized by the shikimate pathway from either tyrosine-deamination or *p*-hydroxylation of cinnamic acid (Dewick, 2002). Nevertheless, despite a large occurrence of simple coumarins oxygenated at the C-7 position, 6-hydroxycoumarin (**8**) was also found in some species like *Bidens parviflora* (Asteraceae), *Paeonia suffruticosa* (Paeoniaceae) and *Hydrangea chinensis* (Hydrangeaceae) (Tommasi et al., 1992; Wu et al., 2002; Khalil et al., 2003)

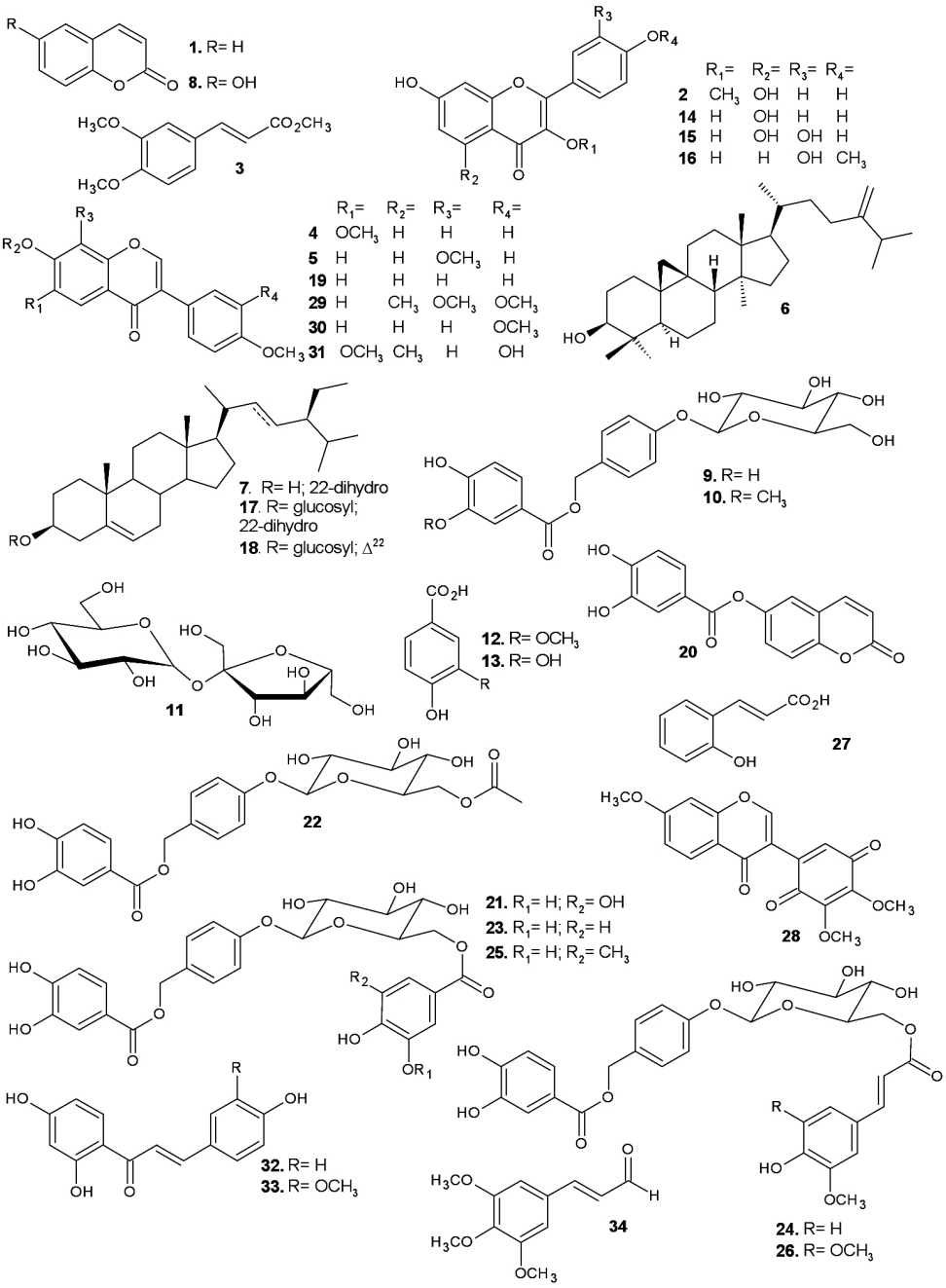

Fig. 5. Chemical structures of constituents isolated from wild *A. cearensis*.

Recently, Bandeira et al (2011) studied a resin exuded from the trunk of *A. cearensis* and found a flavonoid-rich material. The resin ethanol extract was partitioned with water and organic solvents, yielding an ethyl acetate fraction, which was chromatographed on silica gel. From this chromatography, a chloroform fraction was separated by a Sephadex LH-20, resulting in the isolation of a novel compound 3′,4′-dimethoxy-1′-(7-methoxy-4-oxo-4H-cromen-3-yl)-benzo-2′,5′-quinone (**28**), along with six known compounds:, 7,8,3′,4′-tetramethoxyisoflavone (**29**), 3′,4′-dimethoxy-7-hydroxyisoflavone (**30**) and 6,7,4′-trimethoxy-3′-hydroxyisoflavone (**31**), 4,2′,4′-trihydroxychalcone (**32**), 4,2′,4′-trihydroxy-3-methoxychalcone (**33**), 3,4,5-trimethoxycinnamaldehyde (**34**).

5.2.2 Cultivated plant

In order to seek a sustainable alternative for an economic utilization of *A. cearensis*, our research became focused on this *A. cearensis* cultivated plant. The chemical study of the cultivated *A. cearensis* was divided into two parts: (1) a Nuclear Magnetic Resonance (NMR) and the HPLC profiling of ethanol extracts obtained from the aerial part (EEAP) and xylopodium (EEX) of specimens cultivated according to the growing conditions described above; (2) A refined phytochemical analysis of EEAP and EEX extracts produced from specimens in 7 months of growth, where was chosen with basis on pharmacological results.

NMR profiling was performed with extracts of specimens from the 2nd through the 9th month of growth. ^{1}H NMR spectra, recorded in deuterated dimethylsulfoxide, revealed that the extracts from specimens with 2, 4, 7 and 9 months of growth, presented significantly different profiles, requiring further analysis. Hence, these extracts were duly analyzed comparatively with the wild plant extract by Photodiode Array detector (PDA)-HPLC profiling, utilizing constituents previously isolated from the wild *A. cearensis* as an analytical standard. The separations were performed on a C18 analytical column and the mobile phase was a gradient composed of H_2O (pH 3, H_3PO_4-Et_3N)/MeOH. The run time was 40 min and the chromatograms were observed at 254 nm. The qualitative analysis consisted of identification from analytical standards in the chromatograms of ethanol extracts, which were derived from the trunk bark (wild plant), xylopodium and the aerial part (cultivated plant) by retention time and UV spectra. Only 8 out of the 13 standards injected were detected in the samples. Coumarin (**1**) and vanillic acid (**12**) were the only substances present in all extracts of the *A. cearensis*. Amburoside B (**10**) and protocatechuic acid (**13**) were found in all extracts, except in the xylopodium extracts from specimens harvested in the 2nd and 4th month of growth, respectively. (*E*)-*o*-coumaric acid (**27**) and its glucoside (**35**), along with isokaempferide (**2**), afrormorsin (**4**) and kaempferol (**14**) were not detected in any extracts. Ayapin (**36**) and (*Z*)-*o*-coumaric acid glucoside (**37**) were found only in cultivated plants (Table 2).

A quantitative analysis was carried out for four major constituents of the *A. cearensis* [coumarin (**1**), amburoside A (**9**), vanillic acid (**12**), protocatechuic acid (**13**)] in ethanol extracts from trunk bark (EETB) and seeds (EES) of the said wild plant, as well as the whole plant (EEWP), xylopodium (EEX), as well as the aerial part (EEAP) of cultivated specimens in the four following selected months (2, 4, 7 and 9), accounting for 10 samples. The HPLC method was developed in chromatographic condition similar to the once described above and validated according to analytical parameters, defined by the Brazilian Health Surveillance Agency and the Brazilian Institute of Metrology, Standardization and

Substances	Retention time (min.)	Trunk Bark	Xylopodium (month)				Aerial Part (month)			
			2	4	7	9	2	4	7	9
(*Z*)-Coumaric acid glucoside	3,85	-	+	+	+	+	-	-	-	-
p-Hidroxi-benzoic acid	4,99	+	+	+	+	+	-	-	-	-
Protocatechuic acid	7,36	+	+	-	+	+	+	+	+	+
Vanillic acid	10,87	+	+	+	+	+	+	+	+	+
Coumarin	13,98	+	+	+	+	+	+	+	+	+
Amburoside A	14,65	+	+	-	+	+	-	+	+	+
Ayapin	14,85	-	+	+	+	-	+	+	+	+
Amburoside B	17,17	+	-	+	+	+	+	+	+	+

Table 2. Distribution of some constituents in *A. cearensis* (**+**, presence; **-** ,not detected)

Industrial Quality: linearity, selectivity, accuracy, precision as well as the limit of detection and the limit of quantification. As can be noticed on Table 3, amburoside A (**9**) was the most abundant component in EETB, followed by coumarin (**1**), protocatechuic acid (**13**) and vanillic acid (**12**). However, coumarin (**1**) was the only component detected in EES. Among cultivated plants extracts, vanillic acid (**12**) was the principal component in 3 out of 4 periods analyzed through EEW, while coumarin (**1**) appeared as the major compound in the 7th month. Protocatechuic acid (**13**) and amburoside A (**9**) were below the limit of quantification in all extracts, except in the 9th month, whereby amburoside A (**9**) had a considerable content (Leal et al., 2011). In EEAP, vanillic acid (**12**) was the main constituent evaluated in all seasons, reaching the highest concentration in the 7th month (8520 mg/100g ext). On the other hand, coumarin (**1**) was the major component in xylopodium (4th month: 3760 mg/100g ext), except in the last month, when amburoside A (**9**) was the most abundant (680 mg/100g ext). Protocatechuic acid (**13**) presented measurable levels only in the EEAP of 4 months (360 mg/100g ext). Coumarin (**1**) and vanillic acid (**12**) were found preferentially in the aerial part, while amburoside A (**9**) was present mainly in the xylopodium. In comparison with wild plants, EEX of 9 months was the only cultivated plant extract which had amburoside A as the major component like EETB, even so at a concentration being three-fold lower than in the latter.

EEAP and EEX extracts harvested in the 7th month of growth were submitted to partition H_2O/EtOAc, yielding aqueous and ethyl acetate fractions from each extract. Isokaempferide (**2**), amburoside B (**10**), vanillic acid (**12**), *p*-hidroxy-benzoic acid (**38**) and the coumarin ayapin (**36**) were isolated from the ethyl acetate fraction derived from EEAP after being chromatographed on silica and dextran gels, while the aqueous fraction of EEAP yielded (*E*)-melilotoside (**35**) and amburoside B (**10**), again, through C18 solid-phase extraction (SPE) and C18 HPLC. On the flip side, adsorption and exclusion chromatography of the ethyl acetate fraction derived of EEX, afforded the isolation of amburoside A (**9**) and protocatechuic acid (**13**), whereas (*Z*)-melilotoside (**37**) was isolated by Sephadex LH-20 followed with the purification through C18 HPLC (Fig. 6). (*E/Z*)- melilotoside (**35** and **37**) are trivial names for *o*-coumaric acid glucoside in allusion to the genus *Melilotus*, where these compounds were firstly identified. Interestingly, the *E* and *Z*-melilotosides were found in different parts of the *A. cearensis*: (*E*)-stereoisomer exclusively in the aerial part, while (Z)-

Extracts	Concentration (mg/100g extract)– *CV* (%)			
	Protocatechuic acid	**Vanillic acid**	**Coumarin**	**Amburoside**
Wild Plant				
Trunk bark	320 *(6,3)*	200 *(5,6)*	1340 *(6,8)*	2180 *(5,5)*
Seeds	ND	ND	23520 *(3,9)*	ND
Cultivated Plant				
Whole				
2 months	ND	1520 *(1,3)*	1020 *(5,7)*	ND
4 months	ND	2680 *(13,4)*	2000 *(7,0)*	ND
7 months	ND	3440 *(4,3)*	4060 *(6,5)*	ND
9 months	ND	1520 *(6,1)*	660 *(1,8)*	400 *(5,7)*
Aerial Part				
2 months	ND	4780 *(10,6)*	1540 *(3,0)*	ND
4 months	360 (3,3)	6120 *(4,8)*	1660 *(2,0)*	260 *(8,7)*
7 months	ND	8520 *(1,0)*	6060 *(7,9)*	ND
9 months	ND	3460 *(6,3)*	1500 *(3,3)*	ND
Xylopodium				
2 months	ND	780 (5,3)	1320 *(1,0)*	380 *(5,3)*
4 months	ND	760 (7,5)	3760 *(0,9)*	ND
7 months	ND	1380 (2,9)	2500 *(4,2)*	300 *(10,4)*
9 months	ND	540 (9,8)	420 *(10,8)*	680 *(9,4)*

Table 3. Concentrations of four major compounds of *A.cearensis* in different extracts.

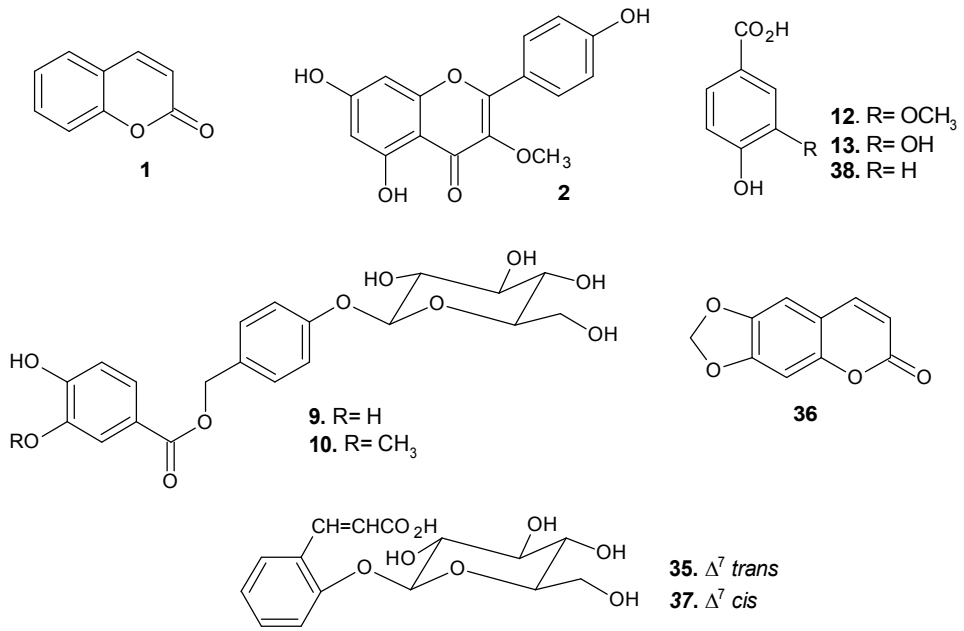

Fig. 6. Chemical structures of constituents isolated from cultivated *A. cearensis*.

stereoisomer was present only in xylopodium. In *Melilotus alba* (a legume), the melilotosides are considered the precursors of coumarin, being one of the major constituents of *A. cearensis*. (*E*)-melilotoside (**35**) exposed to UV radiation (sunlight) may be converted to the less stable stereoisomer (*Z*), which do undergo enzyme-catalyzed lactonization to yield coumarin (**1**) (Dewick et al., 2002).

As part of our effort for finding out which substances are responsible by medicinal properties of *A. cearensis*, isokaempferide (**2**), afrormosin (**4**), amburoside A (**9**), vanillic acid (**12**) and protocatechuic acid (**13**) obtained from this work were assayed in diverse pharmacological tests, which will be discussed briefly. The chemical structures of the new compounds were elucidated by means of spectroscopic techniques such as IR, HRMS, 1D and 2D NMR (COSY, HSQC, HMBC and NOESY).

5.3 Pharmacology of *A. cearensis*

The literature reports several toxicological and pharmacological studies carried out with the extracts and substances isolated from wild and cultivated *A. cearensis.* The focus of them is on the anti-inflammatory, antioxidant, smooth muscle relaxant, antinociceptive, neuroprotector and platelet antiaggregant effects (Leal, 1995; 2006; Leal et al., 1997; 2000; 2001; 2003ab; 2005; 2006ab; 2008).

A toxicological study carried out with the hydroalcoholic extract (HAE) from the trunk bark of the *A. cearensis* administered to rats by the oral route did not show any toxic effects (Leal et al., 2003). Further studies demonstrated that the HAE administered to rats daily for 50 days did not interfere with the pregnancy rate and development during the 1st as well as the 2nd generation of animals (Leal et al., 2003a, Leal et al., 2006a). The cytotoxicity of isokaempferide (**2**), kaempferol (**14**), amburoside A (**9**) and protocatechuic acid (**13**) from the *A. cearensis,* were evaluated on tumor cell lines and on the sea urchin egg development, as well as their lytic properties on mouse erythrocytes. The results showed that isokaempferide (**2**) and kaempferol (**14**), but not amburoside A (**9**) and protocatechuic acid (**13**), inhibited the sea urchin egg development, as well as tumor cell lines. However, only protocatechuic acid (**13**) induced lysis on mouse erythrocytes (Costa-Lotufo et al., 2003).

Previous studies (Leal et al., 1997; 2000) reported the antinociceptive, antiedematogenic and smooth muscle relaxant properties of HAE, coumarin (**1**), and the flavonoid fraction, from wild *A. cearensis*. The antiedematogenic activity was manifested in inflammatory process dependents on polimorphonuclear cells, while the antinociceptive effect of coumarin (**1**) and HAE seems to occur by a mechanism at least in part dependent on the opioid system. Nevertheless, the nitridergic system has also an important role in the coumarin nociception. Additional studies about the pharmacological potential of the HAE, coumarin (**1**) and the flavonoid fraction emphasized the anti-inflammatory potential of these species, which seems to be related to the presence of coumarin (**1**) in the plant (Leal et al., 2003).

Like other medicinal plants containing coumarin (**1**) such as *Justicia pectoralis, Pterodon polygaliflorus, Hybanthus ipecacuanha* and *Eclipta alba*, *A. cearensis,* also has a relaxing activity on isolated guinea pig tracheal muscles (Leal et al., 2000). Confirming this as particular effect, it was recently (Leal et al., 2006) demonstrated the relaxant action of the isokaempferide (**2**). The relaxation of the guinea-pig isolated trachea, induced by isokaempferide (**2**), was a direct and an epithelium-independent phenomenon, resulting

from several intracellular actions through a common pathway e.g., the opening of Ca^{2+} and ATP-sensitive K^+ channels.

Previous studies (Leal et al., 2003; Leal, 2006) showed that the anti-inflammatory activity of HAE, coumarin (**1**), isokaempferide (**2**) and amburoside A (**9**) from *A. cearensis,* seems to occur by an inhibitory action on the release of inflammatory mediators, and/or alternatively by interfering with a certain phase of the neutrophil migration into the inflammatory focus. Other data (Leal et al., 2008) corroborated this hypothesis showing that both the isokaempferide (**2**) and amburoside A (**9**) exert their anti-inflammatory activities mainly by inhibiting the lipopolysaccharide-induced release of TNF-α, although the involvement of other inflammatory mediators cannot be excluded. Furthermore, inhibitions of some biological functions of neutrophils, namely, accumulation of cells and activity of hydrolytic enzymes, as myeloperoxidase, may also play a role.

Amburoside A (**9**) showed a hepatoprotective property in the CCl_4-induced liver toxicity model in rats. This effect may be due to its capacity to modulate the oxidative stress, especially by reducing of the lipid peroxidation, as well as by a significant restoration to normal levels of the catalase activity and GSH contents as observed in CCl_4-intoxicated rats after the amburoside A (**9**) treatment (Leal et al., 2008).

The large-scale usage and demand for the wild *A. cearensis,* as a medicinal plant by communities in the Northeastern of Brazil, governmental programs of phytotherapy as well as the pharmaceutical industry, are contributing to decrease availability on these species, presently considered as endangered ones. In this sense, our laboratory has conducted comparative studies on the pharmacological profile of the ethanolic extract (EtOHE) or vanillic acid (**12**) from the wild and cultivated *A. cear*ensis, by evaluating their antinociceptive and antiedematogenic activities in several experimental models, such as the formalin test, carrageenan or dextran-induced edema and carragenan-induced neutrophil migration into the rat peritoneal cavity (Leal et al., 2010).

The acute treatment with both the EtOHE prepared from all parts of the cultivated *A. cearensis* (4, 7 or 9 months) or the wild *A. cearensis,* present antinociceptive and anti-inflammatory activities (Fig. 7). In addition, vanillic acid (**12**), which together with coumarin (**1**) are the major compounds present in cultivated *A. cearensis*, also showed an antinociceptive activity by inhibiting both phases of the formalin test in mice, and this effect was partially blocked by naloxone. Thus, the data suggest that antinociceptive effect of vanillic acid (**12**) occur by a mechanism at least in part dependent on the opioid system (Leal et al., 2010).

Coumarin (**1**) has been found in several Brazilian medicinal plants including *J. pectoralis*, *M. glomerata* and *A. cearensis*. It has been reported that the antinociceptive and the anti-inflammatory activities of these species seems to be related at least in part to the presence of coumarin (**1**) (Leal et al., 1997; 2003; Leal et al., 1997; Lino et al., 1997; Leal et al., 2000; Freitas et al., 2008). The biological effects of coumarin (**1**) include antibacterial, antiviral, antiedematogenic, antioxidant, lipoxygenase inhibition, lipid peroxidation inhibition, and scavenging of superoxide hydroxyl radicals (Hoult & Paya, 1996; Chang et al., 1996; Casley-Smith et al., 1993; Rajarajeswari & Pari, 2011).

Recently (Leal et al., 2010), it was also determined that the anti-inflammatory effect of vanillic acid (**12**) is isolated from the cultivated *A. cearensis.* This compound orally

administered to rats, was shown to significantly inhibit the carrageenan, but not the dextran-induced edema. It also reduced the accumulation of PMN into the peritoneal cavity of rats and this effect was comparable to that observed with dexamethasone, used as a standard drug (Fig. 7).

Vanillic acid (**12**) is a benzoic acid derivative that is used as a flavoring agent. It is an intermediate in the production of vanillin from ferulic acid (Prince et al., 2011; Kim et al., 2011). Previous studies have shown antifilarial, antibacterial, antioxidant, hepatoprotective and anti-inflammatory (Kim et al., 2011; Prince et al., 2011; Itoh et al., 2009) effects of the vanillic acid. This compound exerts its anti-inflammatory effect by suppressing the production of prostaglandin E2, nitric oxide and cytokines. Furthermore, it also suppressed the activation of nuclear-factor-kappa B and caspase (Kim et al., 2011; Itoh et al., 2009). These findings confirm the anti-inflammatory activity of vanillic acid (**12**) as demonstrated by our laboratory.

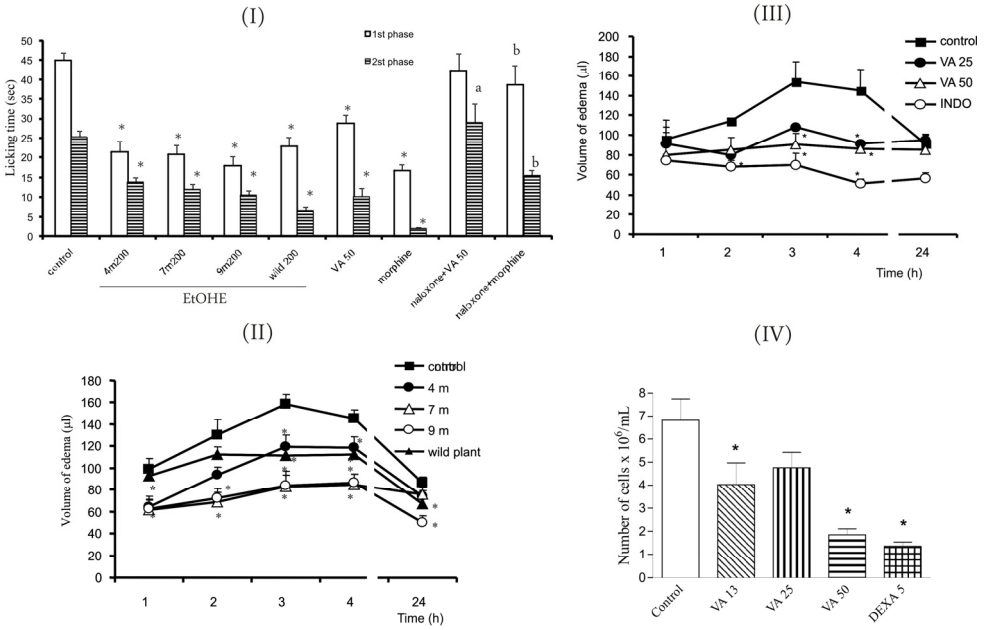

Fig. 7. Antinociceptive and anti-inflammatory effects of ethanolic extracts (EtOHE) and vanillic acid (VA) from *Amburana cearensis* in rodents.[1]

A growing body of evidence suggests that the extract and chemical constituents from the wild *A. cearensis* have pharmacological properties which justify at least in part its traditional use in the treatment of asthma. Among others, the anti-inflammatory activity is possible due

[1] I : effects of EtOHE (cultivated, (4, 7 and 9 months(m) or wild plants: 200 mg/kg, p.o.), VA (50 mg/kg, p.o.) or morphine (MP, 5 mg/kg, s.c.) on the formalin-induced nociception in mice (6-18 animals/group). II, III and IV: anti-inflammatory effects of EtOHE and VA on the carrageenan (Cg)-induced mice paw edema and Cg-inuced rat peritonitis.

to their capacity to modular several responses, especially those related to oxidative stress, the production of inflammatory mediators, and the accumulation and/or activation of inflammatory cells as neutrophils.

The preliminary pharmacological study of the cultivated *A. cearensis* (Leal et al., 2010) showed that both cultivated and wild plants have antinociceptive and anti-inflammatory activities in rodents. Coumarin (**1**) and vanillic acid (**12**) are possibly responsible for the pharmacological activities of the cultivated *A. cearensis* extracts, however the pharmacological importance of other chemical constituents present in the cultivated species cannot be ruled out.

6. Conclusions

The interdisciplinary study of the *A. cearensis* revealed that its ethanol extracts from cultivated and wild sources have similar phytochemical profiles, as consequence, both extracts possess similar pharmacological activities. Hence, these findings support the idea of the utilization of cultivated plants for the manufacturing of herbal drugs preparations by pharmaceutical laboratories, favoring the uniform and constant supply of high quality raw material, as well as the conservation of the wild specimens in the original biome. Indeed, this research indicates promising prospects for the rational use of the *A. cearensis,* however, it is still needed to be advanced in some issues concerning with agronomical, phytochemical and pharmacological knowledge of these species. The influence of some agronomic parameters (plant spacing, shading or sunlight exposure, water supply, etc) on the chemical composition will be performed. Chemical markers or a metabolomic approach will be developed in order to evaluate the influence of the aforementioned agronomic parameters on chemical composition. Pharmacological testing with other types of inflammation experimental models and clinical trials will be carried out aiming to elucidate the mechanisms of action of the *A. cearensis* active principles as well as to evaluate the efficacy in human beings. Additionally, an economic analysis should be performed in order to evaluate the economic feasibility in the production of *A. cearensis* herbal drug preparations from cultivated source.

7. Acknowledgements

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18

General Introduction on Family Asteracea

Maha Aboul Ela, Abdalla El-Lakany and Mohamad Ali Hijazi
Dept. of Pharmacognosy, Faculty of Pharmacy,
Beirut Arab University,
Beirut,
Lebanon

1. Introduction

Asteraceae is the largest family of the plant kingdom, very abundant and also a diverse one. The Asteraceae plants are the most widely distributed of all the families (Porter, C.L. (1969); Evans W. (1989); Hutchinson, J.(1973); Core, E. L. (1955) of the angiosperms. It includes about 1400 genera and over 25000 species (Harborne , J. B., Turner, B.L. (1984); Aboul Ela, M. A.,(1991), forming approximately 10% of the flowering plants.

Asteraceae has characteristic taxonomical characters (Muschler, R. (1912). Members of the family are generally herbs of annul or perennial habits and some tropical forms occur as shrubs. Flowers are grouped in heads known as capitula, surrounded by involucres. It is of two kinds of florets; tubular or disc florets with tubular corolla and mostly hermaphrodite, and ligulate or ray floret, with starp like corolla and mostly female.

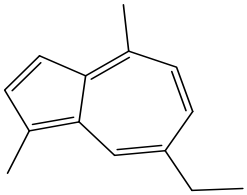
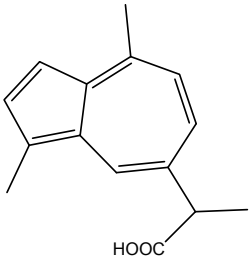
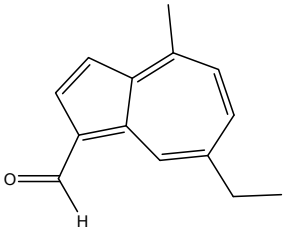
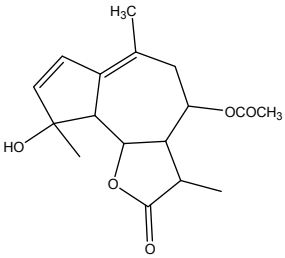
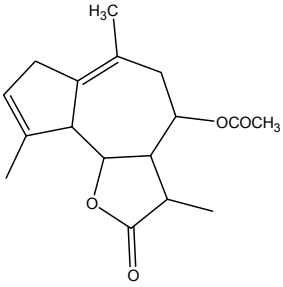
2. Chemistry of genus Matricaria

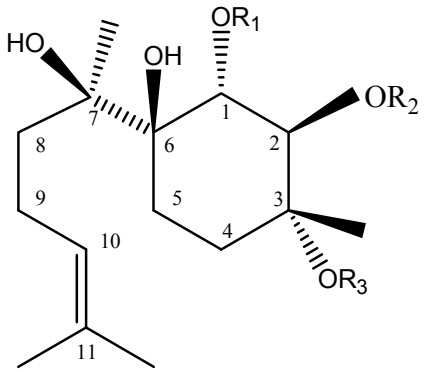
Genus Matricaria comprises plants with various secondary metabolites of different chemical nature recorded mainly in *Matricaria chamomilla*.German chamomile flowers contain 0.24- to 2% volatile oil which is blue in color. Chamomile also contains up to 8% flavone glycosides and flavonol; up to 10 percent mucilage polysaccharides; up to 0.3 percent choline; and approximately 0.1 percent coumarines. The tannin level in chamomile is less than one percent. (Alternative Medicine Review (2008))

Following is a review of the chemical compounds that have been isolated previously from genus Matricaria (Tables 1, 2, 3, and 4).

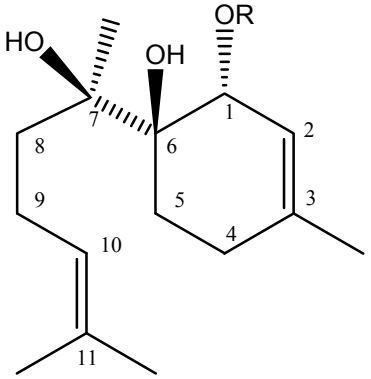
2.1 Volatile oil

Name	Source	Structure	References
a) Azulene derivatives			

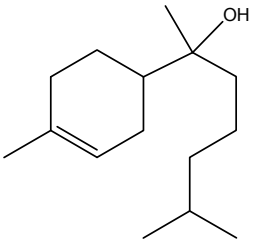
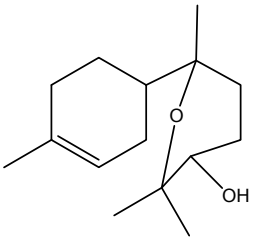
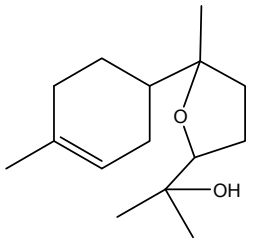
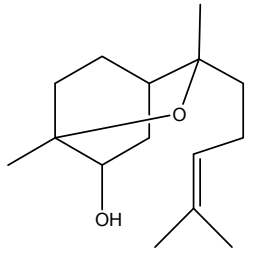
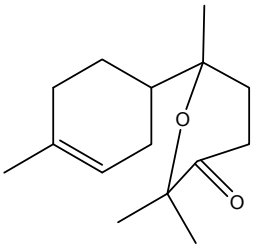
Chamazulene	*M. chamomilla*		Alternative Medicine Review 2008, Ness, A.,Metzger, J. W., Shmidt, P. C. (1996)
Chamazulene Carboxylic acid	*M. chamomilla*		Stahl, E. (1954)
Chamavioline	*M. chamomilla*		Motl, O. ,Repcak, M. (1979), Motl, O. ,Repcak, M. ,Ubik, K. (1983)
Matricin (proazulene)	Ligulate and tubular floret only of *M. chamomilla*		Alternative Medicine Review (2008), S˘orm, F.,Nowak, J., Herout, V.(1953), Cekan, Z., Herout, V., Sorm, F.,(1957)
Matricarin	*M. chamomilla*		Alternative Medicine Review (2008)
b) Sesquiterpenes i) Oxygenated sesquiterpenes			

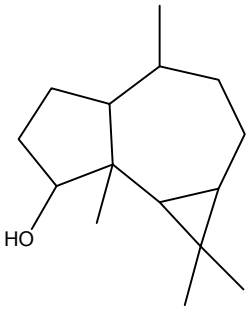
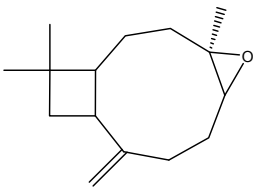
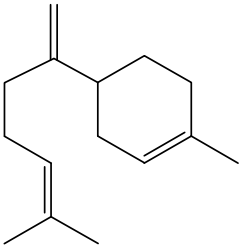
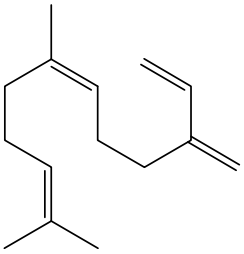
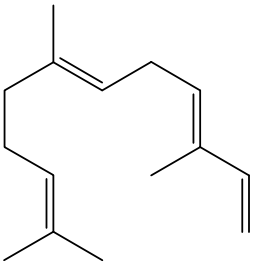


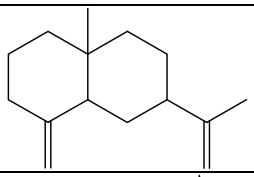
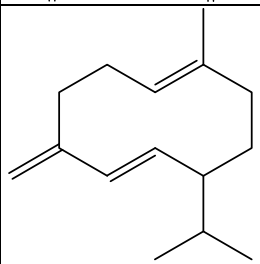
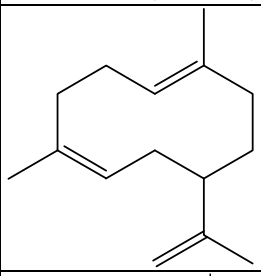
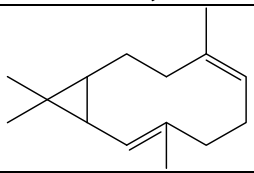
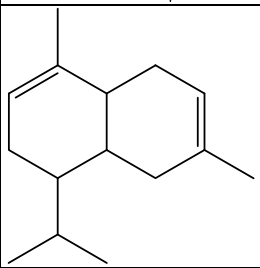
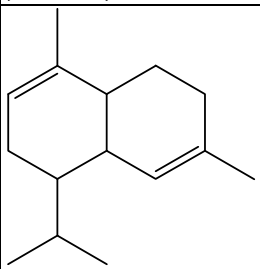
(1R*,2R*,3R*,6R*,7R*)1,2,3,6,7-pentahydroxy-bisabolol-10(11)-ene	*M. aurea*	R1	R2	R3	Ahmed A. Ahmed, Maha A. Abou Elela (1999)
		H	H	H	
(1R*,2R*,3R*,6R*,7R*)1,2,3,6,7-tetrahydroxy-1-acetoxy-bisabolol-10(11)-ene	*M. aurea*	Ac	H	H	Ahmed A. Ahmed, et al.(1993)
(1R*,2R*,3R*,6R*,7R*)1,2,3,6,7-tetrahydroxy-2-acetoxy-bisabolol-10(11)-ene	*M. aurea*	H	Ac	H	Ahmed A. Ahmed, Maha A. Abou Elela (1999)

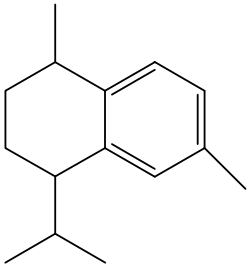
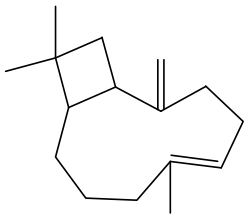
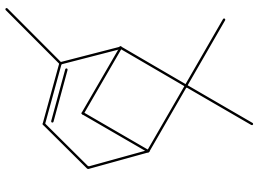
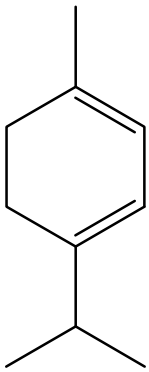


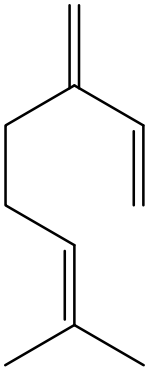
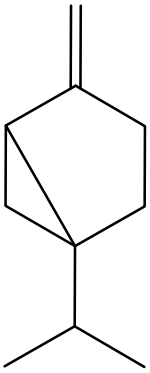
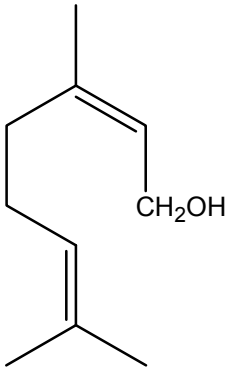
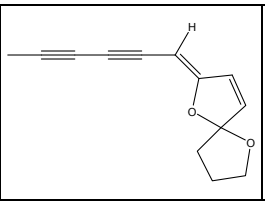
(1R*,6R*,7R*)1,6,7-trihydroxy-bisabolol-2,10- diene	*M. aurea*	R	Ahmed A. Ahmed, et al.(1993)
		H	
(1R*,6R*,7R*)1,6,7-trihydroxy-1-acetoxybisabolol-2,10-diene	*M. aurea*	Ac	Ahmed A. Ahmed, et al.(1993)

(-)-α-bisabolol	*M. chamomilla*		Alternative Medicine Review (2008), S˘orm, F.,Zaoral M.,Herout, V.(1951)
(-)-α-bisabolol oxide A	*M. chamomilla*		Alternative Medicine Review 2008, Sampath , V., et al (1969)
(-)-α-bisabolol oxide B	*M. chamomilla*		Alternative Medicine Review (2008), Sampath ,V.,Sabata, et al ,(1969)
(-)-α-bisabolol oxide C	*M. chamomilla*		Schilcher, H., et al (1976)
α-bisabolone oxide	*M. chamomilla* growing in turkey		Hölzl, J., Demuth, G.(1973)

Spathulenol	*M. chamomilla*		Alternative Medicine Review (2008), Motl, O., et al(1977)
Caryophyllene epoxide	*M. chamomilla*		Reichling, J., et al (1983)
ii) Unsaturated sesquiterpenes			
β-bisabolene	*M. chamomilla*		Anne ORAV, Tiiu KAILAS, and Kaire IVASK (2001)
Trans-β- farnesene	*M. chamomilla*		Alternative Medicine Review (2008), Lemberovics, E. (1979)
Trans -α-farnesene	*M. chamomilla*		Lemberovics, E. (1979)

β-selinene	*M. chamomilla*		A.Pizard, et al.(2006)
Germacrene D	*M. chamomilla*		Anne ORAV, Tiiu KAILAS, and Kaire IVASK (2001) A.Pizard, et al.(2006)
Germacrene A	*M. chamomilla*		A.Pizard, et al.(2006)
Bicyclo germacrene	*M. chamomilla*		A.Pizard, et al.(2006)
Cadinene	*M. chamomilla*		Alternative Medicine Review (2008), Anne ORAV, Tiiu KAILAS, and Kaire IVASK (2001)
α-muurolene,	*M. chamomilla*		Motl, O., Repcak, M.(1979)

Calamemene	*M. chamomilla*		Motl, O., Repcak, M.(1979)
β-caryophyllene	In the root oil of *M. chamomilla*		Reichling, J., et al (1983)
c) Monoterpenes			
α-pinene	*M. chamomilla*		Anne ORAV, Tiiu KAILAS, and Kaire IVASK (2001), A.Pizard, et al.(2006)
α-Terpinene	*M. chamomilla*		A.Pizard, et al.(2006)

Myrcene	*M. chamomilla*		Stransky, K., et al., (1981)
Sabinene	*M. chamomilla*		Anne ORAV, Tiiu KAILAS, and Kaire IVASK (2001) , A. Pizard, et al.(2006)
Gerianol	*M. chamomilla*		Stransky, K., et al., (1981)
Spiroethers			
Cis (Z)-enyne dicycloether *cis*-2-[hexadiyne)- (2,4)-ylidene]-1,6-dioxaspiro-[4,4]-nonene)	*M. chamomilla*		Alternative Medicine Review (2008), Bohlmann, F., Zdero, C. (1982), Bohlmann, F., et al ,(1961)

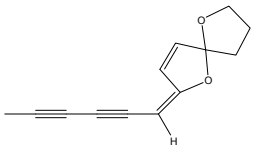
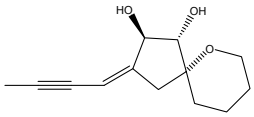
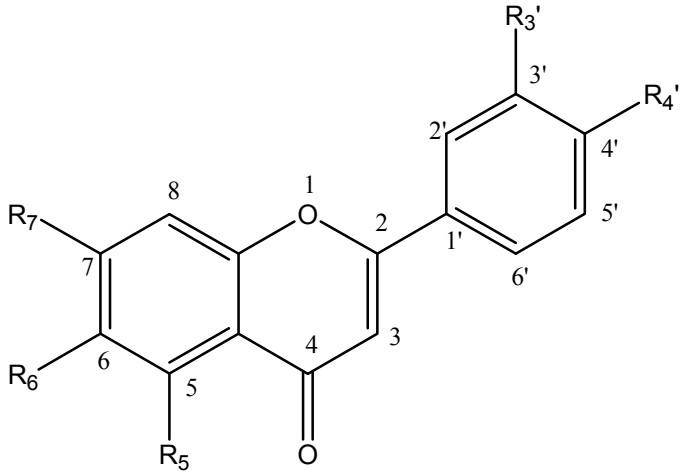
Trans (E)-enyne dicycloether *trans*-2-[hexadiyne)-(2,4)-ylidene]-1,6-dioxaspiro-[4,4]-nonene	*M. chamomilla*		Alternative Medicine Review (2008), Bohlmann, F., et al ,(1961), Bohlmann, F., Zdero, C. (1982)
(3S*,4S*,5R*)-(E)-3,4-dihydroxy-2-(hexa-2,4-diynyliden)-1,6-dioxaspiro-(4,5) decane	*M. aurea*		Ahmed A. Ahmed, Maha A. Abou Elela (1999)

Table 1. Volatile components isolated from Matricaria species

2.2 Flavonoids

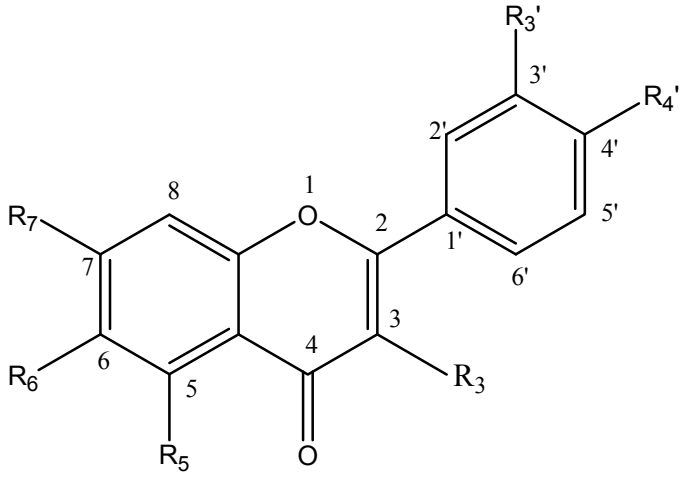
a) Flavone aglycon and glycosides isolated from species *Chamomilla*



Name	Source	R_5	R_6	R_7	$R_{3'}$	$R_{4'}$	Ref.
i) Flavone aglycon							
Apigenin	Ligulate florets flowers	OH		OH		OH	Powe, F., Browning, H. Jr. (1914), S˘orm, P.,et al.,(1952), Kunde, R., Isaac, O.(1979), Carle, R. and Isaac, O. (1985)
Luteolin	flowers	OH		OH	OH	OH	Kunde, R., Isaac, O.(1979), Carle, R. and Isaac, O. (1985)
Chrysoseriol	flowers	OH		OH	OCH_3	OH	Carle, R. and Isaac, O. (1985) , Reichling, J., et al., (1979)

ii) Flavone glycosides							
Luteolin-7-glucoside	Egyptian chamomile floret (leaves) Ligulate florets	OH		OGlu	OH	OH	Kunde, R., Isaac, O.(1979), Elkiey, M. A.,et al., (1963), Greger, H. (1975)
Luteolin-4′-glucoside		OH		OH	OH	OGlu	
Chrysoseriol-7-glucoside	Leaves	OH		OGlu	OCH_3	OH	Greger, H. (1975)
Apigenin-7-glucoside (Apigetrin)	Ligulate floret	OH		OGlu		OH	Kunde, R., Isaac, O.(1979), Lang, W., Schwandt, K. (1957), Hörhammer, L., Wagner, H., Salfner, B. (1963)
Apigenin-7-(6′′-O-acetyl)-glucoside	Ligulate florets	OH		OGlu-ac.		OH	Kunde, R., Isaac, O.(1979), Redaelli, C., Formentini, L., Santaniello, E. (1979)
Apigenin-7-(6′′-O-apiosyl)-glucoside (apiin)	Ligulate florets	OH		OGlu-Apio.		OH	Kunde, R., Isaac, O.(1979), Wagner, H., Kirmayer, W. (1957)

b) Flavonol aglycones and glycosides isolated from *Chamomilla* species



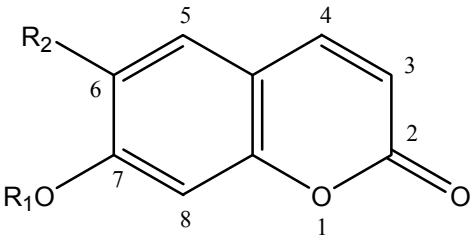
Name	Source	R_3	R_5	R_6	R_7	$R_{3'}$	$R_{4'}$	Ref.
i) Flavonol aglycones								
Lutuletin	Tubular floret flowes	OH	OH	OCH_3	OH	OH	OH	Kunde, R., Isaac, O.(1979), Carle, R. and Isaac, O. (1985) , Ahmed A. Ahmed, Maha A. Abou Elela (1999)
Quercetin	Leaves Tubular floret flowers	OH	OH		OH	OH	OH	Kunde, R., Isaac, O.(1979), Carle, R. and Isaac, O. (1985), Greger, H. (1975)
Chrysosplenol	Chamomile flower	OCH_3	OH	OCH_3	OCH_3	OH	OH	Carle, R. and Isaac, O. (1985), Exner, J., et al., (1981), Hänsel, R., Rimpler, H., Walther, K. (1966)
Chrysosplenitin	Flowers	OCH_3	OH	OCH_3	OCH_3	OCH_3	OH	Carle, R. and Isaac, O. (1985) , Hänsel, R., Rimpler, H., Walther, K. (1966)
Eupatoletin	Chamomile flower Ligulate florets	OH	OH	OCH_3	OCH_3	OH	OH	Kunde, R., Isaac, O.(1979), Carle, R. and Isaac, O. (1985), Exner, J., et al., (1981), Hänsel, R., Rimpler, H., Walther, K. (1966)
Eupalitin	Chamomile flower	OH	OH	OCH_3	OCH_3		OH	Carle, R. and Isaac, O. (1985), Exner, J., et al., (1981), Hänsel, R., Rimpler, H., Walther, K. (1966)
ii) Flavonol glycosides								
Quercetin-7-glucoside (Quercimeritrin)	Tubular floret	OH	OH		OGlu	OH	OH	Kunde, R., Isaac, O.(1979), Lang, W., Schwandt, K. (1957) , Horhammer, L.,

								Wagner, H., Salfner, B. (1963)
Quercetin-3-rutinoside	Chamomile flower	OGlu-Rham	OH		OH	OH	OH	Elkiey, M. A.,et al., (1963)
Quercetin-3-galactoside	Chamomile flower	OGal	OH		OH	OH	OH	Elkiey, M. A.,et al., (1963)

Table 2. Flavone and flavonol aglycon and glycosides isolated from species *Chamomilla*

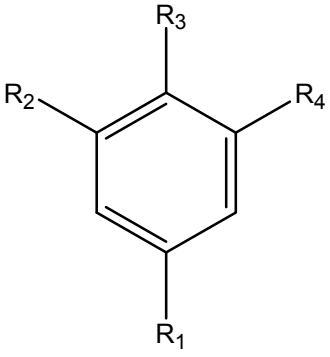
2.3 Coumarines and other polyphenolic compounds

a) Coumarines



Name	Source	R_1	R_2	Ref.
Herniarin	Ligulate and Tubular floret of *M. chamomilla*	CH_3	H	Schilcher, H. (1985)
Umbelliferone	Ligulate and Tubular floret of *M. chamomilla*	H	H	Schilcher, H. (1985)
Isoscopoletin	*M. chamomilla*	CH_3	H	Kotov, A. G., et al., (1991)
Esculetin	*M. chamomilla*	H	OH	Kotov, A. G., et al., (1991)
Scopoletin	*M. chamomilla*	H	OCH_3	Kotov, A. G., et al., (1991)

b) Phenyl carboxylic acid



Name	Source	R_1	R_2	R_3	R_4	Ref.
Synergic acid	Ligulate and tubular floret of *M. chamomilla*	COOH	OCH_3	OH	OCH_3	Reichling, J., et al., (1979)
Vanillic acid	Ligulate and tubular floret of *M. chamomilla*	COOH	H	OH	OCH_3	Reichling, J., et al., (1979)
Anisic acid	*M. chamomilla*	COOH	H	OCH_3	H	Reichling, J., et al., (1979)
Caffeic acid	*M. chamomilla*	$CH_2{=}CH_2COOH$	OH	OH	H	Reichling, J., et al., (1979)

Table 3. Coumarines and other polyphenolic compounds isolated from genus Matricaria

2.4 Miscellaneous substances

Chamomile contains up to 10% mucilage polysaccharides (Alternative Medicine Review (2008)).The main chain of the polysaccharide consists of α-1-> 4 connected D-galacturone acids (Carle, R. and Isaac, O.,(1985)). In addition to xylose, arabinose, galactose, glucose, rhamnnose (Janecke, H., Weiser, W. (1964))(Janecke, H., Weiser, W.(1965)).

Recently, three polysaccharides were isolated and showed remarkable antiphlogistic activity against mouse ear edema induced by crotone oil (Füller, E., (1992)) as fructane (74.3% fructose and 3.4% glucose, similar to inulin), rhamnogalacturonane (28% uronic acid, 3.2% protein, similar to pectin), and arabino-3, 6-galactane glycoproteins.

- Chamomile contains up to 0.3% choline ((Alternative Medicine Review (2008)), (Bayer, J. et al. (1958)) which is supposed to be participating in the antiphlogistic activity of the extract.
- More than 13 amino acids were detected (Schilcher, H.,(1980)) from the fresh chamomilla herb as L-leucine, DL-methionine, DL-α-alanine, glycine, L-histidine, L-(+)-lysine, DL- threonine, DL-serine, and L-glutaminic acid.
- Tannin level is less than 1% (Alternative Medicine Review (2008).

3. Some reported pharmacological activity of the chemical constituents of Matricaria

Several pharmacological actions have been assigned for German chamomile, based primarily on *in vitro* and animal studies. Such actions include antibacterial, antifungal, anti-inflammatory, antispasmodic, anti-ulcer, antiviral, carminative, and sedative effects (Alternative Medicine Review 2008). It is important to mention that therapeutic effectiveness is mainly due to the combined pharmacological and biochemical effects of several chamomile constituents (Schilcher, H.,(1987)).

3.1 Apoptotic effect against cancerous cell

- Darra et al. in 2008 showed that α-bisabolol is able to rapidly, efficiently and selectively induce apoptosis in malignant tumor cells by targeting lipid rafts on cell membranes. Thereafter, α-bisabolol could interact with Bid protein (one of pro-apoptotic Bcl-2 family proteins, analyzed either by Surface Plasmon Resonance method or by intrinsic fluorescence measurement) recruited in lipid rafts region after α-bisabolol treatment, which may be involved in the transduction pathway from plasma membranes to intracellular compartments including mitochondria. However, toxicity towards normal cells or in animals was absent. (Elena Darra, et al., (2008))
- In 2007, Farnesol had been demonstrated by Joo et al. to inhibit proliferation and induce apoptosis in a number of neoplastic cell lines from different origins (J.H. Joo,et al., (2007)) with preferential action in transformed cells versus untransformed cells (Adany, Cancer Lett. (2000)) and (Srivastava JK, Gupta S. (2007))
- Other preliminary study by Srivastava et al. in 2007 recorded that *in vitro* exposure to chamomile results in differential apoptosis in cancerous cells but not in normal cells at similar doses; apigenin and apigenin glycosides appear to be the key components responsible for these effects, (Deendayal patel et al. (2007))
- Moreover, Patel *et al in 2007* identified many mechanisms of action for apigenin-mediated cancer prevention and therapy, including estrogenic/anti-estrogenic activity, anti-proliferative activity, induction of cell-cycle arrest and apoptosis, prevention of oxidation, induction of detoxification enzymes, regulation of the host immune system, and changes in cellular signaling. This suggests that apigenin possesses enormous potential for development as a promising cancer chemopreventive agent in the near future for breast, cervical, colon, lung, prostate, ovarian, skin, endometrial, thyroid, and gastric, hepatocellular, and adrenocortical cancers as well as leukemia. Pre-clinical studies of various animal models of cancer that closely simulate human cancers are still needed (Barton, H. 1959).

3.2 Sedative and anxiolytic effect

- Shinomiya et al. (Kazuaki S. et al., (2005)) investigated the hypnotic activities of chamomile and passiflora extracts using sleep-disturbed model rats. A significant decrease in sleep latency was observed with chamomile extract at a dose of 300 mg/kg. His findings strongly suggested that chamomile is a herbal product possessed both hypnotic and anxiolytic activity in animals.
- (Avallone R.,et al., (2000) showed that apigenin, a flavonoid isolated from *Matricaria chamomilla*, significantly reduced the locomotor activity in the open field test of rats.
- (Viola H., et al.,(1995) , in a study about intraperitoneal administration of chamomile extract in mice, concluded that apigenin functions as a ligand for benzodiazepine receptors, resulting in anxiolytic and mild sedative effects, but no muscle relaxant or anticonvulsant effects. He also reported that apigenin extracted from chamomile flowers inhibited [3H]-flunitrazepam binding in the bovine cerebral cortex.
- Gould L., et al.,(1973) reported that hospitalized patients were given a strong chamomile tea, and ten of the twelve patients immediately fell into a deep sleep lasting 90 minutes.
- Della Loggia, R., et al.,(1981)) also demonstrated that chamomile extract caused a significant prolongation of sleeping time induced by barbiturates in mice.

3.3 Antispasmodic effect

Both flavonoids and essential oil contribute to the musculotropic antispasmodic effect of chamomile. Apigenin, alpha-bisabolol, and the cis-spiroethers appear to provide the most significant antispasmodic effects. (Alternative Medicine Review (2008).

- Maschi *et al.* in 2008 reported the spasmolytic activity of chamomile was through inhibition of cAMP-PDE for the first time. Human platelet cAMP-PDE and recombinant PDE5A1 were assayed in the presence of chamomile infusions. Chamomile inhibited cAMP-PDE activity (IC50) 17.9-40.5 μg/mL), while cGMP-PDE5 was less affected (-15% at 50 μg/mL). Among the individual compounds tested, flavonoids showed an inhibitory effect (IC50) 1.3-14.9 μM), contributing to around 39% of the infusion inhibition.
- Carle, R., Gomaa, K. in 1992 demonstrated that the chamomile oil itself, (-)-α- bisabolol, the bisabolol oxides A and B, and the enyne dicycloethers have a papaverine-like musculotropic spasmolytic activity. In addition, the coumarin derivates umbelliferone and herniarin are also antispasmodically active,(Acheterrath-Tuckermann,et al., 1980)
- In Tests that was performed using rat or rabbit duodenum, where the contractions were induced by barium chloride, acetyl choline, and histamine (Hava M., Janku J. (1957), (Janku, J. (1981), apigenin inhibits the contractions of smooth muscle and those of seminal vesicle of cavy and of rabbit uterus. 10 mg of apigenin were equieffective to about 1 mg of papaverine as for musculotropic effect. (Della Loggia, R. 1985)
- Other flavonoids contribute to the smooth muscle relaxation but to lesser degree. They can be classified in descending activity as follows: apigenin, quercetin, luteolin, kaempferol, luteolin-7-glucoside, and apigenin-7-glucoside. (Hörhammer, L.,et al., (1963)

3.4 Antimicrobial effect

Preliminary *in vitro* studies on the antimicrobial activity of chamomile have yielded promising results.

- Annuk H,et al., in 1999 proved that chamomile extract at concentration 2.5 mg/ml killed trichomonads effectively. It also blocked aggregation of various strains of *Escherichia coli*.
- Shikov, A., et al., in 1999 demonstrated that chamomile oil extract inhibited the production of urease at *H. pylori*. It was suggested that the mechanism of therapeutic action of chamomile oil is based on inhibition of colony activity of *H. pylori* and an inhibiting effect on adhesion of this microorganism of phospholipid — lecithin.
- Turi M. *et al.* in 1997 showed that chamomile extract inhibited the growth of poliovirus and herpes virus while chamomile esters and lactones demonstrated activity against *Mycobacterium tuberculosis* and *Mycobacterium avium*.
- Berry M. in 1995 showed that chamomile oil, at a concentration of 25 mg/mL, demonstrated antibacterial activity against such Gram-positive bacteria as *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus mutans*, and *Streptococcus salivarius*, as well as some fungicidal activity against *Candida albicans*
- The strongest antibacterial activity was recorded for α- bisabolol. It is active in low concentrations against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli,*

Streptococcus faecalis, and *Pseudomonas aeruginosa* and inhibits the growth of strains of *Bacterium phlei* that were resistant against standard anti-infectives (Szabo-Szalontai, M., et al., (1976) and (Szalontai, M.,et al., (1975). Bisabolol, together with enyne dicycloethers, also showed fungistatic activity against *Candida albicans, Trichophytone menthagrophytes,* and *Trichophytone rubrum* at a concentration of 100µg/ml. Chamazulene also had this fungistatic activity, but at higher concentrations (Szalontai.,M., Verzar-petri, G., Florian, E. (1977).

3.5 Anti inflammatory effect

- Recent study in 2009 by Srivastava *et al.* done on aqueous extract of chamomile flowers growing in Egypt, where LPS-activated RAW 264.7 macrophages were used as in vitro model, Chamomile treatment inhibited the release of LPS-induced PGE2 in RAW 264.7 macrophages. This effect was found to be due to inhibition of COX-2 enzyme activity by chamomile. In addition, chamomile extract caused reduction in LPS-induced COX-2 mRNA and protein expression, without affecting COX-1 expression. This suggested that mechanism of action of chamomile on the inhibition of PGE2 production was due to the suppression of the COX-2 gene expression and direct inhibition of COX-2 enzyme activity which is similar to non-steroidal anti inflammatory drugs.
- (-)-α- bisabolol was capable of inhibiting both 5-lipoxygenas and cyclooxygenase (Szelenyi J., Isaac, O., Thiemer, K., (1979)). It has antipyretic activity against yeast-induced pyrexia of the rat (Büchi, O. (1959)). Other experiments showed that (-)-α-bisabolol was capable of inhibiting the formation of ulcers induced by indomethacin, stress, or alcohol (Szelenyi J., Isaac, O., Thiemer, K., (1979))
- Regarding azulenes, their anti inflammatory effect was proven (Zierz, P., Kiessling, W (1953), (Zierz, P., et al. (1957) through inhibition of histamine liberation, inhibition of 5-hydroxytryptamine liberation, anti-hyaluronidase effect, and a decrease of the capillary activity (Uda, T.(1960). This is besides to the activation of the ACTH production (Kato, et al. (1959).
- Chamazulene was identified as the antiphlogistic principle of chamomile oil in a test system of chemosis caused by mustard oil in rabbit and cavy eye (Heubner, et al. (1933) and Pommer (1942)). It was proved by Ammon et al. in 1996 to inhibit 5-lipoxygenase.
- Antiphlogistic activity of flavonoids was proved much later (Baumann, J., et al., (1980)), (Carle, R., Gomaa, K. (1992), (Della loggia, R. (1985)), Della loggia, R., et al., (1984)), (Della loggia R., et al., (1986), Wurm, G., (1982). Apigenin even exceeded the activity of indomethacin and phenylbutazone. The experiments further showed that apigenin had both a positive influence on the vascular phase of the inflammation (e.g., edema) and on the cellular phase (e.g., the migration of leucocytes). Antiphlogistic activity of flavonoids decreased in the following order: Apigenin > luteolin > quercetin > myricetin > apigenin-7-glucoside > rutin.

3.6 Anti ulcerative effect

Torrado S, et al., in 1995 reported that significant protective effect against gastric toxicity of 200 mg/kg acetylsalicylic acid where achieved after oral administration of chamomile oil to rats at doses ranging from 0.8-80 mg/kg bisabolol. Moreover, *in vitro* studies revealed that alpha-bisabolol inhibited gastric ulcer formation induced by indomethacin, ethanol, or stress, Szelneyi I, Isaac O., thiemer K. (1979)

3.7 Other Pharmacological actions

3.7.1 Inhibition of Aflatoxin G1 production

Yoshinari et al. in 2008 showed that the spiroethers of German chamomile inhibited production of aflatoxin G1 AFG1 by *Aspergillus parasiticus* with inhibitory concentration 50% (IC50) values of 2.8 and 20.8 mM respectively. This is through inhibiton of cytochrome P450 monooxygenase CYPA and without inhibiting fungal growth. In addition, it also inhibited production of 3-acetyldeoxynivalenol 3-ADON by *Fusarium graminearum* by inhibiting TRI4. The inhibitory activity of the (E)-spiroether isomer was much stronger than that of the (Z)-spiroether in both cases. Inhibition of TRI4 by the spiroethers showed that TRI4 may be a good target for inhibiting biosynthesis of trichothecene mycotoxins.

3.7.2 Protective effect on diabetic complications

- Kato et al. in 2008 investigated the effects of chamomile hot water extract and its major components on the prevention of hyperglycemia and the protection or improvement of diabetic complications in diabetes mellitus. Results suggested that a suppressive effect of chamomile on blood glucose level was independent of the inhibition of intestinal α-glycosidases but depended on the inhibition of hepatic glycogen phosphorylase (GP). Furthermore, chamomile extract has good inhibitory potency against aldose reductase (ALR2), which plays key roles in the polyol pathway and its activation promotes the progress of diabetic complications. Chamomile components, umbelliferone, esculetin, luteolin, and quercetin, could inhibit sorbitol accumulation in human erythrocytes. Therefore, daily consumption of chamomile tea with meals could be potentially useful in the prevention and self-medication of hyperglycemia and diabetic complications. ATSUSHI KATO,et al ., (2008)

3.7.3 Antioxidant effect

- Lado *et al.* in 2004 studied the volatile oil of several plants and their main components to determine their antioxidant activity. This was done by using the modified method of ferric reducing ability of plasma (FRAP). The reducing ability of juniper, yarrow, and chamomile (145.107 ± 0.007mmol/kg) was very significant and it was twice as high as the average values of the other plants (Lavander, salvia, rosemary, etc). The reducing abilities of the components of volatile oils are lower than those of volatile oils; therefore, the reducing capacities of volatile oils not only attributed solely to terpenes, but also other biologically active compounds may also contribute to ferric reduction and in electron scavenging (Cristina Lado, et al.,(2004)).

3.7.4 Inhibition of morphine dependence

- Gomaa *et al.* in 2003 showed that co-administration of *M. chamomilla* extract containing 0.3% apigenin with morphine not only inhibited dependence to morphine but also prevented the increase in plasma cAMP induced by naloxone-precipitated abstinence. Furthermore, naloxone precipitated morphine withdrawal behavior syndrome was abolished by acute *M. chamomilla* treatment before naloxone challenge, indicating that *M. chamomilla* extract has an inhibitory effect on the expression of naloxone-precipitated morphine withdrawal syndrome.

3.7.5 Tachykinin receptor antagonist

- Yamamoto et al. in 2002 discovered a novel and potent nonpeptide tachykinin NK1 receptor antagonist in the extract of dried flowers of *Matricaria chamomilla.* . It has a unique structure of a polyacylated Spermine which was established as *N*1, *N5, N10, N14*-tetrakis [3-(4-hydroxyphenyl)-2-propenoyl]-1, 5, 10, 14-tetraazatetradecane (tetracoumaroyl spermine). The *K*i values of 1a, estimated from the inhibitory action on the substance P (SP)-induced contraction of the guinea pig ileum and the inhibition of the binding of [3H][Sar9, Met(O2)11]SP to human NK1 receptors, were 21.9 nM and 3.3 nM, respectively.

4. Clinical indications of *Matricaria chamomilla*

German chamomile is a well-known and widely used herb in different parts of the world. Few well designed, randomized, double-blind; placebo-controlled studies are available to fully assess its therapeutic benefit. (Alternative Medicine Review 2008)

4.1 Gastrointestinal effect

- De la Motte S, (1997) conducted a prospective, randomized, multicenter, double-blind, parallel group trial, where 79 children (ages six months to five years) with acute, non complicated diarrhea received either a commercial preparation of apple pectin and chamomile extract or placebo for three days, in addition to a typical rehydration and re-alimentation diet. At the end of three days, significantly more children in the pectin/chamomile group (85%) experienced diarrhea alleviation compared to the placebo group (58%) ($p<0.05$). The pectin/chamomile combination experienced a significant 5.2-hour shorter duration of symptoms compared to the placebo group. Weizman Z. et al. in 1993 in double-blind studies observed the efficacy of a herbal decoction consisting of German chamomile, vervain, licorice, fennel, and balm mint on 68 healthy infants with colic. For seven days the infants (ages 2-8 weeks) received 150 mL of the herbal preparation or placebo with each colic episode, but no more than three times daily. After seven days, 57 percent of the infants receiving the herbal preparation experienced colic relief compared to 26 percent in the placebo group ($p<0.01$).
- Schmid et al. in 1975 showed that chamomile extract is successfully applied in pediatrics due to its carminative and spasmolytic effect with diseases of the gastrointestinal tract and the effect as such is said to set in immediately after taking the preparation. The internal administration of chamomile tea or preparations from chamomile extracts is appropriate in different gastric troubles that can be classed under term of "dyspepsia," as recorded by Weiss in 1987.

4.2 General anti-inflammatory effect

- In 1999 Schilcher demonstrated that chamomile extract therapy is advisable in pediatrics for sensitive skin care of babies, treatment of an inflamed skin or skin defects (as dermatitis ammoniacalis, scald and burn areas and exfoliative dermatitis), and for the treatment of inflammations of the nose and the paranasal sinus by application of a chamomile bath and inhalation.
- Nasemann et al. in 1991 reported about the antiphlogistic effect of Kamillosan® ointment in comparison with a nonsteroidal ointment in case of episiotomies, with

colpitis senilis, and about the improvement of the healing of wounds after surgical operations carried out by laser in gynecology after taking a chamomile (hip) bath.
- Carle et al. in 1987, and according to reports of various gynecological hospitals, showed that chamomile extract is a suitable remedy for the treatment of bartholinitis, vulvitis, and mastitis and in rare cases secondarily healing episiotomies.

4.3 Dermatologyical effect

- Stechele in 1991 and according to a pediatrician's open report showed that very good results could be achieved by using chamomile ointment for the treatment of napkin dermatitis.
- Aertgeerts P et al. in 1985, in an open, bilateral comparative trial, 161 patients with eczema on their hands, forearms, and lower legs initially treated with 0.1-percent diflucortolone valerate received one of four treatments: chamomile cream (Kamillosan), 0.25-percent hydrocortisone, 0.75-percent fluocortin butyl ester (a glucocorticoid), or 5.0-percent bufexamac (a nonsteroidal anti-inflammatory). After 3-4 weeks, the chamomile cream was found to be as effective as hydrocortisone and demonstrated superior activity to bufexamac and fluocortin butyl ester.
- As for Born in 1991, chamomile extract was applied for the irrigation of undermined margins of a wound, pouches, sinus tracts, and hip baths, correspondingly diluted or in concentrated form for swabbing inflammatory lesions of the mucosa.
- Contzen in 1975 proved that the chamomile bath can be used successfully with the local treatment of deep second-degree burns. Apart from an accelerated cleansing process of a wound a significant improvement of the granulation is also observed. Deep necroses are excised; superficial ones heal without proteolytic ferments.
- Glowania HJ et al. in 1987and through a double-blind trial examined the therapeutic efficacy of a topical chamomile extract on 14 patients with weeping dermabrasions from tattoo applications. Those using chamomile noted a statistically significant decrease in the weeping wound area and increased drying compared to the placebo group.

4.4 Sleep enhancement

In an open case study to examine the cardiac effects of two cups of chamomile tea on patients undergoing cardiac catheterization, Gould L. et al. observed that 10 of 12 patients in the study achieved deep sleep within 10 minutes of drinking the tea, Gould L, et al. (1973). The patients had a small but significant increase in mean brachial artery pressure. No other significant hemodynamic changes were observed.

4.5 Radiation therapy

- Fidler P. et al. in 1996 conducted a randomized, double-blind study with 164 cancer patients taking 5-fluorouracil (5-FU) chemotherapy. The patients rinsed three times daily with either a chamomile mouthwash or placebo. After 14 days, no difference was observed between the two groups in the incidence of stomatitis induced by 5-FU.
- Carl W. et al. 1991 examined the effect of 15 drops of Kamillosan Liquidum, a German chamomile mouthwash preparation, in 100 mL of water taken three times daily, for radiation and/or chemotherapy-induced mucositis (characterized by inflammation and ulceration of the gastrointestinal tract including the mouth). Cancer patients (n=98)

were divided into two groups. One group of 66 patients (20 undergoing radiation therapies, 46 undergoing chemotherapy) participated in prophylactic oral care with the mouthwash. The remaining 32 patients underwent chemotherapy and were treated therapeutically after mucositis had developed. Of the 20 patients undergoing radiation, only one developed high-grade (grade 3) mucositis in the final week of treatment, 65 percent developed intermediate grade mucositis, and 30 percent developed low-grade mucositis. Of the 46 patients concurrently receiving chemotherapy and the mouthwash, 36 remained free of any clinically significant mucositis. Of the 32 patients with existing mucositis, all noted immediate relief from mouth discomfort, and within seven days almost all patients had no clinical sign of mucositis.

- Maiche AG et al. in 1991 carried out a double-blind, randomized, placebo-controlled study, where 48 women receiving radiation therapy for breast cancer were treated topically with either chamomile cream or placebo (almond oil) to protect the radiation-treated area. While there were no significant differences between the two groups in objective scores of skin irritation, the patients preferred the chamomile containing cream to the placebo for its rapid absorption and stainlessness.
- According to Bulmenberg, E.-W., Hoefer-Janker, H. (1972), the reactions of mucosa of the rectum resulting from a highly dosed radiation therapy, frequently felt to be unendurable; can also be treated successfully with chamomile extract. For that purpose enema is given three times a week; besides antiphlogistic properties, this also has a mild cleaning effect

4.6 Other uses

- According to Hinz in 1995, a standardized ethanolic-aqueous chamomile flower extract is suitable for the adjuvant therapy of *Angina lacunaris* and for the symptomatic treatment of herpangina often occurring in (early) childhood. In addition has a pain-alleviating effect in cases of inflammatory and painful esophageal diseases.

5. Photograph of the two matricaria specie

Fig. 1. Photograph of *Matricaria aurea*

Fig. 2. Photograph of *Matricaria chamomilla.*

(10-40cm in height, with erect, branching stems .the capitulum (to 1.5cm in diameter) comprises 12-20 white ligulate florets surrounding a conical hollow receptacle on which numerous yellow tubular (disk) florets are inserted (Bruneton J. (1995))

6. Conclusions and recommendations

A lot of studies have been conducted on *Matricaria chamomilla* all over the world where many important biologically active compounds have been separated and identified. However, very few studies are available for *Matricaria aurea* world wide. Nowadays, researches are focusing on exploring the pharmacological profile of compounds from natural origin, where promising results aroused. Challenges remain in finding ways to benefit from these biologically important compounds in treating human health problems.

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Bioavailability of Phytochemicals

Indah Epriliati[1] and Irine R. Ginjom[2]
[1]*Widya Mandala Catholic University,*
[2]*Swinburne University of Technology, Sarawak Campus,*
[1]*Indonesia*
[2]*Malaysia*

1. Introduction

Phytochemicals are increasingly accepted as health promoting, maintaining, and repairing agents in cells, tissues, or the whole human body. Phytochemicals are compounds obtained from plants that exert particular health effects; generally, they are not necessarily basic nutrients (minerals, vitamins, carbohydrates, proteins or lipids), medicines or toxins. The phytochemicals that are frequently associated with human health are phenolics, carotenoids, organic acids, and several miscellaneous bioactive compounds such as saponin and sterols. The contributions of phytochemicals in public health cover various issues world-widely and thus it is seen by researchers, industries, general society, and policy makers as a new tool to manage public health. Ironically, the roles of phytochemicals in health are poorly understood, which warrant the needs for validation as well as scientific database on safety issues and mechanisms of the functions. Even though various genetic-base studies propose mechanisms and health interventions of phytochemicals (Noe et al., 2004), many findings are inconclusive. Hence, the emerging health potentials of phytochemicals are inconclusive; and internationally it has been the reason for new policies/regulations in food trading. This is partly due to limited understanding on phytochemical bioavailability by which the health benefits depend on. Moreover, transport mechanisms for phytochemicals delivery into the target sites, phytochemical metabolisms by the human body, and biomarkers exerting the health benefits are also poorly understood. These complexities call for a new framework on how and to what extent dietary phytochemicals should be recommended in order to reach biologically-safe active dosages.

In the human body, bioavailability is defined as substances obtained from ingested materials that reach circulatory system for further delivery into designated tissues so that the beneficial compounds are biologically available for exerting healthy functions. The normal routes of dietary phytochemicals thus include ingestion, digestions, and transport across gastrointestinal epithelium prior to circulatory vessels. The epithelium in the gastrointestinal tract is a polarized enterocyte cell having two different sides facing luminal hollow (Apical side) and blood capillaries (Basolateral side) where each side is equipped with different transport facilities and barriers. The epithelial cells are critical for bioavailability of target compounds either as entrance gates or as metabolizing machines which release different compounds from the parent molecules. These make further complexing bioavailability routes because the metabolisms and transport processes are also

involved in the orchestrated physiological regulations maintaining homeostasis states of the human body. However, bioavailability of phytochemicals by which the health benefits depend on are not well understood; consequently, it is difficult to be measured.

The difficulties in studies of bioavailability are mainly due to the complexities involved in the biological system, i.e. (a) variation in food materials and the human subjects or surrogate models which are not always representative; (b) complex interactions amongst huge chemicals/food components during postharvest, storage, processing, digestion, and absorption that may alter health benefits; and (c) mechanism pathways. In this paper, fundamental aspects of phytochemical bioavailability are reviewed.

2. Digestibility of phytochemicals

It is known that major phytochemicals are located inside vacuoles of plant cells; and several phenolics form complexes with fibres in the cell wall. These natural existences make the phytochemicals poorly accessed by enzymes or hardly released out from the plant matrices during digestion. Most cell wall materials are indigestible by human enzymic systems. Moreover, it is also poorly permeable for important molecules such as phytochemicals. Therefore, digestibility of the phytochemicals is of great interest, in particular, to reveal how the phytochemicals can affect human health and fight or prevent diseases if the phytochemicals are strongly contained in the food matrices.

2.1 Digestion: principles of human gastrointestinal tract

The digestion compartments in human consist of mouth, gastric, small intestine, and colon (Figure 1). Each has slightly different digestion performances depending on age and gender as listed in Table 1. In the gastrointestinal tract, net nutriome[1] is released as a result of orchestrated secretions, enzymic activities, and physical-mechanical actions of peristaltic movements. The nutriomes will diffuse out from the food particles to chyme solutions. The levels of nutriome in this stage are called availability or accessibility of the components. However, bio-/chemical degradations of the molecules can take place. Hence, digestibility will also provide metabolites/derivatives. Nevertheless, availability and accessibility parameters can only account for intact molecules but not the metabolites.

Architecture and material of the plant tissues is generally unfavourable for activities of enzymic system in the human gastrointestinal tract. As a consequence, limited cell contents of the ingested food materials are released into chyme solution in the gastrointestinal tract. Natural pores and plasmodesmatas may not play predominant roles in diffusion of the nutriome. Nevertheless, according to Stolle-Smits et al. (2009), natural matrices of tomato, mango, apple, and kiwi undergo galactan solubilisation during ripening stage; thus the release of nutriome can be altered. However, processing and chemical compositions of the food matrices themselves may change physicochemical environments of the chyme for nutriome mass transfer. The most recent finding indicates that ingested foods are necessarily designed such that the diffusion of the nutriome favours nutriome absorption by epithelial cells; even for phenolics, it requires lipid-complex called phytosome (Kidd & Head, 2005) to penetrate gut lining and to enter the circulatory system.

[1] Nutriome is a term referring to all beneficial food components

Digestion sites	Infant	Adults	Elderly	Female	Male
Mouth	Improper chewing, imperfect salivary enzymes	Chewing, complete salivary enzymes	Improper chewing, may be with incomplete salivary enzymes	Chewing	Chewing
Gastric	Acid & pepsin digestion	Acid & pepsin digestion	Acid & pepsin digestion	Acid & pepsin digestion	Acid & pepsin digestion
Small intestine	Immature system	Complete tissues and enzymes	Complete tissues and enzymes	Hormonal related digestive secretion, undisturbed by reproductive cycles	Hormonal related digestive secretion , disturbed by reproductive organ cycles
Large intestine	Predominant Bifido bacteria	Lossing Bifido bacteria	Lossing Bifido bacteria	Lossing Bifido bacteria	Lossing Bifido bacteria

Table 1. Summary of digestion characteristics of infant, adult and elderly, female and male

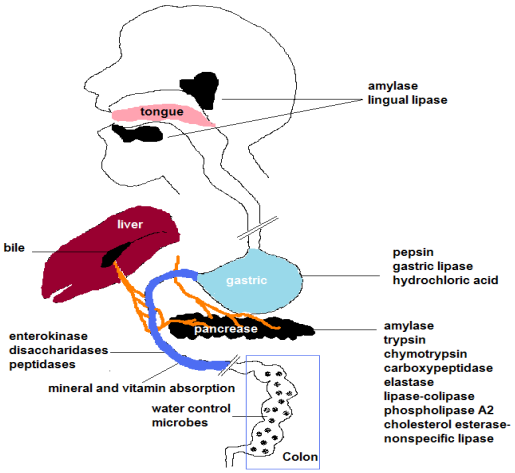

Fig. 1. Principles of human digestion system adapted and modified from Johnson (2001)

2.4 Effects of digestion on phytochemicals

Cell wall materials significantly modulate digestion of plant foods. Nunan et al. (1998) state that in grape berry during its development of the berry fruits the Na_2CO_3-soluble fraction increases before veraison but decreases as the berries softened. It implies that the Na_2CO_3 soluble fraction is the cell wall component which is responsible for firmness and strength. Epriliati (2008) observe that ripe mango, tomato, and papaya behave differently when Na_2CO_3 is added into *in vitro* digestion model mimicking small intestine where not all

aggregated boli from human *in vivo* chewing can be broken down. The diverse resistances of plant cell wall material amongst plant species during digestion may be partly due to Na_2CO_3 soluble fractions. It is more likely that mango and tomato have different levels of Na_2CO_3 soluble fractions which result in diverse *in vitro* digestion effects compared to papaya. Furthermore, processing will affect the way nutriome being released. Meanwhile, heating of the filtered fresh-juices (tomato, mango, and papaya) results in formation of clumpy substances (Epriliati, 2008). Similar clumps are also found as remnant of pectin gel used for taste masking agent of paracetamol and ambroxol (Miyazaki et al., 2005). These imply that consumption of fresh and processed various fruits, rich in pectin, can yield a wide range of phytochemical bioavailability depending on their cell wall material compositions. Furthermore, pasteurisation may render phytochemical release from the clumpy substances.

Phenolics. There are two main routes for digestion of dietary phenolics; i.e. digestion along the gastrointestinal tract and digestion inside the enterocytes. This can happen because hydrolase enzymes, i.e. lactase phlorizin hydrolase are available in intestinal lumen, brush border, and enterocyte (Williamson, 2004). Metabolisms that take place along the gastrointestinal tract are mainly aiming at deglycosilation of glycoside form of dietary phenolics. This deglycosilation is also carried out by microbiota in the colon.

Inside the enterocyte, dietary phenolic glucuronidation of the aglycone form are catalyzed by UGT[2]. Meanwhile, the glycone forms are hydrolyzed and conjugated. The conjugated forms from both glycone and aglycone dietary phenolics are either effluxed into intestinal lumen or translocated into the portal blood vessel. The circulated conjugates of dietary phenolics in plasma can be absorbed by liver and hepatocytes will metabolize them further. For instance, the hepatocyte converts flavonoids into glucuronidated and sulphated forms, which are polar rendering to dissolve in water easier and then be excreted in urine or bile. The pivotal roles of liver indicate that these conjugations are apparently one of physiological needs in the body, for example for bile synthesis in mammalians.

All compounds in wine, which are free from cell wall materials, show clearer responses during gastrointestinal digestion. Flavonol and proanthocyanidin interact with protein in the salivary secretion. However, catechin interacts stronger than epicatechin indicating that molecular characteristics play an important role in this interaction (de Freitas & Mateus 2001). Flavonols and proanthocyanidins remain intact but they may also be broken down when pH is sufficiently low in the stomach. Phenolics stability is strongly affected by pH as studied by Ginjom (2009). For example, syringic and *p*-coumaric acids are stable at pH 2-9 for 24 h. Generally, pH higher than 7.4 is unfavourable for phenolics and the effects of high pH are worsened by lengthy exposures. The number of -OH groups in benzene ring of simple phenolics can also be critical clues for phenolic stability. High pH results in unstable quinones which are oxidized further into diketones and other degradation products. In contrast, the stability of polyphenols such as quercetin, malvidin-3-glucoside, and resveratrol which have more than one benzene ring does not solely depend on their -OH groups. Quercetin is unstable during gastric and pancreatic digestions because quercetin is easily degraded at high pH, yet it is stable at pH 2 and pH 5.5 (Ginjom, 2009). In contrast, *trans*-resveratrol is stable at pH 1-7. Catechin isomers also show different stability at high

[2] UDP glucuronosyltransferase

pH as detected by Ginjom (2009). Similarly, (+)-catechin is stable in *in vitro* digestion up to pH 7.4 at 37 °C for 8 h different from that of (-)-catechin (Friedman & Jurgen, 2000 and Donovan et al., 2006). Overall, phenolics in wine do not undergo significant changes during gastric digestion.

In red wine, anthocyanidin is important component of phenolics. Anthocyanin availability is reduced by 32% after pancreatic digestion compared to that of gastric digestion and undigested sample (Ginjom, 2009). Pancreatic environment, however, decreases monomeric anthocyanin more severely (58.75%) than polymeric anthocyanins (17.72%). Pancreatic condition in the intestine modifies molecular structures of peonidin-3-glucoside, malvidin-3-glucoside, and malvidin glucoside pyruvate as indicated by the changes in their retention time during HPLC/UPLC analyses, although it is unclear why this can happen. However, during *in vitro* digestion, Ginjom (2009) speculates that the losses of monomer are related to their polymerization during the pancreatic digestion. Although non-anthocyanins are insignificantly affected by gastric digestion, pancreatic digestion severely reduces them by ca 88% (equivalent to 22% of total phenolics in red wine). On the other hand, flavan–catechin is speculated to polymerize with anthocyanins or tannin forming precipitates during pancreatic digestion; consequently, they either being eliminated during sample preparation or disposed in aqueous fractions.

Phenolics in tomato products are released into digest solutions more during *in vitro* gastric digestion than during pancreatic digestion and the highest release is from tomato juice (Epriliati, 2008). The main phenolics in tomato are caffeic, catechin, rutin, chlorogenic acid, and coumaric acids. More phenolics are obtained from tomato juice than those from dried and fresh tomato indicating the natural barrier of cell wall has been eliminated. Noticeable changes of phenolic compounds due to processing and digestion are found but the new compounds are not able to be identified. Rutin and catechin are consistently found in fresh, juiced, and dried products. Meanwhile, no *p*-coumaric is found in fresh product whereas *p*-coumaric gradually appears in juiced and in dried products. In contrast, chlorogenic acid is present in fresh products but it gradually disappears in juiced and dried products. This could be caused the different extractability due to different matrices of the products or by chemical changes due to processing and digestion environments (Epriliati, 2008). Gastric digestion does not affect phenolic compounds. However, the phenolic levels are significantly reduced in consecutive gastric-intestinal digestion. Apparently, tomato pectin neither gels nor traps phenolic compounds at lower pH. Altering pH from gastric to intestine may obstruct the molecular phenolic stability.

Similarly, there are different phenolics released from mango during *in vitro* digestion. The phenomena consistently support the possibilities of impermeable pectin where more phenolics are released in a consecutive gastric-intestinal digestion when aggregated boli can be broken down with the addition of Na_2CO_3/$NaHCO_3$ (Epriliati, 2008). Recently, phenolics in gastrointestinal tract markedly behave in a similar way to that of carotenoids incorporated in chylomicrons, thus, all emulsified phytochemical compounds are called phytosome[3] (Kidd & Head, 2005). Therefore, the presence of pancreatic juices and bile extract improve phenolics release during consecutive gastric-intestinal digestions of mango.

[3] Phytosome is a term for vehicles in which phytochemical compounds are bound

Carotenoids. About 50% of extractable carotenoids dominated by lycopene and β-carotene in tomato, mango, and papaya products are released to digest solution in a non-lipidic digestion model (Epriliati, 2008). The release of carotenoids increases significantly in intestinal digestion where bile extract and pancreatic secretions exist. Consecutive gastric-intestinal digestions do not help with higher release of carotenoids. This is more likely due to insufficient emulsifier-water ratios to provide emulsification of carotenoids which are fat soluble. It is concluded that mango, tomato, and papaya carotenoids are released better in intestinal digestion where the model is without addition of oil (Epriliati, 2008).

Organic acids. Pectin content in tomato hinders organic acid release thus the total organic acids in *in vitro* gastric digest solution is lower than that of consecutive gastric-pancreatic digestion. This is evidenced by the changes in pH from highly acidic gastric pH to higher small intestinal pH (~6), that causes disaggregation of boli during *in vitro* digestion. For all types of mango samples, organic acid including ascorbic acid (Vitamin C) is released better during gastric digestion. Apparently, the pectinous materials in mango do not trap organic acids (Epriliati, 2008).

3. Absorption of phytochemicals

Currently, there is no well-established molecular form of absorbed substances in the gastrointestinal tract, i.e. whether they are absorbed intact or as metabolites. On the other hand, it is well known that lifestyle, behavior, diets, and basal metabolism of the subjects are more important affecting factors than age, gender, body weight, and plasma volume (Manners et al., 2003) in bioavailability determination. Therefore, standardized experimental conditions controlling such critical factors of absorption *in vivo* and *in vitro* is a must despite individual human variability.

3.1 Absorptive tissue structures

The main absorptive tissue is the small intestine. In human 81% of the total intestinal lengths is by small intestine and 19% is large intestine. The stretched length of jejunum is around 30.78% of the intestinal lengths. The transit time along human small and large intestine is 3-4 h and 2-4 d, respectively (Vermeulen, 2009). Principles of the intestinal absorptive structures are depicted in Figure 2. A single enterocyte has microvilli and each microvillus has glycocalyx. Such structure considerably increases contact surface areas with luminal contents. Each microvillus also contains a complex structure providing various facilities for uptake/influx and efflux molecules, signalling ports, cytoplasm, and lipid matrix. The glycocalyx and microvilli are the areas where the human body depends on for collecting nutriomes but rejecting hazardous compounds including microbes.

Each enterocyte attaches onto adjacent cells through tight, adherence and gap junctions. Cellular transport from intestinal lumen to portal blood vessel occurs in two ways: paracellular and transcellular. The paracellular entrances for hazards are tightly controlled by those three types of junctions. Molecular weight cut off limits the hazardous substances crossing through both enterocyte lining cells and tight junction. The enterocytes collect compounds that reach apical side. The compounds then traverse into basolateral side where they end up in capillary vessel for circulation into the whole human body.

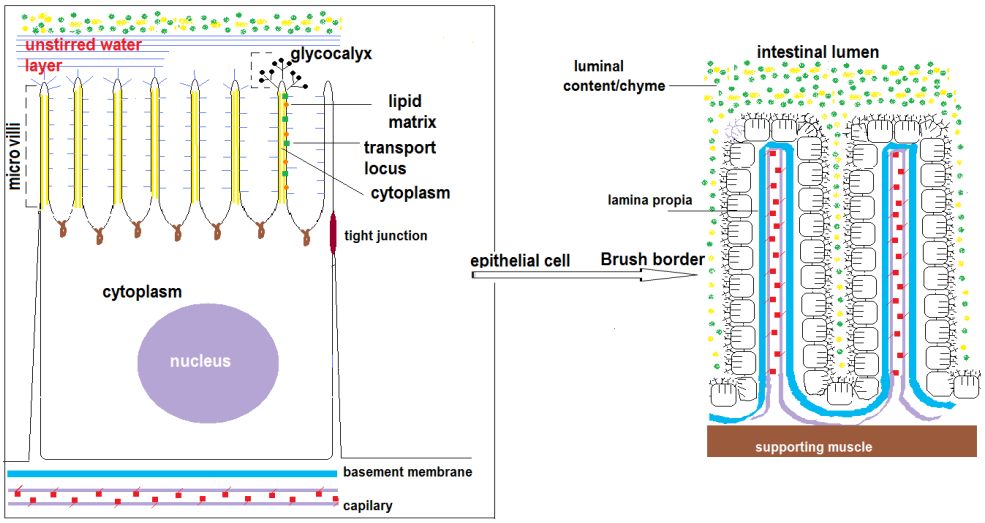

Fig. 2. Absorption tissue: epithelial cell (left) and intestinal brush border (right)

Other barrier in intestine is mucus. Most absorptive tissues comprise of epithelial cells which protect the human body from hazardous components in ingested foods. The epithelial cells are critical gate for human body. Due to its critical roles, the epithelial cells along gastrointestinal tract are covered with mucus secreted by goblet cells making an unstirred water layers so that the coarse particles are not abrasive towards the epithelial cells.

3.2 Transport mechanisms

Epithelial cell membrane is an important part of transport facility. It controls and selectively takes up molecules required for living or treats hazardous molecules. The fate of its work is not well understood despite studies finding many facilities and signaling processes available for regulating transport molecules. The transport modes include passive and active mechanisms. Passive transport is transcellular or paracellular transports and cynocitosis. Active transports are characterized by the use of protein transporters: channels/pump, binding protein transport, and formation of vehicles that is mainly emulsion system incorporating oil soluble compounds, such as chylomicrons. The transporter is able to promote transmembrane movement without hydrolyzing ATP (Johnson, 2001). They are categorized as uniporter (single compound moving down along the electrochemical gradients) and symporter (two molecules at the same time moving in one direction) or antiporter (two molecules at the same time moving in opposite directions).

Several transporters act as cellular efflux port for flavonoids: *P*-glycoproteins, multidrug resistance associated proteins, and breast cancer resistance protein (Johnson, 2001). They generally have loose substrate specificity and also involve in regulating non-nutritional compounds. Several findings point out glucose transporters which allow quercetin glucoside to be absorbed intact besides its aglycone forms. They are SGLT1[4] (SLC5A1[5]),

[4] Sodium dependent glucose transporter

GLUT2[6] (SLC2A2), MCT[7], OAT[8], and OATP[9]. However, results from the *in vitro* cell culture-based experiments are contradictive. Recently, bilitranslocase transport was introduced (Passamonti et al., 2009), that suggests the existence of a uniporter for flavonoids which is assumed to be an analogue of phthalien due to their similar molecular structures. The bilitranslocase is distributed in goblet and parietal cells in gastric, in apical jejunum of rat intestine, and in basolateral site of proximal tubular cell in kidney. However, further research is required for better understanding.

Briefly, bilitranslocase description indicates that target molecules interact with bilitranslocase through hydrogen bonds (hydrophilic properties of the active site); thus, nonionic inhibitors would not interact with it electrostatically. However, a negative charge is found to play an important role for electrogenic movement along the translocation pathway. These are observed through structural analysis (Passamonti et al., 2009). Similarly, the competitiveness of the target compounds can be explained by characteristics of C_4 in C-ring flavon building block where the target molecules are inactively competitive if the sugar moiety is in non-planar position; otherwise, the molecules will be actively competitive. Taking an example of quercetin-3-glucoside, the C_4 carbonyl forces 3-glucosyl moiety is perpendicular to the plane of flavonol aglycone resulting in a non-planar molecule. In contrast, its best competitor cyanidin-3-glucoside has a co-planar sugar moiety to the aglycone. Similarly, comparison of myricetin and delphinidin behave noncompetitively and competitively, respectively. Consequently, noncompetitor and competitor can be in one target molecule if its molecular structure has quinoidal, anionic tautomer, and neutral phenolics. They simultaneously can bind both the noncompetitive and competitive sites of bilitranslocase. Inconclusive role of bilitranslocation is compounded further by noninhibitor responses of other flavonoids, for instance flavonol (+)-catechin and isoflavones—genistin, genistein, daidzin, daidzein, and puerarin (Passamonti et al., 2009).

Bilitranslocase sheds a light for phenolics bioavailability and transport studies. The most striking relevance is that phenolics bioavailabilty is not delivered to blood circulation; instead, it is delivered through lymphatic system. This corrects understanding of hydrophobic nature of phytochemicals. The presence of bilitranslocase also clarifies disappearance of flavonoids in apical side but no basolateral level obtained in Ginjom (2009) and Epriliati (2008), despite GLUT2, MRP[10], organic cation, and amino acid/peptide transporters are available in basolateral domain. This specificity is promising for explaining the diverse bioavailability studies of phytochemicals.

3.3 Phytochemical absorption

There are two groups of nutriome: water soluble and less polar-solvent soluble. The water soluble components diffuse out from the food particles into chyme, traverse across the epithelial lining cells along the brush borders, and enter the portal blood circulation. On the other hand, the lipid soluble nutriome will be emulsified by bile salts and lipidic

[5] Solute carrier
[6] Glucose transporter
[7] Monocarboxylate transporter
[8] Organic anion transporter
[9] Organic anion transporting polypeptide
[10] Multidrug resistance-associated protein

components of diets immediately after diffusing out from the food particles. The emulsion acts as vehicles moving along the intestinal lumen. Contacting with the epithelial brush border and unstirred water layer on the top of the epithelial lining cells, rearrangement of vehicle emulsion take place which eventually releases the lipid soluble compounds into the cells. These compounds then traverse across the epithelium cells and end up in the lymph circulation. Nevertheless, many studies show losses material balances during transport across the epithelial lining gut. Moreover, the proportion of traversing compounds which are both water soluble and lipid soluble nutriomes that survive intact entering the circulatory system is not well understood. Similarly, proportion of metabolized nutriome used up by the epithelial cells as energy source is unclear.

Phenolics. Many studies support evidences that aglycone polyphenols are not only absorbed in the small intestine but also in the large intestine after microbial digestions. The steps may involve hydrolysis of sugar moiety by intestinal enzymes.

In the human small intestine and stomach, 95% of caffeic acid is absorbed while 62% of its ester form (called chlorogenic acid) is reduced. All are absorbed intact, except chlorogenic acid which mostly enters the human body from colon. Proanthocyanidins are pH sensitive thus it is likely to be broken down in stomach so that they may be readily absorbable. Meanwhile, catechin and epicatechin is poorly absorbed in the small intestine (≤20%) in a dose dependent manner. However, enterocytes can act differently; for instance, in intestinal jejunum it metabolizes flavanols into glucuronidated conjugates whereas in ileum it translocates flavanols intact. In the large bowel, most microflora metabolize flavonols and proanthocyanidins; for example, catechin metabolites include (-)-5[3′4′5′-trihydroxyphenyl]-γ-valerolactone; (-)-5[3′4′-dihydroxy phenyl]-γ-valerolactone; 3-hydroxyphenylpropionic acid; 3-hydroxybenzoic acid; or 3-hydroxyhippuric acid (Ginjom, 2009).

With a new bilitranslocase transport mechanism it is likely that the determinations of bioavailability of phytochemicals are necessarily being revised. pH and temperature are necessarily taken into account in order to avoid underestimation/overestimation regarding its stability. Several issues include absorption of quercetin and anthocyanin, glycone and aglycone forms, and conjugation/glucuronidation of phytochemicals as well as the presence of alcohol. Quercetin absorption varies from one food source to another. Its absorption from wine is enhanced by alcohol presence. Resident time of quercetin expressed as half-life clearance is 11-28 h (Manach et al., 2005). A very low level of intact anthocyanins is found in plasma after administration of anthocyanins. Resveratrol is absorbed well in the small intestine and being glucuronidated. Consumption of red wine would provide a good level of resveratrol bioavailability can be questioned whether this is because of alcohol presence.

Flavonoid is one of the group molecules with molecular weights >500 Da and has bioavailability level of <1%. Such molecules are unlikely to be transported through passive diffusion pathways. Further study found that influx membrane transporters cannot recognize flavonoid (signalling) whereas the efflux transporters do. Consequently, potential of flavonoids to be expelled is higher than that of influxed into the cells (Johnson, 2001).

In determination of phytochemical bioavailability, researchers should not limit their detection for ingested molecular forms only based on reported presence in the diets. It has been proven that at plasma levels many phytochemicals have been conserved by digestion and by hepatic activity. Fitting the mass balance of ingested phytochemical is challenging.

For instance, total metabolites in plasma levels are found reaching 4 mmol/L when intake is 50 mg aglycone equivalent whereas urinary excretion levels are 0.3-43% of the ingested doses, depending on polyphenol types. Flavonol such as quercetin in broccoli is rarely found as free quercetin. Human who consume 21-100 mg/d of quercetin show exclusive form of methyl, sulphate, or glucuronic acid conjugates totally amounted to maximum 1-5 μmol/L aglycone equivalent (Moreno et al., 2006). However, several phytochemicals are found intact, especially those which are absorbed easily. The ranks of phytochemical absorption is gallic acid and isoflavones > catechins and flavanones, quercetin glucoside > proanthocyanidins, galloylated tea catechins, and anthocyanins (Moreno et al., 2006).

Carotenoids. Carotenoids of mango, tomato, and papaya in caco-2 absorption model are not detected (Epriliati, 2008) in spite of *in vivo* data indicates that carotenoid plasma level increase after consumption of carotenoid-rich foods. Processing altered matrices of ingested food system and more likely degraded carotenoids which caused variation in bioavailability of carotenoids. A comparative study of organic and inorganic carrot found that apparently organic farming practices do not affect bioavailability of carotenoids in carrot consumption. Ingestion of total carotenoids of 24.3±1.4 mg organic carrot and 23.2±2.5 mg inorganic carrot results in 700 nmol/L β-carotene and 350 nmol/L α-carotene, and 150 nmol/L lutein after 2 weeks interventions (Stracke et al., 2009).

Organic acids. Organic acid provides organic anion important for metal binding and counteracting acidosis as well as preventing chronic diseases (Sabboh et al., 2011). Particular organic acids are apparently absorbed into plasma. Most organic acids in tomato, mango, and papaya products are absorbed in *in vitro* caco-2 model but they are not found in the basolateral sides (Epriliati, 2008). On the other hand, citric acid and oxalic from banana and sweet potato are consistently found to be absorbed and translocated into basolateral sides in *in vitro* caco-2 model (Sabboh et al., 2011). The absorbed organic acids are much lower compared to the original levels in food materials, thus, the retained organic acids in particles may be useful for controlling pH in colonic fermentation because selection of microbes in the large bowel is important.

Miscellaneous. Phytosterol could be absorbed at very low level using the same transport facilities for cholesterol due to structural similarities. It needs emulsion vehicle to diffuse in the aqueous lumen system, crossing the lipid membrane, and, finally, entering circulatory system. This requires evaluation because absorption is closely connected to which mechanisms are involved in health function, which is still debatable (Kang et al., 2010).

Triterpenoids citrus limonin glucoside is one of metabolites in citrus plant. Generally, it is water soluble; yet few aglycone forms of liminoids are insoluble. According to Manners et al. (2003) liminoid metabolites are found in human after ingestion of citrus juice containing limonin glucoside which may undergo epimerization from limonin glucoside to epilimonin (m/z 471.2). This may be from reaction pathways of hydrolization of glucoside moiety followed by lactonization. Although low level of limonin is ingested, it is eventually available in plasma after 6 h (Manners et al., 2003). During the first 3 h the higher ingestion level of limonin results in more significant changes in plasma epilimonin levels, regardless of age and gender. However, after 6 h, all volunteers show increased levels of plasma epilimonins at any ingestion levels of 0.25 g/200 mL–2 g/200 mL that is equivalent to 7 glasses of natural juices. The authors conclude that ingestion of limonin glucoside produces

epimer limonin at C_{17}. It is clear that the human body does not necessarily control levels of plasma limonin and its absorption in the gut whereas limonin glucoside enters blood plasma through GLUT pathways, but it is necessarily hydrolyzed and lactonized. If it is absorbed through GLUT pathways without being metabolized, it should enter blood plasma at the same rate with sugars. The problem is that variation of individuals cannot be ignored since by the time it shows accumulation or decrease of detected limonin levels. The consequence of this accumulation is also not understood. Overall, limonin aglycone form is apparently safer than that of limonin glucosides; therefore, the high level of limonin glucoside form is controlled. Based on transit time of chyme in the gut, 6 h will be long enough to bring the chyme completely passing the small intestine. Therefore, lower level of ingestion results in limonin absorption after microbial glucoside hydrolisis in bowel. These speculations remain to be elucidated.

Interactions involve in various phytochemicals and nutrient transports. Since phytochemical are generally reactive molecules they can interact with various compounds in the chyme and this will affect phytochemical bioavailability and vice versa. Phytochemicals that interact with vitamin E include lignans, curcumin, anthocyanins, phenolic acid and catechin, as well as cereal alkylresorcinol (Frank, 2004). Interaction of vitamin E and plant lignans significantly increases vitamin E bioavailability as much as 900% in plasma level; 1,350% in liver; and 1,556% in lung using rat model. On the other hand, using human and rat model tocopherol-ω-hydrolase activity is effectively inhibited by sesamin[11]. Sesamin also reduces degradation of γ-tocopherol and urinary secretion so that it increases γ-tocopherol level in plasma. However, not all lignans show similar effects. For instance, sesamin or flaxseed lignan secoisolariciresinol diglucoside, either its monomer or oligomers decrease tocopherol by 50%. Experiment using rat model indicates that flaxseed lignan decreases α and γ tocopherol availability in a dose dependent manner. However, it presence increases lipid peroxidation. The majority of flaxseed lignan is converted into mammalian lignan allowing them to be absorbed (Frank, 2004).

In contrast, the effect of curcumin studied using rat model on α tocopherol bioavailability is less apparent when compared to flaxseed or sesame lignans where it is only detected in lung. In fact, curcumin is absorbed, metabolized, and secreted as glucuronidated metabolites. Similarly, the effect of anthocyanins on tocopherol bioavailability is neglected. Using the same rat model, it is found that caffeic acid increases γ-tocopherol in the liver and it is also converted into its metabolites 5-caffeoylquinic acid which in turn increases the levels of α-tocopherol in lung. However, when ingested as 5-caffeoylquinic acid, it is metabolized into caffeic acid and quinic acid; and caffeic acid is absorbed and found in plasma both in human and rat models. In contrast, ferulic acid is found to form complexes with albumin in blood plasma and LDL; hence, it does not affect tocopherol bioavailability. Interestingly, (+)-catechin and (-)-catechin isomers similarly improve α-tocopherol bioavailability in plasma and liver (Frank, 2004). There is a slight difference regarding their effects on γ-tocopherol where 2R,3R-isomer(-)-epicatechin enhances γ-tocopherol bioavailability whereas 2R,3R-(+)-catechin has no effect on it. The differences between γ– and α– tocopherol is estimated due to (i) antioxidant activity of catechin isomers on a tocopherol and (ii) different effects of the isomers on cytochrome P450 enzymes such as

[11] Lignan exists in sesamin

CYP1A1, CYP1A2, CYP2B1, AND CYP3A4 as well as CYP4F2. Alkylresorcinols in outer layer of wheat and rye is also absorbed and metabolized. Its presence improves γ-tocopherol in liver and lung but not α-tocopherol observed in rat. The various effects on tocopherol isomers are unclear although molecular differences of alkylresorcinol and tocopherol is known.

Addition of citric acid affects iron uptake. In reverse, citrate reduction improves iron bioavailability (Glahn et al., 1998). Iron bioavailability is also influenced by purple and brown pigments in rice; apparently, the pigment behaves similarly to tannin, phenolic, anthocyanin, or phytic acid (Glahn et al., 2002).

Interactions amongst carotenoids (Kostic et al., 1995; van den Berg, 1999) show that β–carotene inhibits lutein uptake. These interactions perhaps also occurred at the micelle formation and transport levels, or their combination (van het Hof et al., 2000). Similarly, β–carotene shows competitive inhibition to lycopene transport (Johnson, 1998). Meanwhile, carotenoids can interact with proteins and pectin decreasing absorption the carotenoids (Williams, 1998). Moreover, the cathecol structure in the B–ring of flavonols and 2,6-di-*tert*-butyl-4-mehtylphenol inhibits the dioxygenase enzyme and conversion of β–carotene (Nagao et al., 2000; Nagao, 2004). On the other hand, metabolites of bio-oxidation may act as pro-oxidants in the body (Nagao, 2004). Konishi found that tea phenolics inhibit other dietary phenolics (Konishi et al., 2003).

Among several fruits and vegetables, papaya and tomato consumption are found to be benefecial in hypolipidemic diet components, with similar mechanisms observed during *in vivo* experiments using rats (Kumar et al., 1997). Here, soluble and insoluble fibers can bind bile acids, thus influencing micelle formation and absorption of lipophilic substances by the brush border. Lignin and guar gum are apparently better bile binders than cellulose, which is relatively inert. Interaction also occurs between fiber and intestinal mucin, which probably alters absorption and nutrient diffusion from bulk lumen content (Vahouny & Cassidy, 1985). Moreover, fiber bound health promoters include lycopene in tomato peel (Awad et al., 2002) and antioxidants in mango peel (Larrauri et al., 1996), where the antioxidants found in mango peel, pulp and seed include gallotannins (Berardini et al., 2004). Consumption of fiber-rich food products can reduce minerals and vitamin (Schneeman & Gallaher, 1985). Generally, those authors agree that pectin and cellulose play important roles, especially in reducing the activity of digestive enzymes, or hormones such as insulin (Schneeman & Gallaher, 1985; Vahouny & Cassidy, 1985).

4. Kinetics simulation of phytochemical bioavailability

Kinetics is a study observing changes of the phytochemicals after ingestion including elimination period. To understand kinetics of phytochemicals after ingestion, kinetics simulation is frequently carried out. The limitations of simulations should be acknowledged in interpreting the results. Moreover, bioavailability closely relates to absorption and metabolism, yet there are limited understanding of bioavailability markers. Furthermore, the markers need validating, i.e. the molecular forms selected as bioavailability markers are necessarily those which actually cause health effects.

Affecting factors of phenolic bioavailability include matrix of food sources, processing condition during food preparations, chemical compositions, and molecular physicochemical

properties of the target molecules. Molecular forms of phenolics such as glycone or aglycone definitely make diverse variations on bioavailability levels. In addition to these factors, individual gastrointestinal tract of the human also affects bioavailability. Gastrointestinal pH, level of secretions, microbiota, and age have been established as crucial factors affecting digestion and absorption of phytochemicals. Equally, the role of interactions amongst food components and their interactions with gastrointestinal secretions contribute significant effects in determining bioavailability of phytochemicals.

Tannin-protein interactions occur starting from mouth and food systems. The interaction depends on size, conformation, and charges of proteins; molecular size, flexibility, and water solubility of phenolics; and environmental conditions such as pH. Proteins with higher molecular weights or loose conformational structures or rich in proline/hydrophobic amino acids, increase its potential to be precipitated by tannin. On the other hand, flavonols (three orthohydroxyl groups on the B-ring) has higher affinity to protein compared to those with two orthohydroxyl groups. Similarly, the affinity increases with increasing galloylation degrees. The order of flavonols affinity is (-)-epigallocatechin gallate >(-)-gallocatechin gallate >(-)-epicatechin gallate >(-)epigallocatechin or (-)-epicatechin or (+)-catechin (Ginjom, 2009). Interestingly, tannin also plays pivotal roles in its capability to act as health protective antioxidant.

4.1 *In vitro* model of digestion and transport

Effects of *in vitro* digestion on wine phytochemicals are significant during pancreatic digestion step, especially for nonpolar compounds. Therefore, water solubility level is crucial in generating an appropriate *in vitro* digestion model. In contrast, acid does not significantly affect the phytocemical components in wine.

In vitro model for absorption using a monolayer cell culture can help bioavailability determinations with human surrogates; however, the results should be carefully considered. More importantly, the results cannot be liberately generalized for human system biology. Yun et al. (2004) propose a constant to equalize *in vitro* measurement using caco-2 monolayer with human *in vivo* measurement for iron. Furthermore, there are critical factors in utilizing such *in vitro* model for a bioavailability study that should be carefully considered. For instance, the original composition of digest containing bile salts decreases TEER (transepithelial electrical resistance) indicating serious detrimental effect on the cell monolayer integrity. In addition, alcohol content in wine also affects the monolayer integrity so that alcohol removal is required although alcohol enhances phenolic absorption. This is unrealistic wine samples. Furthermore, the delicate properties of the monolayers may result from lacking of mucus/unstirred water layer protecting the epithelium. The development of an appropriate and standardized *in vitro* model needed to be persued continuously.

4.2 Kinetics of phytochemical bioavailability

Kinetics study of phytochemicals is scarce. Several experiments are reviewed below to understand phytochemical kinetics after ingestion.

Quercetin. Quercetin is more likely to be absorbed quickly in the human gut after ingestion, e.g. quercetin-3-glucoside from blackcurrant juice is 4 h or pure quercetin glucoside capsule is ca 30 min. Quercetin-3-rutinoside takes longer time to reach peak plasma levels compared

to the two previously mentioned, i.e. after 5-10 h. Short- and long- term studies show kinetics absorption of quercetin is quick and easy; and there are no interactions with other food components. Moreover, bioavailable quercetin can be obtained from normal diet regardless of whether it contains the berries or not. Therefore, it is proposed that fasting quercetin bioavailability is used as a biomarker of high fruit and vegetable intakes for all plant based foods (Erlund et al., 2006).

Soyasaponin. Soyasaponin has a very low bioavailability when investigated using *in vivo* experiments involving animals and human (Kang et al., 2010). However, it is also found that possible metabolites of soyasaponin are detected in *in vitro* and *in vivo* studies, although it is found several days after ingestion (Kang et al., 2010). The metabolites include soyasapogenol B, which is secreted into faeces in human *in vivo* experiments. However, the metabolism is more likely due to microbiota in the colon which is supported by *in vitro* data using fresh faecal microbiota. *In vitro* data show sequential metabolism of sapogenin by the microbiota as follows: soyasaponin I after 48 h incubation at 37 °C, and it is converted into soyasaponin III after 24 h and disappeared at 48 h where the predicted final metabolite is soyasapogenol B. These sequential metabolisms take place through sugar hydrolysis which results in the formation of more hydrophobic metabolites and smaller molecules (Kang et al., 2010).

Lignan. Low lignan bioavailability is recovered in plasma in human after ingestion of lignans (Kang et al., 2010). It is interesting that lignan is easily absorbed into plasma after ingestion. The available information is for secoisolariciresinol diglucoside and its aglycone secoisolariciresinol and matairesinol. Based on the studies, at least 40% of ingested lignans are metabolized by intestinal bacteria and these metabolites can be detected in the plasma. Metabolites of lignans appearing in the human plasma after ingestion follows the sequences: (i) at 14.8±5.1 h enterodiol is maximally found in plasma, (ii) at 19.7±6.2 h enterolactone is maximally bioavailable, and (iii) at 8-10 h enterolignans is bioavailable. Resident time of lignan metabolites in plasma is 20.6±5.9 h for enterodiol and 35.8±10.6 h for enterolactone, respectively (Kang et al., 2010).

Phytosterol. Low phytosterol bioavailability is observed in human plasma after ingestion. Definite small amount (0.6-7.5%) of phytosterol is transported through gut epithelial cells *in vivo*. Chemically, phytosterol is similar to cholesterol; yet cholesterol is absorbed at much higher levels than phytosterol. This is because of side chain differences, i.e. ethyl/methyl group in C_{24} which increases hydrophobicity but reduces absorption; and the presence of Δ_5 double bond. The similarity of absorption mechanism of phytosterol and cholesterol is that (i) they need to be in emulsion system and (ii) to be facilitated by Niemann-Pick C1 like 1 (NPC1L1) protein. Surprisingly, it is just recently acknowledged that many of bioactive compounds need to be in emulsion system to make them more bioavailable (Kang et al., 2010).

Phytate. Phytate bioavailability is low in human plasma levels after ingestion. Plasma myo-[inositol-2-H^3(N)]hexakisphosphate in human after ingestion is dose-dependent and it only reaches 3-5 times higher than that of diet poor in myo-[inositol-2-H^3(N)]hexakisphosphate. Further study using rat found that absorbed phytate is quickly distributed into tissues such as brain, kidneys, liver, and bone in its original dietary molecular forms. The highest level is in brain reaching 10 times compared to average of tissues (Kang et al., 2010). This is beyond

conventional nutrition believes that phytic acid and phytate is not traversed across lipid bilayer.

Sulfur compounds. Bioavailability of isothiocyanates is better than glucosinolate in the human gut. In spite of different cruciferous origins and types, isothiocyanate is always found in plasma and its metabolites in urine is consistently found as dicarbamate or mercapturic acid. It is important to note that not all glucosinolates behave similarly. Generally, heated or cooked glucosinolate is less bioavailable (1.8-43%) than raw (8.2-113); and it is quickly absorbed in the gut and quickly excreted in urine (24 h). The exceptions are from pak choi (butenyl and pentenyl isothiocyanates, 8%), garden cress (benzyl isothiocyanate, 14%), and water cress (phenylethyl isothicyanate, 50%) compared to 100% isothiocyanate. Critical factors of the study remain: (i) individual variations (different microflora in the bowel, metabolism, and chewing ability), (ii) natural cruciferous matrices so that strongly entrapped glycosinolate in the cells will be hardly released during chewing, and (iii) types of glucosinolates (Vermeulen, 2009).

Vegetable consumption during lunchtime shows a general lag phase for excretion of mercapturic acid at 4 h after ingestion (Vermeulen, 2009). The sulfocompounds (isothiocyanate) in the body is conjugated. Raw vegetable consumption results in a fast excretion whereas cooked vegetable has longer resident time of conjugated form. Elimination for half-life of the compound is 2-4 h, which is longer than that of other study (1.8 h) (Ye et al., 2002), with excretion rate of 0.18-0.33 h^{-1} by assuming the first order reaction.

Food source of sulfur compounds is a determinant factor in absorption in addition to processing and physiological conditions. Sulforaphane content in raw and cooked broccoli is 9.92 and 61.4 μmole/kg, respectively; and 37 and 3.4 % of them are recovered in urine in the form of sulforaphane mercapturic acids. On the other hand, 54% of benzyl isothicyanate from garden cress is found in urine but phenylethyl isothicyanate from watercress after chewing is 47%. When cooked watercress is administered, only 1.2-7.3% of glucosinolates is recovered; this is much lower than sulforaphane (17.2-77.7%). Monitoring dithiocarbamate in urine shows 12% recovery from boiled broccoli sprout. The recovery increased to 80% when the boiled broccoli sprout is treated with myrosinase. About 68% of allyl isothicyanate from mustard is excreted in urine as mercapturic acid while sinigrin is present at 15% and 37% from cooked and raw cabbage[12], respectively (Vermeulen, 2009). Generally, the routes of metabolisms in the human body vary depending on the target molecules and food sources. Glucoraphanin and sulforaphane from cooked and raw broccoli peak for maximum 6 and 1.6 h, respectively (Vermeulen, 2009). The half-life clearance of sulforaphane in the human body from the aforementioned vegetables is 4.6 and 3.8 h, respectively. These are different from half-life time of mercapturic acid which is 2.4 and 2.6 h, respectively. Further investigations using human subjects show inconclusive results that particular polymorphism S-glutathione transferase (GST M1, T1 and P1) and N-acetyl transferase (NAT2) gene affect the variations (Vermeulen, 2009). It is important to view these phenomena under the holistic affecting factors.

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[12] Data is calculated from : First order kinetics

k_a: intercept and slope with a residual method; k: natural log of plasma amounts plotted against time; k_e: natural log of absolute excreted amounts vs time; area under curve (concentration vs time) extrapolated using trapezoid method; lag phase is determined from empiric curve obtained; base line is not used

Phenolics. Generally, the least absorbed polyphenols are proanthocyanidins, galloylated tea catechins, and anthocyanins (Epriliati, 2008 and Ginjom, 2009).

Caffeine. Using pharmacological principles, absorption simulations of pure compound in intestine is mimicked by caco-2 monolayers. During the simulated transit method (model A), unchanged caffeine was transported across epithelial cells (Figure 3). This indicates that caffeine is directly transported to the basolateral compartment without damaging the tight junctions. This transport is selectively occurring in the apical to basolateral direction over the bioassay time (240 min). The apical caffeine levels from simulation of transit method even after 120 min (Figure 3, top panel) are higher than that of semi dynamic apical solution method (B model) (Figure 3, middle panel). Caffeine was transported by the enterocytes in the apical to basolateral direction apparently without an equilibrium state being generated. Uptake of caffeine was rapid and basolateral secretion possibly required a certain amount of caffeine intracellularly is retained. When a high gradient concentration was maintained, continuous basolateral secretion of caffeine took place at a constant rate. As a result, the final level of basolateral caffeine was higher than the apical levels, even when it was subjected to a 22 h bioassay (C model) (Figure 3, bottom panel). The transport mechanism of caffeine may be a simple passive diffusion. However, another study shows caffeine can also be transported by the transcellular route (Mao, 2007). In addition, caffeine is found interacts with glucose uptake sensitivity (Pizziol, 1998).

Catechin. The simulation transit method of catechin indicates that it is retained in the apical compartment at about one-third of the initial amount (86.8 nmol) and remains at about the same level throughout the experiment (Figure 4, top panel). However, basolateral compartment analysis did not indicate equal amount of translocated catechin. In contrast, most basolateral samples contain very little catechin. In the static apical solution method (Figures 4, middle and bottom panels), apical catechin was reduced to 39 nmol after 22 h, but there were no indication of transported catechin in the basolateral compartment. In the present study, there may have been some metabolism of catechin based on apical losses which require further study to identify possible metabolites of catechin.

Lycopene. Lycopene is neither transported (Figure 5) nor chemically changed during bioassay using all three bioassay methods for all time periods. Its hydrophobicity and unfavorable molecular geometry apparently prevents lycopene from passing through monolayers via either paracellular or transcellular routes. This was confirmed by the decrease of TEER values for all monolayers, which is not accompanied by lycopene translocation into the basolateral compartment from the apical solutions. In the present study, the apical lycopene do not show disappearance in the transit model (Figure 5, top panel). Instead, lycopene shows apical accumulations with renewal solutions. Similar results are obtained from the semi dynamic model (Figure 5, middle panel) and confirmed in the 22 h static model (Figure 5, bottom panel). Lycopene absorption has been shown to be affected by the presence of other carotenoids, the lipid status, and plasma antioxidant capacity (Bohm & Bitsch, 1999). However, another study found that lycopene plasma levels after consumption of cherry tomatoes are insignificantly different from the plasma base line (Bugianesi et al., 2004). Further absorption from micelles has been shown to be slow (e.g. lycopene absorbed by LNCaP and Hs888Lu cells took approximately 10 h; Xu et al., 1999). This suggests that epithelial cells may have specific mechanisms that are not micelle dependent.

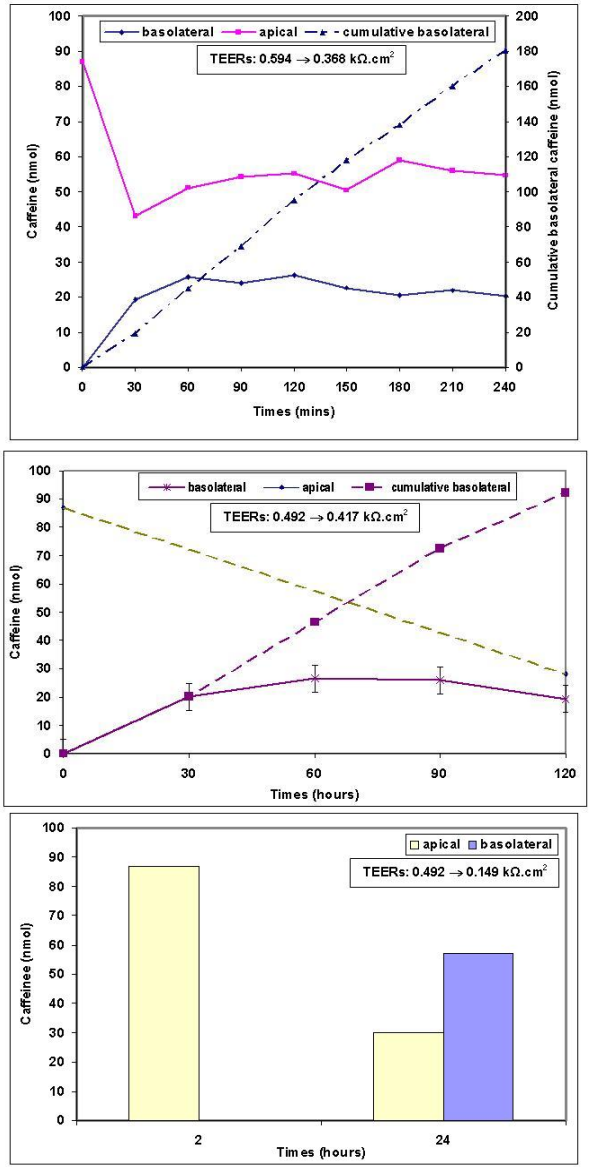

Fig. 3. Bioassay of caffeine using simulated transit method (top panel: model A; using 2 transwell-inserts), static apical solution method (middle panel: model B; using 4 transwell-inserts), and static apical and basolateral solution procedures for 22 h (bottom panel: model C; using 2 transwell-inserts)[13]

[13] Model A: apical side is replenished every 30 min, Model B: basolateral side is refreshed every 30 min, Model C: both apical and basolateral are not replenished for 22 h

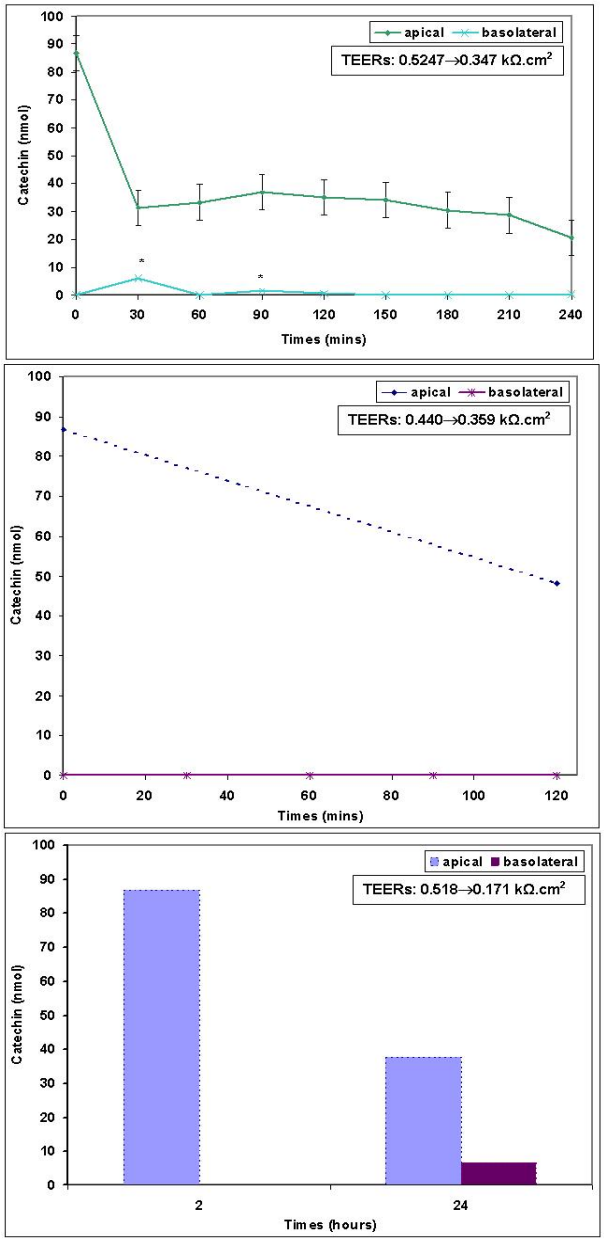

Fig. 4. (+) Catechin transport using simulation of transit chyme (top panel: model A; using 2 transwell inserts), static apical solution methods (middle panel: model B; using 4 transwell inserts), and static apical and basolateral solution after 22 h (bottom panel: model C; using 4 transwell inserts)

β–carotene. There are always reductions of apical levels but not necessarily accompanied by release into the basolateral side (Figure 6). Meanwhile, β-carotene completely disappears in the 22 h static model, both from the apical and basolateral sides although TEER values drops from 0.497 to 0.125 kΩ.cm². β-carotene may diffuse better than lycopene, as indicated by the β-carotene apical disappearances; however, neither is translocated. This may be related to intrinsic solubility, as β-carotene is more soluble than lycopene in the mixed aqueous/organic solvents. In the semi dynamic model after 120 min, apical β-carotene decreases and in the static model after 22 h, β-carotene disappears completely.

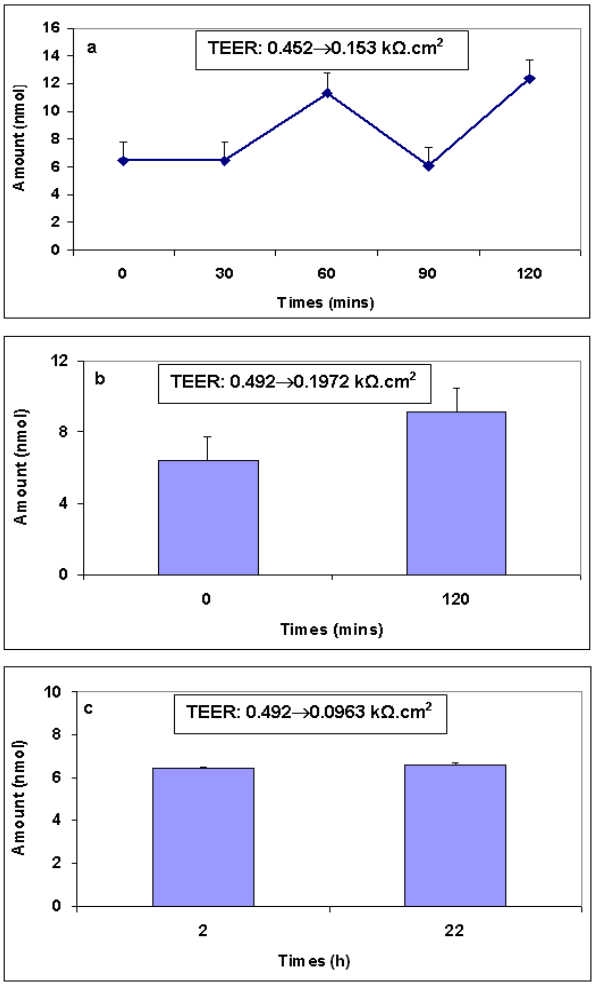

Fig. 5. Apical lycopene bioassay; **a** transit model (model A), **b** basolateral renewals (model B), **c** static model (model C) in buffer-0.5% DMSO

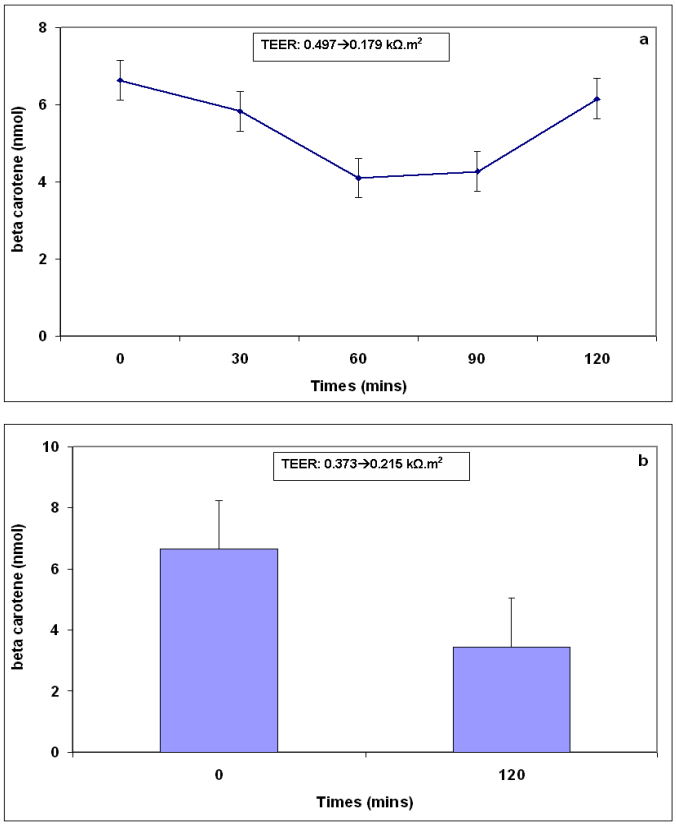

Fig. 6. Apical β-Carotene in HBSS-25 mM HEPES contained 0.5 % DMSO: **a** transit chyme (model A), **b** static apical solution (model B)

4.3 Dosages

Establishing the most suitable dosages for an optimal health benefit of a phytochemical is not an easy task. As an antioxidant, phytochemicals are generally required in small doses due to its ability to become pro-oxidant. Based on its traditional usage, the doses are commonly determined from folklores, thus the key compounds mostly responsible for their health functions and their mechanisms remain to be explored through epidemiological studies. Table 3 lists what doses studied *in vitro* and folklores.

Phytochemicals	Effects	Dosages
Soya saponin (Kang et al., 2010)	• Inhibits metastasis HT-1080 cells • Decrease HT cell growth • Inhibit AFB_1-DNA adduct formation in HepG2 liver cells • Induce apoptosis in SNB 19 glioblastoma cells	• 100-300 µl/mL; 24 h • 150, 300, 600 ppm; 72 h • IC_{50} at 30 µg/mL; 48 h • 25-75 µM; 48 h

Phytochemicals	Effects	Dosages
	• Suppress HTC 15 cell proliferation • Induce macroautophagy • Decrease migratory ability/increase adhesion of B16F10 cells • Enhance adhesion of MCF7 cells	• 25-500 ppm • 100 ppm; 24 h • 25-75 μM; 12 h • 50 μM; 24 h
Soyasapogenol A and B (Kang et al., 2010)	• Suppress HT 29 cell growth	• 6-50 ppm; 72 h
Soyasaponin – soyasapogenol B monoglucuronide mixture (Kang et al., 2010)	• Suppress HT 29 cell growth	• 50 ppm; 72 h
Phytate (Kang et al., 2010)	• Decrease expression of TNF-a and TNF II in Caco-2 cells • Inhibit proliferation of HT 29 cells • Inhibit growth of MCF-7/Adr cells • Inhibit growth of MDA-MB 231cells • Inhibit growth of MCF-7 cells • Inhibit growth of HepG2 cells • Inhibit growth of LNCaP cells • Inhibit growth of DU145 cells	• 1, 2.5, and 5 mM; 12 h • 13 mmol.L; 12 h • IC_{50} at 1.26 mM; 96 h • IC_{50} at 1.32 mM; 96 h • IC_{50} at 4.18 mM; 96 h • 0.25-5 mM; 6 d • 0.5-4 mM; 24 h • 0.25-2 mM; 24 h
Phenolics in colourful potatoes	• Treating gastric ulcer	• 2-3 times a day for no more than 4-6 weeks; drinking diluted water extract of fresh potato (Tan & Rahardja, 2010)
Phytochemicals from luffa [*Luffa cylindrica* Roem.; *Luffa Aegyptica* Mill.; *L. Cattupincina* Ser.; *L. Pentandra* Roxb.]	• To treat intestinal inflammation • To improve breast milk production • To treat asthma	• 1-2 times a day; drinking mature seed extract made from 20 g powder in a ½ cup of hot water • Drinking extract of a 6 g of seed and flesh powder • 2-3 times a day; drinking young luffa juice sweetened with sugar
Phytochemicals from Indian champor weed [*Pluchea indica* (L.)	• For fever and emetic sweat removal • For body odour removal	• once a day; consuming 10 g of boiled leaves • regularly 3 times a day; consuming 10-15 pieces of

Phytochemicals	Effects	Dosages
Less.]		raw or steamed leaves with rice
	• For relieving gastrointestinal disorders in children	• Consuming 3-5 pieces of crushed leaves mixed with soft rice (porridge) (Dalimartha, 2005; Hariana, 2006).
Phytochemicals from Watercress [*Nasturtium officinale* R. Brown, *N. officinale* W.T. Aiton, *N. nasturtium-aquaticum* (L) H. Karst., *Radicula nasturtium Cav.*,]	• For tuberculosis	• 2-3 times a day; consuming soup made from 250 g watercress and pig bone, added with sufficient salt
	• For inflamed lung and coughing	• Consuming soup made from 60 g of watercress and sugar
	• For skin irritation	• several times a day; consuming soup of boiled watercress
	• For urinary problems	• Consuming soup made from 250 g of watercress and palm sugar (Muchlisah & Hening, 2009)
Phytochemicals from bilimbi [*Averrhoa bilimbi* Linn]	• For hypertension	• once every three days; drinking extract of 3 bilimbi fruits in 3 glasses of water, concentrated 3 times
	• For acne	• 3 times a day; applying a mixture of 6-8 ground bilimbi fruits, a ½ tea spoon of salt, a ¼ glasses of water onto acne
	• For muscle pain	• Applying a mixture of 25 pieces of bilimbi leaves, 10 clove, and 15 pepper, ground finely, added with a small amount of vinegar, at suffering body/tissues
	• For teeth cavities	• Chewing 5 pieces of bilimbi fruits with a little salt and at the suffering teeth (Hariana, 2006).
Phytochemicals from Glossy nightshade, Black nightshade [*Solanum americanum*	• For urethra infection	• twice a day; drinking a ½ glasses of extract of 30 g of black nightshade fruits with *Hedyotis diffusa* grass, and *Phyllantus urinearia* in a 3 glasses of water,

Phytochemicals	Effects	Dosages
Miller, *Solanum nodiflorum* Jacq, *Solanum ningrum* auct non L.]		concentrated twice
	• For eczema or dermatitis	• twice a day; consuming 60 g of boiled shrub
	• For xeropthalia	• 3 times a day; chewing around 15 black nightshade fruits
	• For cervical erosion	• 1-2 times a week for 8 weeks; applying a mixture of ground boiled-black nightshade fruits at the suffering tissues
	• For pektay	• 3 times twice a week; drinking a ½ glasses of decoction of 30 g ground black nightshade fruits and *Celosia cristata* flowers in a 3 glasses of water, and concentrated (Dalimartha, 2008)
Phytochemicals from Waxy gourd [*Benincasa hispida* (Thunb) Cogn., *B. cerifera savi, Cucurbita hispida* Thunb. *Lagenaria dasystemon* Miq]	• Curing hemorrhoid	• once a day for 14 days for adult; given infusion liquid of 1 waxy gourd fruit as big as a palm hand, added by 10 pieces of anises/fennels, a ±1cm length of *Alixa stellata*, and a tea spoon of honey; (Kementerian Lingkungan Hidup, 2011)
	• To treat diabetes	• Consuming 100-150 g boiled or juiced waxy guard (Wijayakusuma, 2008)
Phytochemicals from Lemon basil [*Ocimum americanum, O. citriodorum, O. africanum, O. canum Sims, O. brachiatum Blume*]	• To ease people suffering from early ejaculation, late menstruation, breast milk and gas cleanser in the human body, and for removing fever	• twice a day; drinking a ½ glasses of decoction of 15 g lemon basil grass in a 2 glass of water for 15 minutes (Hariana, 2006)

Table 2. Resume of dosages used in studies regarding phytochemicals health effects and in folklores[14].

[14] The information of Indonesian medicinal folklores is obtained through a collaboration project between Korean Food Research Institution and Bogor Agricultural University, Indonesia in 2011.

5. Recommended daily allowance

5.1 Recommended daily allowance

Recommended uses and maximum limits of uses in modern public health management are limited. The ancient uses are based on folklores and old documents. This information should be followed up with proper scientific investigations and documentations. Even for broccoli which is extensively studied, the recommended daily intake has not been officially established. US national cholesterol education program recommends adult subject to consume 2 g of phytosterol/d for optimally lowering LDL-C and coronary heart disease risks by 10% (Kang et al., 2010). The mechanism of this is still vague but it is known that phytosterol/phytostanol does not necessarily present simultaneously with cholesterol to control cholesterol absorption.

5.2 Public health management

There is limited information on detailed diet prescription aiming at treating a particular disease, except those recorded in ancient medications. Dieticians usually arrange diets for patients not aiming for disease treatments but to meet certain nutritional requirements to improve their stamina or immune system to combat their physiological problems. Mechanism for phytochemical health benefits have been studied extensively. Current understanding shows that public health would take benefits from diet management for prevention and maintaining public health instead of treating it. Many research results found scientific base of phytochemicals. For example, liminoids has increasingly proven positive health effects including induction of glutathione S-transferase, inhibiting cancers growth, and lowering cholesterols (Kang et al., 2010), yet officially, this still has not been established for recommended daily allowance. On the other hand, information from ancient medicinal prescriptions as listed in Table 3 is mostly in the form of decoction of the phytochemical sources and the boiled water is drunk. Interesting research area is to establish whether such preparation preserve biological functions of the phytochemicals or, instead, the methods modify molecular form of the phytochemicals that is a much safer and/or more bioactive than its original forms.

5.3 Phytochemicals incorporation in diets

Phytochemicals are commonly consumed as supplements either in capsules, tablets, or powders. The incorporation of such ingredients in food products may or may not face problems of stability, especially at its extraction step and formulation and food processing in which heating is one of predominant aspects for generating food palatability. Most conventional food preparations are of high risks on phytochemical instability. Attempt to improve food technology remains inconclusive. Health effect study indicates that enriched ground beef with soy phytosterol reduces total cholesterol, LDL-C, and TC/HDL cholesterol by 9.3, 14.6, and 9.1%, respectively (Vermeulen, 2009). Such attempts require standardization for establishment of functional food regulations.

5.4 Phytochemical demands per capita

In order to maintain health where phytochemicals are involved, a daily recommended allowance similar to other nutrients is required. Therefore, prior to daily recommended

allowance establishment, there is a need for dosage allowance for each bioactive. Similarly, when recommended allowance has been established, food chain supply needs to provide necessary quantity of the phytochemical sources for people. Such data are currently unavailable, and thus, a database and information system for it needs to be established.

6. Conclusion

Phytochemicals bioavailability is strongly dependent on cell wall compositions of the food matrices they originate from, structural chemistry of the phytochemicals, history of processing, as well as individual human gastrointestinal system. Determination of phytochemical bioavailability is increasingly developed using both *in vitro* and *in vivo* approaches, and yet the results are still inconclusive. The main challenge is to develop an *in vitro* model that can represent human *in vivo* condition for practical uses. On the other hand, many aspects of bioavailability is not well understood, prompting further research and database for recommended dosages and consequently per capita phytochemical demands for public health management. Currently, folklores are the main sources of public health management using phytochemicals and database remains to be pursued for better scientific base of folklores practices.

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Ximenia americana: Chemistry, Pharmacology and Biological Properties, a Review

Francisco José Queiroz Monte[1], Telma Leda Gomes de Lemos[1], Mônica Regina Silva de Araújo[2] and Edilane de Sousa Gomes[1]
Programa de Pós-Graduação em Química Universidade Federal do Ceará, Fortaleza - Ceará
Depatamento de Química, Universidade Federal do Piauí, Teresina - Piauí, Brasil

1. Introduction

The use of plants as medicinal agents to the treat of many diseases has been investigated for a long time since the antique civilizations. Several plants are used in traditional medicine against inflammatory diseases as well as various types of tumors on the base the potential of their chemical constituents. Although many compounds are extremely toxic, when we have the relation between the toxicity of a compound and its chemical pattern of substitution that can result in a more in-depth understanding of these compounds (Atta-ur-Rahman, 2005). Today, even after more than 200 years, the chemistry of natural products remains a challenge and an important field of research in several science areas (chemistry, biology, medicine, agronomy, botany and pharmacy). The reasons for it's large use are the considerable pharmacological potential observed in natural products, in the great development in the process of detection, isolation, purification and, especially, the advances in spectrometric techniques [infrared (IR), mass spectrometry (MS) and nuclear magnetic resonance (NMR ^{1}H and ^{13}C) for structural elucidation of new and complex compounds. These advances were outstanding in both NMR and MS spectrometry. The NMR allows the complete ^{1}H and ^{13}C NMR spectral assignments (chemical shifts and coupling constants) which serve to build a data base to support computer assisted structure elucidation. These data are also useful in the fuller understanding of the correlations between molecular conformation and biological activity of natural substances with biological importance (Loganathan *et al.*, 1990). Mass spectrometry has a huge application in chemistry, biochemistry, medicine, pharmacology, agriculture and food science. Although the mass spectrometric ionization techniques EI (electron impact) and CI (chemical ionization) required the analyte molecules to be present in the gas phase and were thus suitable only for volatile compounds, the development of several desorption ionization methods [FD (field desorption), FABMS (fast atom bombardment), ESIMS (electrospray), MALDI-MS (matrix assisted laser desorption ionization)] allowed the hight-precision mass spectrometric analysis of different classes of biomolecules.

The genus Ximenia belongs to the Olacaceae and comprises about 8 species (Brasileiro et al., 2008): Ximenia roiigi, Ximenia aegyptiaca, Ximenia parviflora, Ximenia coriaceae, Ximenia aculeata, Ximenia caffra, Ximenia americana and Ximenia aegyptica. X. caffra stands out for

being used in Tanzania for the treatment of irregular menstruation, rheumatism and cancer (Chhabra & Viso, 1990) and, in Limpopo Province, South Africa, for treatment diarrhea (Mathabe, 2006). However, X. americana Linn. is the most common, being native to Australia and Asia where is commonly known as Yellow Plum or Sea Lemon. It is found mainly in tropical regions (Africa, India, New Zealand, Central America and south America), specially Africa and Brazil. The plant is characterized as a small tree spinose 3-4 feet tall, gray or reddish bark, with leaves small, simple, alternate, of bright green color and with a strong smell of almonds. The flowers are yellowish-white, curved and aromatic. Fruit are yellow-orange, aromatic, measuring 1.5 to 2.0 cm in diameter, surrounding a single seed and have a pleasant plum-like flavor (Matos, 2007). In Asia, the young leaves are consumed as a vegetable, however, the leaves also contain cyanide and need to be thoroughly cooked, and should not be eaten in large amounts.

X. americana, commonly called "ameixa do mato", "ameixa de espinho" and "ameixa da Bahia", is widely distributed in northeast Brazil. A tea obtained from its barks has been used in popular medicine as cicatrizing, adstringent and as an agent against excessive menstruation. As a powder, it treats stomach ulcers and the seeds are purgative (Braga, 1976; Pio-Correia, 1984). This specimen has been recently examined (Araújo *et al.*, 2008,2009) and the stem ethanolic extract afforded steroids (stigmasterol and sitosterol), triterpenoids (betulinic acid, oleanolic acid, 28-O-(-D-glucopyranosyl) oleanolic acid, 3-oxo-oleanolic acid, 3β–hydroxycicloart-24(*E*)-ene-26-oic acid and sesquiterpenoids (furanoic and widdrane type). A large number of sesquiterpenes are constituents of essential oils of higher plants and seem to intervene in the pharmacological properties attributed to these volatile fractions (Bruneton, 1999). It has been clarified that the biological activities of the liverworths are due to terpenoids and lipophilic aromatic compounds (Atta-ur-Rahman, 1988). Steroids and triterpenes with therapeutic interest and manufacturing employment, are a group of secondary metabolites of outstanding importance (Bruneton, 1999). Considerable recent work strongly indicates the great potential of the triterpenoids as source of use medicinal (Mahato *et al.*, 1992).

Investigations in the past 10 years showed that the constituents of *X. americana* have shown several biological activities such as, antimicrobial, antifungal, anticancer, antineoplastic, antitrypanosomal, antirheumatic, antioxidant, analgesic, moluscicide, pesticidal, also having hepatic and heamatological effects.

In general, the compounds found in *X. americana* were saponins, glicosydes, flavonoids, tannins, phenolics, alkaloids, quinones and terpenoids types. In addition, the plant is potentially rich in fatty acids and glycerides and the seeds contain derivatives cyanide. The identified compounds did not demonstrate a representative pattern of each class. For example, the sesquiterpene were furanoic and widdrane while, triterpenes exhibited oleanane and cycloartane skeletal type. Concerning the fatty acids, in addition to common C16, C18 and C22, a distinctive feature is the presence of acetylenic, as well as, very long chain fatty acids.

We can see, from all the information summarized above, that work on plants of the genus Ximenia is justified, particularly *Ximenia americana* species, where systematic study is still not satisfactory, specially, relative to specific biological activity of their chemical constituents.

The present review compiles the published chemical and pharmacological information on the species *X. americana* and update important data reported in the last ten years in the scientific literature.

2. Biological activity

2.1 Antimicrobial and antifungal activities

To evaluate the scientific basis for the use of numerous plants species used to treat diseases of infectious origin, crude extracts of these plants were investigated. The antimicrobial activity of the extracts of the various parts of the investigated plants such as roots, leaves, seeds, stem barks and fruits, appears to be due to the presence of secondary metabolites such polyphenols, triterpenes, sterols, saponins, tannins, alkaloids, glycosides and polysaccharides (Geyid *et al*., 2005; James *et al*., 2007; Maikai *et al*.,2009; Ogunleye *et al*., 2003).

X. americana is a plant used in traditional medicine for the treatment of malaria, leproutic ulcers and infectious diseases of mixed origin by natives in Ethiopia, Guinea, Sudan and in the Northern part of Nigeria (Geyid *et al*., 2005; James *et al*., 2007; Magassouba *et al*., 2007; Maikai *et al*., 2009; Ogunleye *et al*., 2003; Omer & Elnima, 2003).

The crude extracts of *X. americana* show antimicrobial and antifungal activities. The crude aqueous, methanolic, ethanolic, butanolic and chloroform extracts from different parts (leaves, root, stem and stem bark) of the plant were subjected to phytochemical screening and from the test carried out, it was observed that the secondary metabolites contained were saponins, flavonoids, tannins, terpenoids, sterols, quinones, alkaloids, cyanogenetic glycosides, cardiac glycosides and carbohydrates in the form of sugars and soluble starch. The results of phytochemical screening of various parts solvent extracts of *X. americana* are presented in Table 1.

The MeOH extract from leaves of *X. americana* inhibited or retarded growth of *Neisseria gonorrhea* organism at dilution as low as 250 µg/ml. This same extract showed antifungal effect against *Candida albicans* and *Cryptococus neoformans* in concentration of 4000 µg/ml. Chemical screening conducted on the extract showed the presence of several secondary metabolites as tannins, sterols, terpenoids, flavonoids and saponins (Geyid *et al*., 2005). The antimicrobial activities of ethanol extract of the leaves were evaluated against six common bacterial isolates (*Pseudomonas aeruginosa*, *Proteus vulgaris*, *Bacillus subtilis*, *Escherichia coli*, *Staphylococus aureus* and *Candida albicans*) and was active against all of them. The highest degree of activity was for *P. aeruginosa* (inhi bition zone: 20 mm), followed by *B. subtilis* and *C. albicans* (inhibition zone: 10 mm). Activity of the organic extract of the plant was comparable to that of commercially available penicillin disc (2 µg) which was more active against *P. aeruginosa* but less effective against *S. aureus*. The results of phytochemical analysis indicated the presence of saponins, flavonoids, tannins and cyanogenetic glycosides. Alkaloids and anthraquinones were not present (Ogunleye *et al*., 2003). The root, stem bark and leaves aqueous and methanolic extracts of *X. americana* were tested against five bacteria and they inhibited the growth of *Staphylococus aureus* and *Klebsiella pneumoniae* while *Shigella flexineri* was inhibited by only methanolic leaves, aqueous bark and aqueous leaves extracts. *Salmonella typhi* and *Escherichia coli* were not affected by these extracts. The

Plant part	Solvent	Class of Compounds									Ref.
		Tannins	Steroids	Terpenes	Saponins	Flavonoids	Alcaloids	Cardiac	Glycosids	Quinones	
Leaves	MeOH	+		+	-	+	-	-		-	Geyid *et al.*, 2005
Leaves	H_2O	+	-	-	+	+	-	+		-	Ogunleye *et al.*, 2003
	EtOH	+	-	-	+	+	-	+		-	
Leaves	H_2O	+	-	-	+	+	-	+		+	James *et al.*, 2007
	MeOH	+	-	-	+	+	-	+		-	
Stem bark	H_2O	+	-	-	+	+	-	+		+	
	MeOH	+	-	-	+	+	-	+		+	
Root	H_2O	+	-	-	+	+	-	+		+	
	MeOH										
Stem bark	BuOH	+	-	+	+	+	+	+		-	Maikai *et al.*, 2009
	MeOH	+	-	+	+	+	+	+		+	
	H_2O	+	-	+	+	+	-	-		+	
Root	$CHCl_3$										Omer & Elnima, 2003)
	MeOH				+	+					
Stem	EtOH		+	+							Araújo *et al.*, 2008,2009

+: present; -: absent; Ref.: references
Some extracts showed the presence of carbohydrates in the form of sugars and soluble starch (James *et al.*, 2007 & Ogunleye *et al.*, 2003); few extracts showed also the presence of cyanogenetic glycosides (Ogunleye *et al.*, 2003). Quinones are of the anthraquinone type; terpenes are sesquiterpenes and triterpenes type (Araújo *et al.*, 2008, 2009).

Table 1. Phytochemical screening of stem bark, leaves, root and stem extracts of *X. Americana.* (Placed on the table 1)

Minimum Inhibitory Concentration (MIC) was only evident for the methanolic extracts at $1.25x10^4$ μgmL^{-1} (1:4) against *Staphylococus aureus* while the Minimum Bactericidal Concentration (MBC) of the extracts was obtained at $2.50x10^{-4}\mu g$ mL^{-1} (1:2) (James *et al.*, 2007). From the results, inhibitory activity of extracts (methanolic root) was more pronounced on *Klebsiella pneumonia* whereas it shows no activity against *Escherichia coli*, *Salmonella typhi* and *Shigella flexineri*. The methanolic root extract showed highly significant ($p<0.05$) activity on *Klebsiella pneumonia* when compared with leaf extracts and methanolic bark extract. The phytochemical constituents present in the extracts were carbohydrates in the form of sugars and soluble starch (except for aqueous and leaves extracts), cardiac

glycosides, saponins, tannins and flavonoids while alkaloids were absent in all the extracts. It was concluded that the extracts of methanolic roots, stem bark and leaves have bacteridal activities over the concentration of $2{,}5x10^4$ - $1{,}25x10^4$ μgmL^{-1} and that the presence of carbohydrates, glycosides, flavonoids and tannins in the diferent extracts are responsible for their antibacterial activity. The antimicrobial properties of the bark, leave, root and stem extracts of *Ximenia americana* were screened against *Bacillus subtilis*, *Staphyllococus aureus*, *Escherishia coli* and *Pseudomonas aeruginosa* (Table 2) using the cup-plate agar diffusion method and the minimum inhibitory concentration by agar dilution method (Omer *et al*., 2003).

Part used	Solvent system	% Yield	Inhibition zone (mm)				MIC (mg/ml)			
			B.s	S.a	E.c	Ps.a	B.s	S.a	E.c	Ps.a
Bark	$CHCl_3$	1.1	13	12	11	15	N.D	N.D	N.D	N.D
	MeOH	21.1	23	30	19	22	0.31	0.62	19.79	19.79
	H_2O	8.9	18	18	16	14	0.40	1.62	3.24	1.62
Leaves	$CHCl_3$	10.7	13	14	-	12	N.D	N.D	N.D	N.D
	MeOH	26.6	23	22	-	25	1.55	0.77	9997	12.45
	H_2O	5.0	17	19	16	22	0.59	1.19	>25.5	19.11
Root	$CHCl_3$	2.2	15	13	12	13	N.D	N.D	N.D	N.D
	MeOH	3.7	15	21	19	15	3.27	6.54	>34.88	>34.48
	H_2O	5.7	13	13	-	-	2.68	10.74	28.65	28.65
Stem	$CHCl_3$	2.7	-	11	11	-	N.D	N.D	N.D	N.D
	MeOH	11.8	20	25	-	24	>72.75	3.41	>72.75	>72.75
	H_2O	2.7	17	17	13	13	5.12	5.12	>13.65	>13.65

B.s, *Bacillus subtilis;* S.a, *Staphyllococus aureus*; E.c, *Escherichia coli*; Ps.a, *Pseudomonas aeruginosa;* concentration of extracts 100 mg/ml, 0.1 ml/cup; inhibition zones are the mean of three replicates. MIC, minimum inhibitory concentration; N.D, not detected.

Table 2. Antibacterial activity of *Ximenia americana* extracts against standard organisms. (Placed on the table 2)

The methanolic extract was the most active one. The aqueous extract also exhibited high activity which justifies its traditional use. *Staphyllococus aureus* was the most susceptible bacterium among the tested organisms. The table 3 show the antibacterial activity of *Ximenia Americana* against the pharmaceuticals patterns.

Several other studies to determine the presence of antimicrobial activity in crude extracts of *Ximenia americana* were performed (Magassouba *et al*., 2007; Maikai *et al*., 2009). In all, the various extracts were found to have broad spectrum effect against standard organisms (*Escherichia coli*, *Pseudomonas aeruginosa, Staphylococus aureus, Proteus vulgaris, Candida albicans, Bacillus subtilis, Salmonella typhi* and *Shigella flexineri*) and supports the traditional usage of this plant as remedy in treatement of microbial infections.

In general, the antimicrobial activity of extracts of the various parts of the plants appears to be due to presence of secondary metabolites. In some experiments, was remarked that the

Reference drugs	Concentration µ/ml	MDIZ			
		B.s	S.a	E.c	Ps.a
Ampicillin	40	14	25	-	-
	20	13	22	-	-
	10	-	19	-	-
	5	-	18	-	-
Benzyl penicillin	40	-	37	-	-
	20	-	33	-	-
	10	-	28	-	-
	5	-	24	-	-
Cloxacillin	40	-	29	-	-
	20	-	27	-	-
	10	-	22	-	-
	5	-	18	-	-
Gentamicin	40	24	18	25	22
	20	22	16	17	15
	10	17	14	16	12
	5	15	13	11	-

Interpretation of sensitivity test results: Gram(+) bacteria*; Gram(-) bacteria **;
>18 mm (M.DIZ)= sensitive; >16 mm (M.DIZ)=sensitive;
14-18 mm (M.DIZ)= intermediate; 13-16 mm (M.DIZ)= intermediate;
<14 mm (M.DIZ)= resistant; and < 13mm (M.DIZ)= resistant.

Table 3. The activity of *Ximenia Americana* against the clinical isolates. (Placed on the table 3)

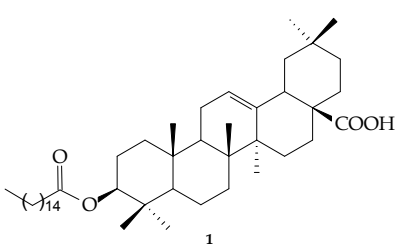
plants which accumulate polyphenols, tannins and unsaturated sterols/terpenes showed to inhibit or significantly retard growth of eight of the ten test organisms; the species, which constitute polyphenols and unsaturated sterols/terpenes; and polyphenols, tannins, unsaturated sterols/terpenes, saponins and glycosides inhibited six organisms each while, those with polyphenols, tannins, unsaturated sterols/terpenes, saponins; and alkaloids and unsaturated sterols/terpenes inhibited growth of five bacterial strains each (Geyid *et al.*, 2005). Cyanogenetic glycosides are reported to possess antimicrobial activity (Finnermore *et al.*, 1988). Tannins have been traditionally used for protection of inflamed surfaces of the mouth and treatment of catarrh, wounds, haemorrhoids and diarrhea and as antidote in heavy metal poisoning. They have the ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins and also complex with polysaccharide (Maikai *et al.*, 2009; Scalbert, 1991; Ya *et al.*, 1988). Flavonoids are naturally occurring phenols, which posses numerous biological activities including anti-inflamatory, antiallegic, antibacterial, antifungal and vasoprotective effects and, also have been reported to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Dixon *et al.*, 1983; Geyid *et al.*, 2005; Hostettman *et al.*, 1995; James *et al.*, 2007; Maikai *et al.*, 2009; Ogunleye *et al.*, 2003). Terpenoids have also been reported to be active against bacteria, the mechanism of action involve membrane disruption by the lipophilic compounds (Geyid *et al.*, 2005; James

et al., 2007; Maikai *et al.*, 2009; Ogunleye *et al.*, 2003). Although it is difficult to speculate on the mechanism of action of the constituents of the extracts on the basis of studies conducted to date, the antimicrobial activity of these extracts is due, no doubt, the presence of these secondary metabolites. In the case of extracts of *Ximenia americana,* probably, due the presence of tannins, flavonoids, triterpenes/steroids, saponins or cyanogenetic glycosides.

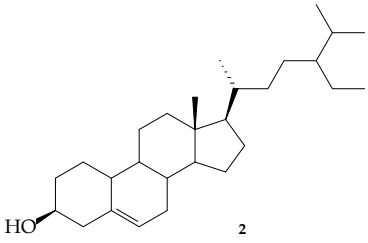
In summary, the results justified the use of *X. americana* as having antibacterial properties and support its use as agent in new drugs for therapy of infectious diseases caused by pathogens.

2.2 Pesticidal activity

Olecaceous seed oils are a rich source of acetylenic lipids and unsaturated fatty acids (Badami & Patil, 1981 & Sptizer *et al.*, 1997). Acetylenic metabolites show some biological activities including, insecticidal activity (Jacbson, 1971). *X. americana* was recorded to contain octadec-11-en-9-ynoic acid, named xymeninic acid as well as icosenoic-triacontenoic acids, all of which belong to the ω-9 series (Rezanka, & Sigler, 2007). Bioactivity-driven fractionation of the $CHCl_3$ extract of the root of *X. americana* using the Brine Shrimp Lethality Test (BST) and hatchability test with *Clavigralla tomentosicollis* eggs yielded two fractions (F006, soluble in petroleum ether and F005, soluble in 10% H_2O in MeOH) as the most actives (F005, BST LC_{50} 78 (129-48) µg/mL and F006, BST $LC\%_{50}$ 76(121-49) µg/mL) (Fatope *et al.*, 2000). A combination of F005 and F006 was submitted to hatchability test (inhibition of hatching = 68 % of control) and successive BST-dircted fractionation on silica gel column and preparative TLC yielded oleanene palmitates (**1**), β-sitosterol (**2**) and C_{18} acetylenic fatty acids (**3** and **4**) as yellow oils.



1



2

The substance **4** suppressed the hatchability of *C. tomentosicollis* eggs at 92 % of control when tested at 4 x 10^4 µg/mL (correcting for unhatched eggs in the control using Abbott's formula):

% control = [(% unhatched of treated group - % unhatched of untreated group)/ (100 - % unhatched of untreated group)] x 100

These acetylenic fatty acids show characteristic spectrometric data. The ^{13}C NMR spectrum of **3** displayed absorptions diagnostic of acetylenic carbons at δ_C 80.4 (C) and 80.1 (C) and of carboxylic carbon at δ_C 189.1 (C), in agreement with its IR spectrum which exhibited bands at 2200 and at 1713 cm^{-1}, characteristic of acetylenic and acid groups, respectively. Compound **3** had molecular formula$C_{18}H_{32}O_2$, as established by HREI-MS (*m/z* 280.2378 for $[M^+]$) in combination with its ^1H and ^{13}C NMR spectra. From analysis spectral data compound **3** was thus established as octadeca-5-ynoic acid (tariric acid). Compound **4** had a

mol wt 6 mass units less than that of **3** with molecular formula $C_{18}H_{26}O_2$ as revealed by HREI-MS (*m/z* 274.2021 for [M+]) in combination with its ^1H and ^{13}CNMR spectra. The ^{13}C NMR spectrum of **4** displayed absorptions diagnostic of acetylenic carbons at δ_C 83.4 (C) and 74.1 (C) and of carboxylic carbon at 179.3 (C), in agreement with its IR spectrum which exhibited bands at 2232 and 1702 cm^{-1}, characteristic of acetylenic and acid groups, respectively. The ^{13}C RMN spectrum also exhibited six resonance at δ_C 148.2 (CH), 140.9 (CH), 136.9 (CH), 129.8 (CH), 109.3 3(CH) and 108.6 (CH), revealing the presence of three double bonds. From a detailed spectral analysis considering, especially, the multiplicity of signals and coupling constants in the ^1H NMR spectrum, as well as the presence of diagnostic peaks in the mass spectrum, compound **4** was thus established as 10*Z*,14*E*,16*E*-octadeca - 10,14,16-triene-12-ynoic acid, a ene-ene-yneene acetylenic fatty acid (Fatope *et al*., 2000).

$CH_3—(CH_2)_{11}C\equiv C—(CH_2)_3CO_2H$ **3**

$CH_3CH=C_{16}HCH=C_{14}HC\equiv C_{12}CH=C_{10}H(CH_2)_8CO_2H$ **4**

In addition, *Ximenia* seed oil have been found to contain fatty acids with more than 22 carbon atoms (very long fatty acids) which are found only rarely in nature. Using liquid chromatography in combination with mass spectrometry was founded that *Ximenia* oil to contain fatty acids with chain length C_{34} and C_{36} (Rezanka & Sigler, 2007). Effectively, two very long chain unsaturated fatty acids C_{40} and C_{35} (**5** and **6**) were isolated (Saeed & Bashier, 2010) from *X. americana* seeds and fruits, respectively. The mass spectrum of the major component (**5**) showed a molecular ion at m/z 604 corresponding to the molecular formula $C_{40}H_{76}O_3$. The IR spectrum of **5** showed a broad absorption band at 3600-3200 cm^{-1} (OH) and the presence of strong absorption at 1742 cm^{-1} attributed to ester group. The base peak appeared at m/z 55 (C_4H_7+) due to allylic bond cleavage and peaks at m/z 479 and 151 furnished from fragmentation in C_{28}-C_{29} and C_{26}-C_{27}, respectively. In addition, the peaks at m/z 31, 59, 73 and 74 (McLafferty rearrangement) were compatible with unit $CH_3OCO(CH_2)_3$-. The compound **5** was identified as methyl-14,14-dimethyl-18-hydroxyheptatracont-27,35-dienoate. The mass spectrum of **6** showed a molecular ion at 578, corresponding to the molecular formula $C_{35}H_{62}O_6$. The IR spectrum showed bands at 3500, 1731 and 1645 cm^{-1} corresponding to OH, C=O and C=C groups, respectively. The base peak appeared at m/z 73 ($C_3H_5O_2$+) which is characteristic for the methyl ester, reinforced by additional peaks at m/z 31, 59 and 74 (McLafferty rearrangement). An peak at m/z 479 was due to M-$C_5H_7O_2$ and one at m/z 339 is due to the cleavage C_{13}-C_{14} while, those at m/z 126 and 265 were due to $C_7H_{10}O_2$ and M-$C_{17}H_{28}O_2$, respectively. The compound **6** was identified as dimethyl-5-Methyl-28,29-dihydroxydotriacont-3,14,26-triendioate.

$[CH_3OCO(CH_2)_{12}C(CH_3)_2(CH_2)_3CHOH(CH_2)_8CH=CH(CH_2)_6CH=CHCH_3]$

5

$[CH_3OCOCH_2)CH=CHCH_3(CH_2)_8CH=CH(CH_2)_{10}CH=CH(CHOH)_2(CH_2)_2COOCH_3]$

6

2.3 Analgesic activity

The aqueous extract of stem bark of *X. american* has analgesic properties that justify its use popular in countries such as Tanzania, Senegal, Zimbawe and Nigeria. The extract of *X.*

americana in doses containing 10 to 100 mg/kg P.C, inhibits contractions of the abdomen with analgesic effects comparable to those of phenylbutazone. In fact, at doses of 100 mg / kg P.C, phenylbutazone causes an inhibition of pain in 45.2±2%. The percentage of inhibition by extract of *X. ameriacana* is 61.1±% in the same concentration. These properties are probably due to the presence of flavonoids and saponins, detected in the extract (Soro *et al*., 2009). The analgesic activity of the methanol extract of *X. americana* leaf was investigated in chemical models of nociception in mice. The extract at doses of 200, 400 and 600 mg/kg i.p. produced an inhibition of 54.13, 63.74, and 66.4% respectively, of the abdominal writhes induced by acetic acid in mice. In the formalin test, the administration of 200, 400 and 600 mg/kg i.p. had no effects in the first phase (0 to 5 min) but produced a dose dependent analgesic effect on the second phase (15 to 40 min) with inhibitions of the licking time of 29.3, 47.8 and 59.8%, respectively. These observations suggested that methanol extract of *X. americana* leaf possesses analgesic activity (Siddaiah *et al*., 2009).

2.4 Antipyretic activity

The bark of stem of *X. americana* has been used in West Africa for the treatment of pain and fever. To verify this second property, the treatment of rats in hyperthermia with *Ximenia americana* stem bark aqueous and with beer yeast was compared to those obtained with lysine acetylsalicylate (Aspegic). The study showed an antipyretic action of the extract. Moreover, the toxicological study of the stem extract indicated a LD_{50} of 237.5 mg/kg P.C according to the classification of Diezi this plant is relatively toxic. The experiments show that the properties of *X. americana* could due to the presence of saponosides, as show by screening tests performed in this study. These results justified the use of *X. americana* in traditional cure of fever treatment (Soro *et al*., 2009).

2.5 Antitrypanosomal activity

The in vitro antitrypanosomal activity of methanolic and aqueous extracts of stem bark of *Ximenia americana* was evaluated on Trypanosoma congolense. Blood obtained from a high infected mice with *T. congolense* (10(7) was incubated with methanolic and aqueous extracts at 20, 10 and 5 mg/ml and Diminal(R) (diminazene aceturate) at 200, 100 and 50 µg/ml in a 96 micro plate. The results revealed that methanol and aqueous extracts had activity at 20 and 40 mg/ml however, the methanolic extracts were more active than aqueous extracts at 10 and 5 mg/ml. Phytochemical screening of the methanolic and aqueous extracts of the bark showed that they both had flavonoids, anthraquinones, saponins, terpenes and tannins. The aqueous and methanolic extracts appears to show some potential activity against *T. congolense* (Maikai *et al*., 2008).

2.6 Anticancer activity

Plants have been show to provide a useful source of natural products that are effective in the treatment of human neoplastic diseases. Information recorded from ancient civilizations has demonstrated the use of plants in search of treatment for various types of cancer (Hartwell, 1967-1971). An analysis of plant materials that had been studied at the National Cancer Institute (NCI), USA for discovering new anticancer drugs showed that if ethnopharmacological information had been used, the yield of plants harboring antineoplastic activity would have been significantly increased (Spjut & Perdue, 1976). The

list of natural products stored for study as more effective drugs for the treatment of human cancers (NCI) were generated by searching for specific structural types (Steven & Russel, 1993). However, the presence of some large class cannot be ruled out. Examples of anticancer agents developed from higher plants are the antileukemic bis-indole alkaloids vinblastine and vincristine from the *Catharantus roseus* (Apocynaceae); diterpene taxol, used to treat breast cancer, lung cancer, and ovarian cancer and also used to treat AIDS-related (Kaposi's sarcoma) from *Taxus breviflora* (Taxaceae); pyrrolo[3,4,b]-quinoline alkaloid camptothecin (antileukemic) from *Camptotheca acuminate* (Nyssaceae) and pyridocarbazole alkaloid elipticine (antitumor) contained in *Ochrosia elliptica* (Apocynaceae). A large number of other active natural products with toxicity to cells in culture (Walker carcinosarcoma 256, mouse L-1210 leukemia, Ehrlich ascite tumor, sarcoma 180 and mouse P-388 leukemia cell lines) have been detected (Geran *et al.*, 1972 & Lee *et al.*, 1988).

Cell line				
Tumor cell lines	IC_{10}[a](ug/ml medium)	IC_{50}[b] (ug/ml medium)	IC_{90}[c] (ug/ml medium)	IC_{90}/IC_{10}[d] medium)
MCF7	0.6	1.7	10	16.7
BV173	0.4	1.8	7.0	17.5
CC531	0.8	3.3	12	15.0
U87-MG	1.0	9.0	100	100
K562	5.0	11	180	36
SKW-3	3.1	20	700	226
HEp2	5.0	21	100	20
NC1-H460	4.0	21	150	38
PC3	3.5	26	>1000	>300
MDA-MB231	5.0	33	100	20
HT29	8.0	40	350	44
U333	7.0	65	300	43
SAOS2	20	66	1000	50
LAMA84	10	90	600	60
HL60	30	90	1000	33
CML-T1	2.5	160	1000	400
AR230	17	170	700	41
Non tumor cell lines				
MCF10	35	>100	>100	>2.0
MDCK	12	27	60	5.0
N1H/3T3	2	33	>100	>50
PNT-2	2	20	>100	>50

[a]Inhibitory concentration 10 (concentration inhibiting the cell growth by 10%), as accessed by MTT assay;
[b]Inhibitory concentration 50 (concentration inhibiting the cell growth by 50%), as accessed by MTT assay.
[c]Inhibitory concentration 90 (concentration inhibiting the cell growth by 90%), as accessed by MTT assay.
[d]Ratio of IC_{90} and IC_{10} values.

Table 4. Antiproliferative activity of an aqueous extract from *X. americana* in 16 human and one rodent tumor cell lines and in 4 immortalized non-tumor cell lines.

The antineoplastic activity in vitro of various extracts from *Ximenia americana*, plant used in African traditional medicine for the treating cancer, was investigated (Voss *et al.*, 2006, 2006). The most active, aqueous extract was subjected to a detailed investigation in a panel of 17 tumor cell lines (Table 4) originating from human (16 lines) and rat (1 line), showing a averageI C_{50} of 49 mg raw powder/ml medium. The majority of cell lines (11 out of 17) were classified as sensitive (the sensitivity varied from 1.7 mg/ml in MCF7 breast cancer cells to 170 mg/ml in AR230 chronic-myeloid leukemia cells) and three of these (MCF7 breast cancer, BV173 CML and CC531 rat colon carcinoma) showed a particularly high sensitivity, with ratios lower than 0.1 of the average IC_{50}. The *in vivo* antitumor activity was determined in the CC531 coloretal rat model and significant anticancer activity was found following peroral administration, indicating a 95% reduced activity.

A comparison of the antineoplastic activity of the extract with three clinically used agents is given in Table 5. The cytotoxicity profiles of four cell lines are illustrated by the respective IC_{10}, IC_{50} and IC_{90} values, as well as by the corresponding IC_{90} to IC_{10} ratio, describing the slop of the concentration-effect curve. Most prominently, the ranking in sensitivity differed between the extract and the positive controls. In variance to the extract, which resulted in the lowest IC_{50} and IC_{90}/IC_{10} ratio in MCF7 cells, miltefosine and cisplatinum caused the lowest IC_{50} and IC_{90}/IC_{10} ratio in HEp2 cells. Similar to the extract, the lowest IC_{50} following gemcitabine exposure was seen in NCF7 cellls. However, this agent differed from all the others by its lack in effecting 90% growth inhibition, were the HEp2 cells; notably, the cells were most resistant to the agent. In contrast, SAOS2 cells were found to best most resistant to the extract as well as to miltefosine and cisplatinum.

Cell line	Treatment	IC_{50}	IC_{50}	IC_{90}	IC_{90}/IC_{10}
MCF7	Extract (µg/ml)	0.6	1.8	10	16.7
	Miltefosine (µM)	6.5	40	80	12.3
	Cisplatinum (µg/ml)	0.22	2.2	10	45
	Gemcitabine (µM)	0.001	0.012	>100	$>10^5$
U87-MG	Extract (µg/ml)	1.0	9.0	100	100
	Miltefosine (µM)	4.7	27	70	14.9
	Cisplatinum (µg/ml)	0.12	1.6	18	150
	Gemcitabine (µM)	0.002	0.014	>100	$>5x10^4$
HEp2	Extract (µg/ml)	5.0	21	100	20
	Miltefosine (µM)	1.2	2,8	8.0	6.7
	Cisplatinum (µg/ml)	0.09	0.4	1.4	15.6
	Gemcitabine (µM)	0.2	0.47	17	85
SAOS2	Extract (µg/ml)	20	66	1000	50
	Miltefosine (µM)	5.0	40	120	24
	Cisplatinum (µg/ml)	0.11	3.1	10	91
	Gemcitabine (µM)	0.007	0.034	>100	$>10^4$

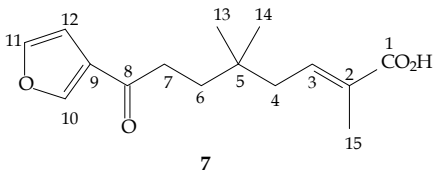
Table 5. Cytotoxicity profiles of the extract and three standard antineoplastic agents in a subpanel of the cell lines

In order to define the substance class of the active component(s) (Voss *et al.*, 2006) experiments were carried out on physicochemical properties. In the process, lipids and lipophilic plant secondary metabolites could be excluded, since the biological activity was only extracted by strongly polar solvents. Large amounts of tannins were identified in the aqueous extract. However, extracts prepared in methanol or 70% acetone, both solvents known to efficiently extract tannins from plant materials, had only a low (methanol) or no (70% acetone) cytotoxic activity. Molecules smaller than 10 kDa were excluded by ultrafiltration. Out of the known class of plant cell macromolecules, DNA and RNA were not found in the aqueous extract and digestion experiments with DNase or RNase had not effect biological activity. However, proteins and polysaccharides were shown to be present in the aqueous extracts and could not be further separated by physicochemical methods. Digestion experiments with trypsin and proteinase K hinted at a protein being responsible for the cytotoxic activity.

A well-defined family of cytotoxic plant proteins is that of the type II ribosome-inactiving proteins (RIPs). These proteins with molecular weight of about 60 kDa, consist of two polypeptide chain, termed A- and B- chain, with an MW of about 30 kDa each, being held together by disulphide bridge. Cumulative evidences (cytotoxic effects, MW, two-chain structure of the proteins in the affinity-purified fraction and one mass-spectrometrically sequenced tryptic peptides) strongly suggests that the active components of the plant material are so far unknown proteins belonging to the type II RIP family.

By a combination of preextraction, extraction, ion exchange and affinity chromatography, a mixture of two cytotoxic proteins was isolated. The eluted peptides were analyzed by electro-spray ionization mass spectrometry (MS/MS). The MS/MS mass spectrum is a method in which a first analyzer isolates a precursor ion which then undergoes a fragmentation yielding a product ions and neutral fragments. A second spectrometer analyzes the product ions. MS/MS applications are plentiful in the study of fragmentation mechanisms, observation of ion-molecule reactions, applications to high-selectivity and high-sensitivity analysis and determination of elementary compositions. Thus, it is a rapid selective analysis method for the components of a complex mixture and macromolecules in biological fluids. The homology of the translated protein sequence from isolated peptides to known type II RIP precursor protein sequence demonstrates that the new protein termed "riproximin" is a so far unknown member of this class. In conclusion, from biological activity of each of the two proteins as well as from MS/MS sequence analysis, showing the presence of two B-chain and two A-chain in the mixture, the *X. americana* extract analyzed contains a mixture of two new proteins, riproximin, belongs to the family of type II ribosome-inactivating proteins.

Two sesquiterpenes (**7** and **8**) isolated from the EtOH extract of the stems of *X. americana* did not inhibit the growth of HL-60 (human leukemia), HTC-8 (human colon) and MDA-MB-435 (human breast cancer) cell lines.



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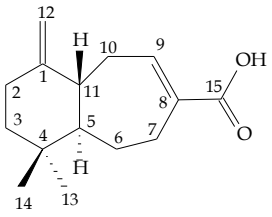


8

The compounds **7** and **8** were recently isolated and their structures were elucidated on the basis of spectral analysis (IV, MS and NMR) and the complete assignment of the ^{1}H and ^{13}C NMR signals were achieved by 1D(^{1}H, ^{13}C and DEPT) and 2D (^{1}H - ^{1}H COSY, ^{1}H - ^{13}C HMQC, ^{1}H-^{13}C HMBC and ^{1}H - ^{1}H NOESY) NMR experiments. The sesquiterpene **7**, isolated as a white powder, has molecular formula$C_{15}H_{20}O_4$ deduced from its EIMS ($M^{+\cdot}$ 264) in combination with its ^{1}H and ^{13}C NMR spectra. The ^{1}H and ^{13}C NMR spectra combined with distortionless enhancement by polarization transfer (DEPT) technique exhibited signals that allowed characterize the three isoprene units (C-1, C-2, C-3, C-4 and C-13; C-8, C-9, C-10, C-11 and C-12; C-5, C-6, C-7, C-14 and C-15) of **7**. Thus, the ^{13}C NMR spectra exhibited signals for six sp^2 carbons [olefinic bond: C-2 (δ_C 128.9), C-3 (δ_C 141.7) and furan ring: C-9 (δ_C 127.9), C-10 (δ_C 147.1), C-11 (δ_C 144.4), C-12 (δ_C 108.9)], two carbonyl [conjugated ketone, C-8 (δ_C 195.3) and conjugated carboxylic acid, C-1 (δ_C 173.1)]), three methylene [C-4 (δ_C 41.2), C-6 (δ_C 36.5) and C-7 (δ_C 35.9), three methyl [C-13 (δ_C 12.4), C-14 (δ_C 25.9) C-15 (δ_C 25.9) and one quaternary carbon [C-5 (δ_C 34.3)]. One conjugated ketone (δ_C 195.3) was also evident from the absorption at 1682 cm^{-1} in the IR spectrum. In the HMBC spectrum, obvious long-range connectivities between the methylene group 2H-7 (δ_H 2.71, dd, 7.9, 6.0 Hz) and C-8 (δ_C 195.57) and between the methylene group 2H-4 (δ_H 2.20, d, 7.7 Hz) and C-5 (δ_C 34.56) allowed the assembly of the molecule and show it to consist of a furanoid sesquiterpene. Others diagnostic ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC and ^{1}H-^{13}C HMBC correlations permitted to assign all the hydrogen and carbon atoms. The sesquiterpene, **8** isolated as a white solid, has molecular formula$C_{15}H_{22}O_2$ deduced from its EIMS ($M^{+\cdot}$ 234) in combination with its ^{1}H and ^{13}C NMR spectra. The ^{1}H and ^{13}C NMR spectra combined with distortionless enhancement by polarization transfer (DEPT) technique exhibited signals that allowed characterize the three isoprene units (C-1, C-2, C-3, C-11 and C-12; C-4, C-5, C-6, C-13 and C-14; C-7, C-8, C-9, C-10 and C-15) of **8**. The ^{13}C NMR spectra exhibited signals for four sp^2 carbons [three substituted, C-8 (δ_C 132.34) and C-9 (δ_C 145.01) and disubstituted, C-1 (δ_C 154.71) and C-12 (δ_C 111.63) bonds; one conjugated carboxylic acid, C-15 (δ_C 173.71), besides signals to five methylene, two methyne, one quaternary and two methyl carbons. The possibility of himachalano type structure was eliminated based on the interpretation of spin-spin interactions revealed by ^{1}H-^{1}H COSY spectrum, which clearly showed the presence of cross peaks corresponding to the couplings of two atoms of hydrogen 2H-6 [δ_H 1.68 (m) and 1.50 (m)] with H-5 hydrogen [δ_H 1.81 (m)] and with the two hydrogen atoms 2H-7 (δ_H 2.45 and 2.35) besides interaction of H-5 (δ_H 1.81) with H-11 (δ_H 2.50, q). This sequence does not appear in the skeleton type himachalano. The *trans* configuration fusion ring was supported by correlations observed in NOESY NMR spectrum, that exhibited the presence of nOes indicating that the hydrogens 3H-13 (δ_H 1.01, s), H-5 (δ_H 1.81) and H-3ax (δ_H 1.58, t, 10.8 Hz) are oriented on the same side (á) of the molecule, while the hydrogens 3H-14 has the same orientation (â) that the hydrogens H-11 (δ_H 2.50, q), H-6ax (δ_H 1.50) and H-3eq (δ_H 1.74, dd, 10.8 , 8.9). Others diagnostic ^{1}H-^{1}H COSY, ^{1}H ^{13}C C HMQC and ^{1}H ^{13}C ^{13}C HMBC correlations permitted to assign all the hydrogen and carbon atoms.

2.7 Others activities

2.7.1 Antiviral effect

The stem bark MeOH extract of *X. americana* as well as several others plant species used by the Maasai pastoralis of East Africa showed antiviral effect against measles virus *in vitro* by

plaque reduction neutralization assay. Potentially active constituents from extracts of all the plants include polyphenols, alkaloids, tannins, sterols, terpenes, saponins and glycosides, between others (Parker *et al*., 2007).

2.7.2 Hepatic and heamatological effects

A study (James *et al*., 2008) was conducted from the leaves, stem bark and root aqueous extract of *X. americana* with albino rats. The results of this work shows that the extracts significantly ($P<0.05$) increasing the level of serum alanine transaminase (ALT) and aspartate transaminase (AST), results indicative of hepatocellular damage. The result also shows that the root has the ability to impair albumin synthesis as observed by the decrease of level of serum albumin. The weight of the animal showed a significant ($P<0.05$) reduction on administering the leaves extract as compared to the control and the others extracts. This reduction might be due to poor intake and utilization of food by the animals in the leaves extract group. The significantly ($P<0.05$) higher content of hydrogen cyanide, saponins, and oxalates in the root extracts indicates that the root extracts may be more toxic. Hydrogen cyanide is known to cause gastrointestinal inflammation and inhibition of cellular respiration. Saponins are known to have haemolytic properties and the ability to reduce body cholesterol by preventing its reabsorption. The high saponin content in the root may lead to gastroenteritis manifested by diarrhea. Oxalates have been known to cause irreversible oxalate nefrosis when ingested in large doses. Thus, there is need to isolate the specific component(s) responsible for the toxicity in the root extract in order to standardized the preparation for maximum therapeutic benefit.

2.7.3 Toxicity

The stem bark of *X. americana* was evaluated for its phytochemical constituents and acute toxicity effect on the Swiss albino mice (Maikai *et al*., 2008). The results from the extracts administered intraperitoneally/orally at doses of 10, 100 and 1000 mg/kg body weight revealed no death with doses up 5000 mg/kg body weight. Post mortem, hematological and histopathological examination did not show any significant ($P<0.05$) weight changes. Phytochemical screening of the aqueous extract stem bark revealed the presence of cardiac glycosides, flavonoids, saponins and tannins. The results suggested that the aqueous extract is not acutely toxic to the mice.

2.7.4 Food composition and cosmetic use

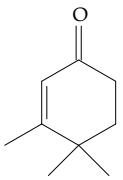
Glyceride blends containing ximenynic acid (**9**) (found in *X. americana*) are useful for the preparation of food compositions or food supplements, including margarine, chocolate, ice cream, mayonnaises, cheese, dry soups, drinks, cereal bars and sauces and snack bars. The blend provides a composition providing health benefits consisting of insulin resistance, or related disorders such as diabetes, delaying the onset of symptoms related to development of Alzheimer's disease, improving memory function, lowering blood lipid levels, anticancer effects or skin antiageing effects (Koenen *et al*., 2004). Food *X. americana* flowers are a replacement for orange blossoms with similar fragrance and soothing cosmetic properties (Paolo, 1979).

$$CH_3(CH_2)_5CH\overset{E}{=}C\equiv C(CH_2)_7CO_2H$$

3. Others constituents isolated from *X. americana*

Besides the substances mentioned in the text of this chapter, several other originated from *Ximenia americana* were isolated.

Isoprenoids



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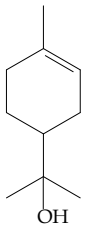

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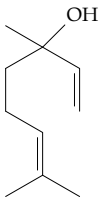

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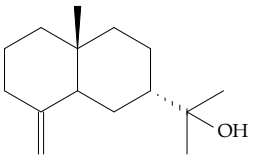

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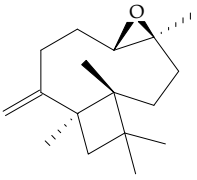

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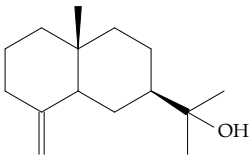
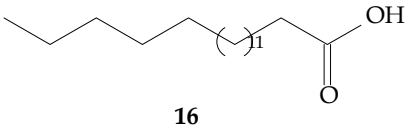


15

Fatty acids



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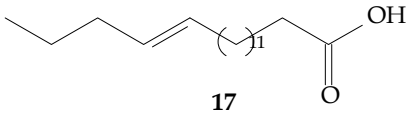
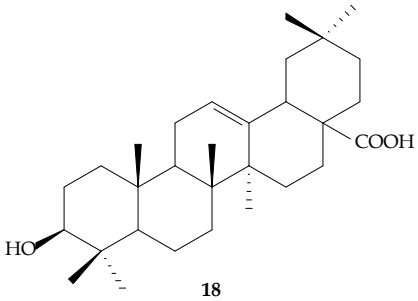


17

Triterpenes



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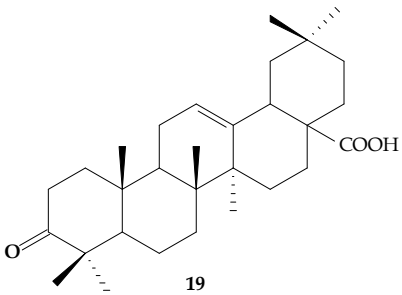

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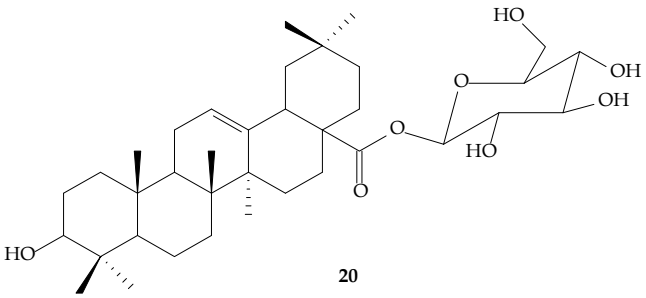
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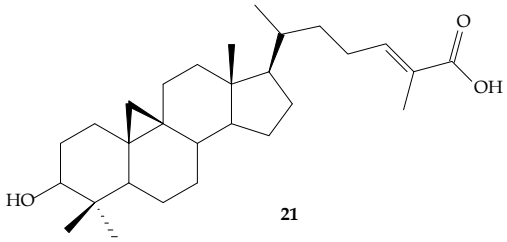
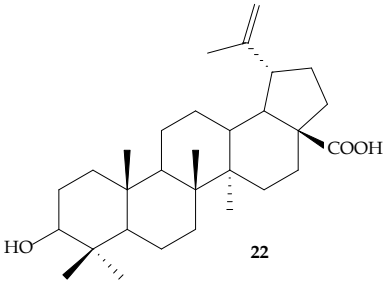
OH
O
OH
O
HO
O
HO

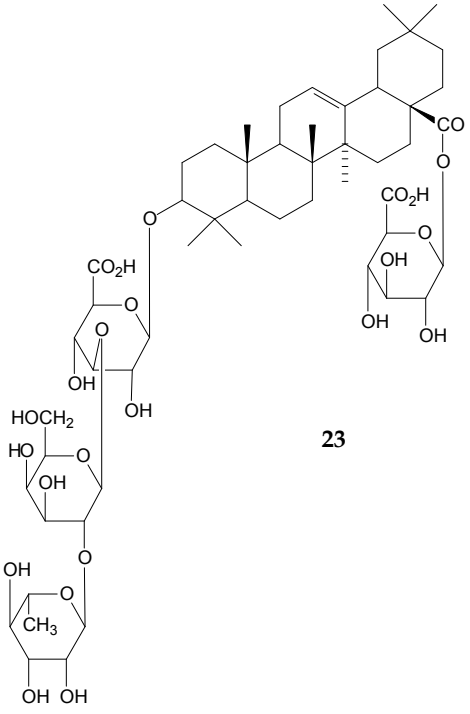
20


O
OH
HO

21

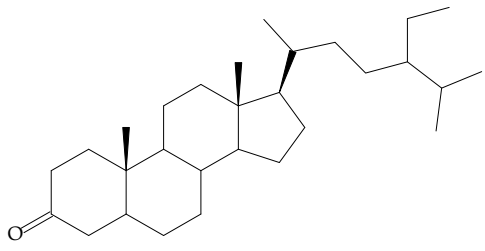

COOH
HO

22


CO
O
O
CO2H
O
OH
OH
OH
CO2H
O
OH
OH
OH
HOCH2
HO
O
OH
O
OH
O
CH3
OH OH

23

Steroids



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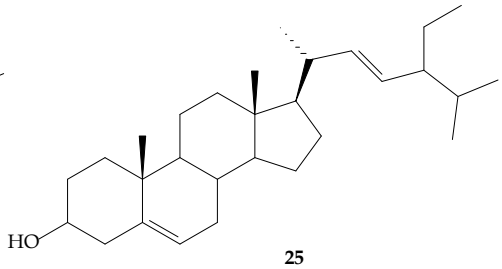

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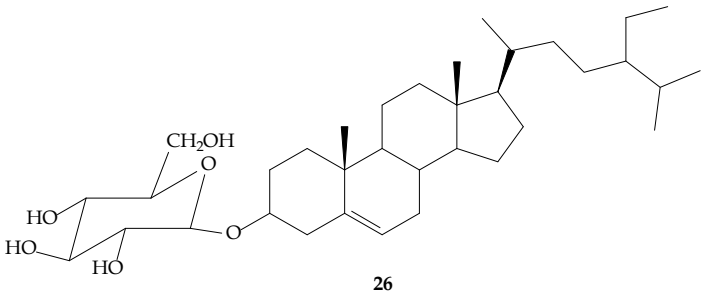

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4. Summary/conclusion/future directions

From an extensive literature review was observed that the *Ximenia americana* is widely used as a popular alternative remedy in certain regions of some countries of the Africa (Guinea, Ethiopia, Nigeria, Sudan) and in the Brazil. The plant, used by their crude extracts, especially, aqueous and methanolic, showed several biological activities such as antimicrobial, antifungal, anticancer, antitrypanosomal, antirheumatic, antioxidant, analgesic, moluscicide, pesticidal, antipyretic, antifugal, among others. There are several papers in the literature confirming these activities. The crude extracts consist of complex mixture of compounds called secondary metabolites produced by plants, which include, mainly, flavonoids, saponins, alkaloids, quinones, terpenoids, phenols, glycosides and sterols.

Many plants have a prolonged and uneventful use that may serve as indirect evidence to their efficacy. However, in the absence of objective proof of efficacy and without the knowledge of the constituents responsible for the physiological actions, the validity of the remedies is questionable and its use restricted. It generally was observed that the more the constituents in a given species, the more diverse the micro-organisms it acts upon. The difference of activity appears to be directly related to the qualitative and/or quantitative diversity of the compounds that are being accumulated by the plants investigated.

However, detailed studies on the toxicity of extracts revealed through phytochemical screening showed that many constituents chemicals can affect the animal positively or negatively as a result of prolong usage. Thus, was founded that tannins and anthraquinones are thought to have both proxidant and antioxidant effects on the body. While the antioxidant protects, the proxidant damage the tissues and organs. Also, was observed that the presence of tannins and other compounds interferes with absorption of nutrients such proteins and minerals resulting in weight loss. The extracts contained the presence of saponins has been reported to produce free radicals and hydrogen peroxide during its oxidation to semiquinone in the body, is thought to damage the cells of the body. The results of several studies conducted so far have produced a scientific basis that can justify the use of *Ximenia americana* in medicine. As we see the many works on *X. americana* show its effectiveness in treating various diseases. In all studies, were highlighted the participation and importance of secondary metabolites produced by them. However, there are still many details to be clarified. As mentioned above, in general, it was observed that the more the constituents in a given species, the more diverse the micro-organisms it acts upon. Moreover, the activity of plant extracts seems to be related to quality and quantity of metabolites present, possibly due to the possibility of synergism while, different types of metabolites appear to be related to specific biologic actions. In this context it is important to point out that the norisoprenoid isophorane (**10**), shown to be carcinogenic agent (Mevy *et al.*, 2006), was identified in the leaves of *X. americana*, which would conflict with its use in treating cancer. The last report about compounds isolated from *X. americana* up to date were the sesquiternes **7** and **8**, triterpenoids **18-22** and steroids **24-26,** all from ethanol extract of stems (Araújo *et al.*, 2008, 2009). Some of them have not yet been exhaustively investigated from the point of view of biological activity.

Future studies should be performed using chromatographic methods such as HPLC (high performance liquid chromatography) and LC-MS (Liquid chromatography coupled to mass spectroscopy) to obtain the chromatographic profile of the chemical composition of the extracts. Then carry out guided study (biological activity) in order to isolate and identify the pure constituents. Finally, as reported, many compounds may exhibit both carcinogenic and anticarcinogenic effects but it is not excluded that the occurrence of compounds other than volatile constituents may act in the anticarcinogenic process. Consequently, these results encourage further investigations to extracts and identify the active chemical compounds responsible for the specific biological activity in order to standardized the plant preparation for maximum therapeutic benefit.

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21

Phytochemicals and Their Pharmacological Aspects of *Acanthopanax koreanum*

Young Ho Kim, Jeong Ah Kim and Nguyen Xuan Nhiem
Chungnam National University,
South Korea

1. Introduction

Botanical medicines have been applied for the treatment of various human diseases with thousands of years of history all over the world. In some Asian and African countries, 80 % of population depends on traditional medicine in primary health care. On the other hand, in many developed countries, 70 % to 80 % of the population has used some forms of alternative or complementary medicine. The long tradition of using plants for medicine, supplemented by pharmaceutical research, has resulted in many plant-based Western medicines. Traditional medicine has provided Western medicine with over 40 % of all pharmaceuticals (Samuelsson & Bohlin, 2004). In the past decades, therefore, research has been focused on scientific evaluation of traditional drugs of plant origin.

Acanthopanax species (Araliaceae) are widely distributed in Asia, Malaysia, Polynesia, Europe, North Africa and the America. There are about 40 species of *Acanthopanax* to be found in over the world. *Acanthopanax* species have traditionally been used as a tonic and sedative as well as in the treatment of rheumatism, and diabetes. *A. koreanum* Nakai is an indigenous plant prevalently distributed throughout South Korea. It is deciduous shrub with upright to slightly arching stems, small, fresh green, trilobed to palmately divided leaves and several axillary as well as terminal round clusters of decorative, bluish black berries in late summer and autumn. Extensive investigation of chemical components in *A. koreanum* has been reported by many researchers in the worldwide. Several types of compounds have been isolated from this plant. Major active constituents are reported as lupanes and their glycosides, diterpenes, monoterpenes, lignans, phenylpropanoids, flavonoids from whole parts of *A. koreanum.* Of these, lupane triterpenes were reported as major components of leaves and *ent*-kauranes are main components of the roots of *A. koreanum.* They showed significant biological effects by several bioassay systems such as 1) anti-inflammatory activities: inhibit lipopolysaccharide (LPS)-stimulated TNF-α, IL-6, and IL-12 p40 productions in bone marrow-derived dendritic cells, decrease the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) proteins, and reduce iNOS and COX-2 mRNA in a dose-dependent pattern, 2) anticancer, and 3) anti-osteoporosis by effects on the differentiation of osteoblastic MC3T3-E1 cells. The desired target of this chapter is to introduce explanations of structures and pharmacological activities of novel compounds, which have been isolated and identified from *A. koreanum* since 1985. Those studies have reported and focused on bioactivities of unambiguous

compounds from *A. koreanum*, therefore we discuss new pharmacological findings on these compounds.

The depth and breadth of research involving this plant has been organized into easily accessible and comparable information. Using Chemical Abstracts, Scifinder Scholar, and BIOSIS databases, relevant research papers were selected by based on pertinence and specificity to ethnopharmacology and phytochemistry, as well as readability. This collection was then carefully reviewed, extracted, and corroborated with available characterization data from other sources.

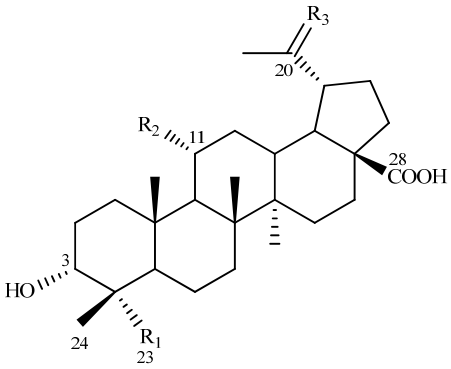
2. Phytochemistry and pharmacology of *A. koreanum*

2.1 Lupane aglycones

Impressic acid (**1**) was isolated for the first time from *Schefflera impressa* by (Srivastava, 1992) and it was found in the roots (Cai et al., 2004b) and the leaves (Kim et al., 2010) of *A. koreaum*. Impressic acid exhibited potently NFAT inhibitory activity with IC_{50} value of 12.6 μM. In the studies of (Kim et al., 2010), impressic acid (**1**) and (20*R*)-3α-hydroxy-29-dimethoxylupan-23,28-dioic acid (**4**) showed significantly anti-inflammatory activities by inhibiting TNF-α, IL-6, and IL-12 p40 productions in bone marrow-derived dendritic cells with LPS-stimulated. Furthermore, impressic acid was found to inhibit TNF-α-induced NF-κB activity by inhibiting the induction of COX-2 and iNOS in HepG2 cells. Impressic acid also up-regulated the transcriptional activity of PPAR by elevating the expression of PPARγ1, PPARγ2, and SREBF-2, and by suppressing the expression of Insig-2 (Kim et al., 2011). One new norlupane, 3α,11α-dihydroxy-20,23-dioxo-30-norlupane-28-oic acid (**2**) as well as two known lupane aglycones, impressic acid (**1**), 3α,11α-dihydroxy-lup-20(29)-en-23-al-28-oic acid (**3**) were isolated and determined by (Park et al., 2010). They were evaluated for the differentiation of osteoblastic MC3T3-E1 cells. Among of them, compound **1** significantly increased osteoblastic cell growth and differentiation as assessed by MTT assay and collagen content. Compound **2** significantly increased the growth of MC3T3-E1 cells and caused a significant elevation of osteoblastic cell differentiation as assessed by the alkaline phosphatase activity (Park et al., 2010). Other compounds, 3α,11α-dihydroxy-lup-20(29)-en-23-al-28-oic acid, 3α-hydroxylup-20(29)-en-23,28-dioic acid (**5**), and 3α,11α,23-trihydroxy-lup-20(29)-en-28-oic acid (**6**) were also isolated from steamed leaves (Kim et al., 2010). However, they showed weak anti-inflammatory activity. 3α-Hydroxylup-20(29)-en-23,28-dioic acid (**5**) possessed broader antiviral activity against respiratory syncytial, influenza (H1N1), coxsackie B3, and herpes simplex virus type 1 viruses with IC_{50} values of 6.2, 25.0, 12.5, and 18.8 μg/mL, respectively (Li et al., 2007).

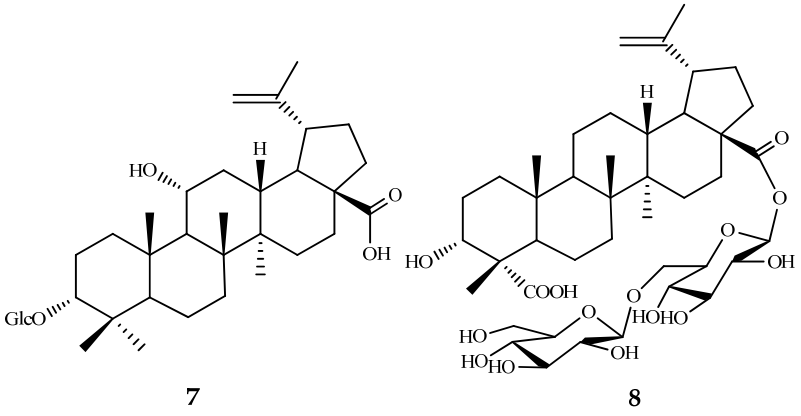
2.2 Lupane-triterpene glycosides

Up to date, eighteen lupane-type triterpene glycosides have been isolated from this plant and almost of them from the leaves of *A. koreanum.* They are main saponin components of the leaves of *A. koreanum.* The first lupane triterpene glycoside, acantrifoside A (**1**) was isolated from both *A. koreanum* and *A. trifoliatus* in a year of 1998 by (Yook et al., 1998). And then, two new saponins, acankoreoside A (**10**) and acankoreoside B (**11**) were isolated from the leaves of this plant (Chang et al., 1998). Our group reported seven new lupane-type triterpene glycosides, acankoreosides I-O. Their biological activities were evaluated for



Name	Parts	R_1	R_2	R_3	Reference
Impressic acid (**1**)	leaves roots	CH_3	OH	CH_2	(Cai et al., 2004b)
3α,11α-Dihydroxy-20,23-dioxo-30-norlupane-28-oic acid (**2**)	leaves	CHO	OH	=O	(Park et al., 2010)
3α,11α-Dihydroxy-lup-20(29)-en-23-al-28-oic acid (**3**)	leaves	CHO	OH	CH_2	(Park et al., 2010)
(20R)-3α-Hydroxy-29-dimethoxylupan-23,28-dioic acid (**4**)	steamed leaves	COOH	H	$CH(OCH_3)_2$	(Kim et al., 2010)
3α-Hydroxylup-20(29)-en-23,28-dioic acid (**5**)	steamed leaves	COOH	H	CH_2	(Kim et al., 2010)
3α,11α,23-Trihydroxy-lup-20(29)-en-28-oic acid (**6**)	steamed leaves	CH_2OH	OH	CH_2	(Kim et al., 2010)

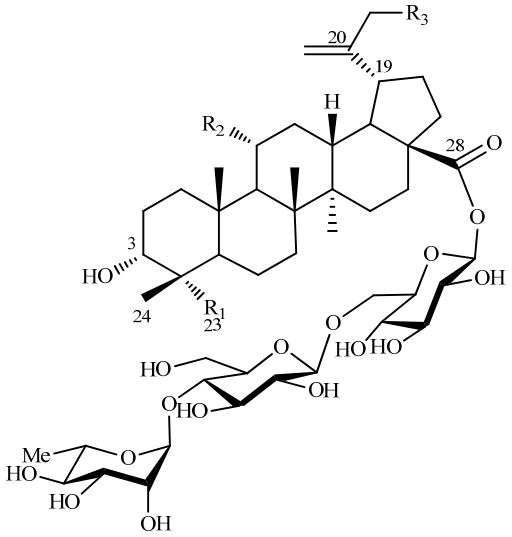
Fig. 1. Structures of main lupane-type triterpenes isolated from *Acanthopanax koreanum*



7 **8**

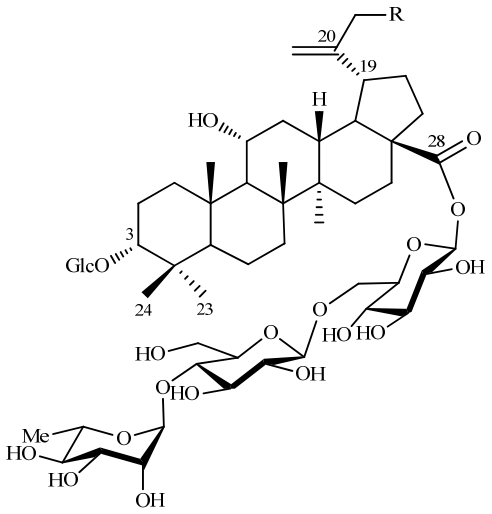
Fig. 2. 3-*O*-β-D-glucopyranosyl 3α,11α-dihydroxylup-20(29)-en-28-oic acid (**7**) and 3α-hydroxylup-20(29)-en-23,28-oic acid 28-*O*-[β-D-Glucopyranosyl-(1→6)-β-D-Glucopyranosyl] ester (**8**) (Kim et al., 2010)

cytotoxic activities including A549 (lung), HL-60 (acute promyelocytic leukemia), MCF-7 (breast), U937 (leukemia) cancer cell lines; immune enhancement activity (INF-γ and IL-2 release in spleen cells); anti-inflammatory (inhibitory TNF-α, IL-6, and IL-12 p40 productions in bone marrow-derived dendritic cells with LPS-stimulated, and RAW 264.7). Searching for anticancer activities from natural compounds, several acankoreosides showed significantly cytotoxic activities in various cancer cell lines (A549, HL-60, MCF-7, and U937). The effects of three new lupane glycosides, acankoreosides F-H (**13**, **14**, and **21**) on the LPS-induced production of nitric oxide and prostaglandin E_2 were evaluated in RAW 264.7 macrophages. Among of them, acankoreoside F (**13**) showed the most potent inhibitory PGE_2 (59.0 %) and NO (42.0 %) production at concentration of 200.0 μM. Furthermore, eleven lupane triterpene glycosides from *A. koreanum,* including three new compounds acankoreoside M-O (**16**, **24**, and **25**) were evaluated for Con A-induced splenolytic production of IL-2 and IFN-γ. The results indicated that acankoreosides A (**10**), D (**12**), L (**24**), and acantrifoside A (**9**)

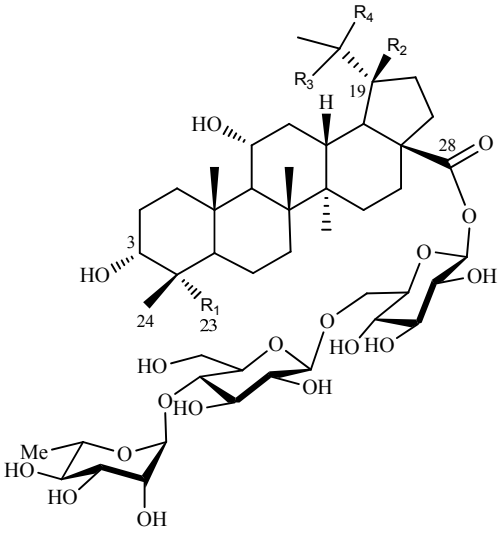


Names	Parts	R_1	R_2	R_3	Ref.
Acantrifoside A (**9**)	leaves	CH_3	OH	H	(Yook et al., 1998)
Acankoreoside A (**10**)	leaves, roots	COOH	H	H	(Chang et al., 1998) (Cai et al., 2004b)
Acankoreoside B (**11**)	leaves	CH_2OH	OH	H	(Chang et al., 1998)
Acankoreoside D (**12**)	leaves	CHO	OH	H	(Chang et al., 1999)
Acankoreoside F (**13**)	leaves	COOH	H	OH	(Choi et al., 2008)
Acankoreoside G (**14**)	leaves	CHO	H	OH	(Choi et al., 2008)
Acankoreoside I (**15**)	leaves	CHO	OH	OH	(Nhiem et al., 2009)
Acankoreoside M (**16**)	leaves	COOH	OH	OH	(Nhiem et al., 2010b)
3-Epibetulinic acid 28-*O*-glc-glc-rha (**17**)	leaves, roots	CH_3	H	H	(Cai et al., 2004b)

Fig. 3. Structures of lupane-type triterpene glycosides from *A. koreanum*



Name	Part	R	Reference
Acankoreoside C (**18**)	leaves	H	(Chang et al., 1999)
Acankoreoside N (**19**)	leaves	OH	(Nhiem et al., 2010b)



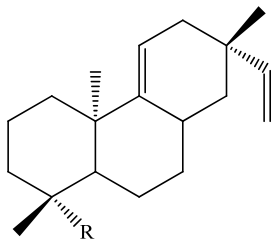
Name	Part	R_1	R_2	R_3	R_4	Reference
Acankoreoside E (**20**)	leaves	COOH	H	H	CHO	(Park et al., 2005)
Acankoreoside H (**21**)	leaves	CHO	H	H	COOH	(Choi et al., 2008)
Acankoreoside J (**22**)	leaves	COOH	H	=O	-	(Nhiem et al., 2010a)
Acankoreoside K (**23**)	leaves	COOH	H	OH	Me	(Nhiem et al., 2010a)
Acankoreoside L (**24**)	leaves	COOH	H	OH	CH_2OH	(Nhiem et al., 2010a)
Acankoreoside O (**25**)	leaves	COOH	OH	H	CH_3	(Nhiem et al., 2010b)

Fig. 3. Structures of lupane-type triterpene glycosides from *A. koreanum* (continued)

significantly increased both IL-2 and IFN-γ. The structure-activity relationship of these compounds was also discussed. Moreover, lupane aglycones and lupane glycosides were assayed for LPS-stimulated pro-inflammatory cytokine production. These results suggested lupane aglycone inhibited pro-inflammatory cytokine production stronger than lupane glycosides (Kim et al., 2010). This was further confirmed by the study of (Cai et al., 2004b).

2.3 Pimarane-type diterpenes

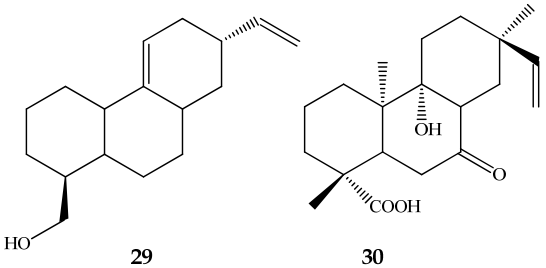
A number of pimarane-type diterpenes have been isolated and associated with significant biological activity. There are seven pimarane-type diterpenes from *A. koreanum*. All of them were isolated from the roots. Acanthoic acid was presented in roots and leaves of this plant, and was one of compounds having potent anti-inflammatory activity. Acanthoic acid, a pimarane diterpene ((-)-pimara-9(11),15-dien-19-oic acid), was isolated for the first time from *A. koreanum* in a year of 1988 by (Kim et al., 1988b) and was proved with high content of this plant. Acanthoic acid has widely exhibited of biological activities. In study of (Kang et al., 1996), acanthoic acid has potent anti-inflammatory effects by reducing the production of proinflammatory cytokines such as IL-1 and TNF-α. It was also effective in supressing experimental silicosis and cirrhosis. Furthermore, acanthoic acid was found to suppress TNF-α gene expression (Kang et al., 1998) and TNF-α-induced IL-8 production in a dose-dependent manner. Acanthoic acid also inhibited TNF-α-induced MAPKs activation, IκB degradation, NF-κB nuclear translocation, and NF-κB/DNA binding activity (Kim et al., 2004). Furthermore, acanthoic acid significantly inhibited production of both TNF-α and tryptase in trypsin-stimulated human leukemic mast cell-1 at concentrations of 10 and 100 μg/mL with a dose-dependent manner. Acanthoic acid inhibited ERK phosphorylation and NF-κB activation induced by trypsin treatment without blocking of trypsin activity even though 100 μg/mL. These results suggested that acanthoic acid may inhibit the production of inflammatory mediators through inhibition of ERK phosphorylation and NF-κB activation pathway in human mast cells (Kang et al., 2006).



Name	Part	R	Reference
(-)-Pimara-9(11),15-dien-19-ol (**26**)	root barks	CH_2OH	(Kim et al., 1988b)
Acanthoic acid (**27**)	roots, leaves	COOH	(Kim et al., 1988b)
(-)-Pimara-9(11),15-dien-19-ol 19-acetate (**28**)	root barks	CH_2OAc	(Kim et al., 1988b)
(-)-Pimara-9(11),15-diene (**29**)	root barks	CH_3	(Kim et al., 1988b)

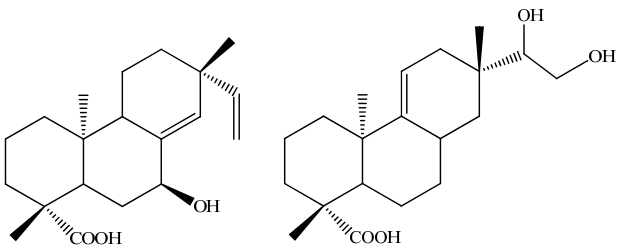
Fig. 4. Structures of pimarane-type diterpenes from *A. koreanum*

The hepatoprotective effects of acanthoic acid were evaluated in a D-galactosamine/ lipopolysaccharide-induced fulminant hepatic failure mouse model. The effects were likely associated with a significant decrease in serum TNF-α levels, which are correlated not only with those of alanine aminotransferase and aspartate aminotransferase but also with the reduced number of apoptotic hepatocytes in the liver as confirmed using the terminal deoxynucleotidyl transferase-mediated (dUTP) nick end-labeling method and DNA fragmentation assay (Nan et al., 2008). The protective effect of acanthoic acid was investigated in acetaminophen-induced hepatic toxicity. These results indicated that acanthoic acid protected liver tissue from oxidative stress elicites by acetaminophen-induced liver damage (Wu et al., 2010). Acanthoic acid markedly suppressed the protein expression of TNF-α, COX-2, NF-κB and chymase as well as the mRNA expression of TNF-α and COX-2 (Kang et al., 2010).



29 **30**

Isopimara-9(11),15-dien-19-ol (**29**)	roots	(Chung & Kim, 1986)
Acanthokoreoic acid A (**30**)	roots	(Cai et al., 2003a)



31 **32**

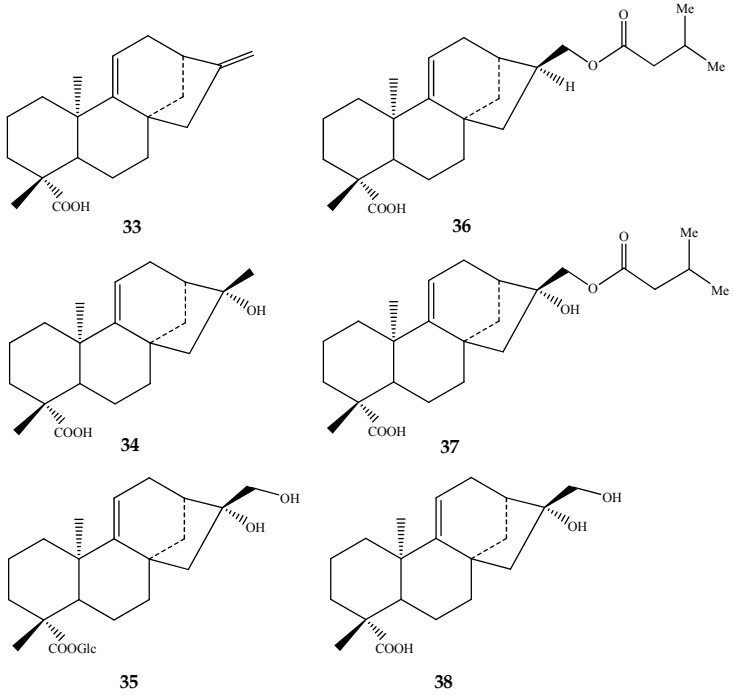
7β-Hydroxy-*ent*-pimara-8(14),15-dien-19-oic acid (**31**)	roots	(Cai et al., 2004a)
Sumogaside (**32**)	roots	(Cai et al., 2004a)

Fig. 4. Structures of pimarane-type diterpenes from *A. koreanum* (continued)

In study of (Cai et al., 2003a), a new compound, acanthokoreoside acid A (**30**) as well as acanthoic acid (**27**), (-)-pimara-9(11),15-dien-19-ol (**26**), and sumogaside (**32**) were isolated from CH_2Cl_2 fraction of *A. koreanum* roots. They were evaluated for inhibitory activity on IL-8 secretion in TNF-α-stimulated HT-29 and TNF-α secretion in trypsin-stimulated HMC-1. In the TNF- α-stimulated HT-29, acanthoic acid and sumogaside significantly inhibited IL-8 secretion at concentrations of 1, 10, and 100 μM and at concentrations of 10 and 100 μM, respectively.

2.4 *ent*-Kaurane-type diterpenes

ent-Kaurane, a tetracyclic diterpene, has been proven to excert various biological activities like cytotoxicity, anti-inflammation, and so on. From the roots of *A. koreanum*, (Kim et al., 1988b) and (Cai et al., 2003b) isolated six *ent*-kaurane-type diterpenes, including *ent*-kaur-16-en-19-oic acid (**33**), 16α-hydroxy-*ent*-kauran-19-oic acid (**34**), paniculoside IV (**35**), 16αH,17-isovaleryloxy-*ent*-kauran-19-oic acid (**36**), 16α-hydroxy-17-isovaleryloxy-*ent*-kauran-19-oic acid (**37**), and 16α,17-dihydroxy-*ent*-kauran-19-oic acid (**38**). (Cai et al., 2003b) evaluated five *ent*-kauranes for TNF-α secretion from HMC-1, a trypsin-stimulated human leukemic mast cell line. The results indicated that 16αH,17-isovaleryloxy-*ent*-kauran-19-oic acid (**36**) exhibited potently an inhibitory activity with IC_{50} value of 16.2 μM. Furthermore, these compounds were assayed for their inhibitory effect against NFAR transcription factor and 16α-hydroxy-17-isovaleryloxy-*ent*-kauran-19-oic acid (**37**) was found to significantly inhibit NFAT transcription factor with IC_{50} of 6.7 μM (Cai et al., 2004a). The authors also found that remain compounds containing a hydroxyl group at C-16 or a glycoside at C-4 showed no activity.



Name	Part	Reference
ent-Kaur-16-en-19-oic acid (**33**)	roots	(Kim et al., 1988b)
16α-Hydroxy-ent-kauran-19-oic acid (**34**)	roots	(Cai et al., 2003b)
Paniculoside IV (**35**)	roots	(Cai et al., 2003b)
16αH,17-Isovaleryloxy-*ent*-kauran-19-oic acid (**36**)	roots	(Cai et al., 2003b)
16α-Hydroxy-17-isovaleryloxy-*ent*-kauran-19-oic acid (**37**)	roots	(Cai et al., 2003b)
16α,17-Dihydroxy-*ent*-kauran-19-oic acid (**38**)	roots	(Kim et al., 1988b)

Fig. 5. Structures of *ent*-kaurane-type diterpenes from *A. koreanum*

2.5 Other class compounds

Two lignans were found from the roots of *A. koreanum*. Those were acanthoside D (**39**) (Hahn et al., 1985) and ariensin (**40**) (Kim et al., 1988a). Beside these lignans, the first

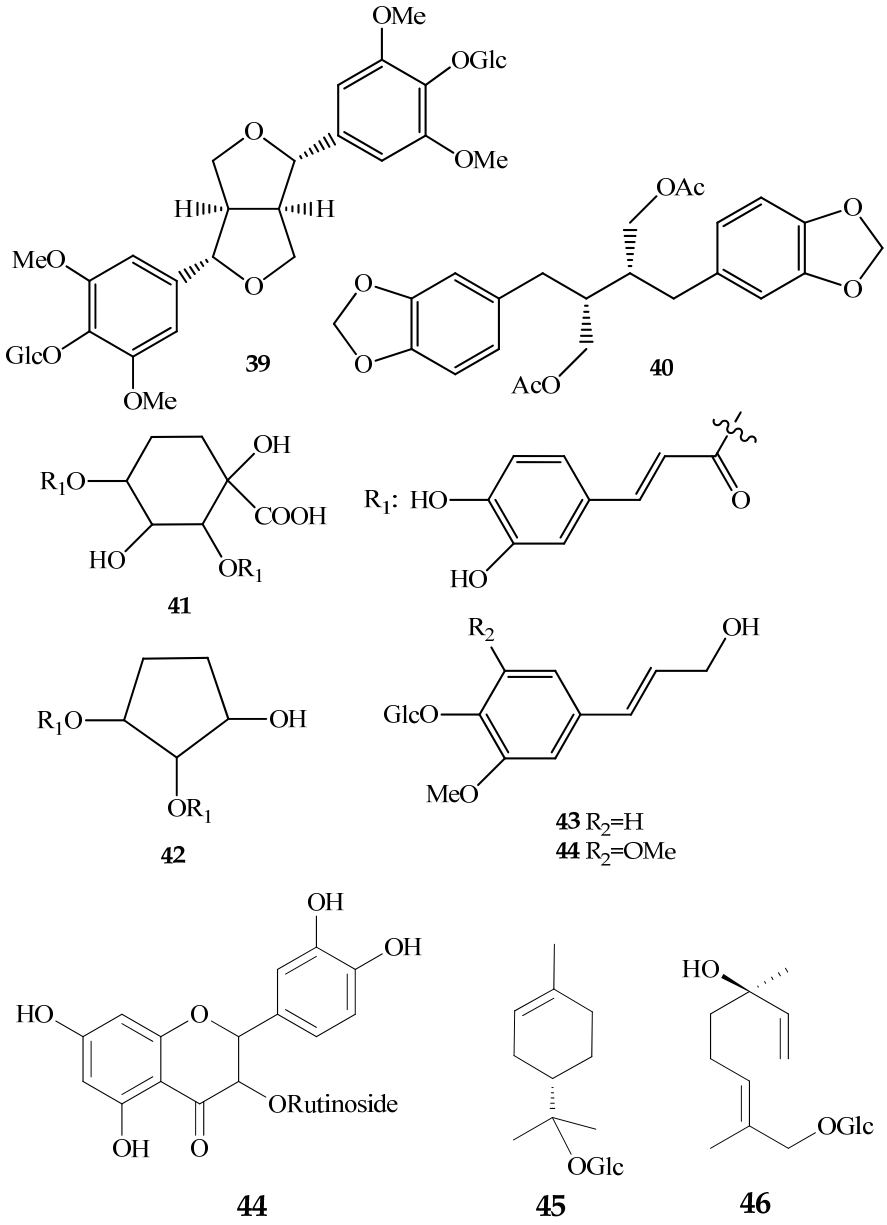


Fig. 6. Structures of compounds isolated from *A. Koreanum*

Pos.	1	2	3	4	5	6	7	8	9	10	11	12
1	35.8	35.7	35.1	32.8	32.6	35.9	36.2	33.1	36.2	33.1	35.9	34.9
2	26.8	26.9	25.5	26.0	25.9	27.1	21.9	26.2	26.9	26.2	27.1	26.8
3	74.8	73.7	75.8	72.8	72.7	75.9	81.5	71.5	75.3	73.0	75.7	72.7
4	38.4	53.5	37.7	51.8	51.7	41.2	37.9	51.9	38.5	52.8	41.1	52.6
5	49.4	44.4	48.8	44.7	44.8	43.9	50.6	45.3	49.6	45.0	43.8	43.9
6	19.2	21.8	19.3	21.6	21.0	18.3	18.4	21.7	18.6	21.8	18.3	21.0
7	36.0	35.7	35.4	23.8	34.5	35.6	35.7	34.6	35.7	34.6	35.4	35.2
8	42.7	43.2	42.2	41.6	41.5	42.8	42.7	41.8	42.8	42.8	42.7	42.4
9	55.9	56.2	55.6	50.7	50.8	56.2	55.9	51.0	56.2	51.2	55.6	55.6
10	39.8	39.6	39.1	38.2	37.2	39.6	39.7	37.4	39.9	37.6	39.6	38.7
11	69.8	70.7	70.4	21.1	21.5	69.9	69.9	20.9	69.8	21.2	69.8	69.4
12	38.4	39.4	37.7	27.7	25.8	38.4	38.3	26.0	38.3	26.0	38.3	37.8
13	37.5	37.5	37.2	28.6	38.4	37.6	37.7	38.4	37.4	38.4	37.4	37.0
14	42.9	44.1	42.6	43.0	42.7	42.8	42.9	42.9	43.0	43.0	42.9	43.0
15	29.9	30.6	29.5	30.2	30.0	30.1	30.2	30.1	30.0	30.1	30.0	29.6
16	32.8	32.7	32.0	34.7	32.8	32.8	32.9	32.2	32.3	32.4	32.2	31.9
17	56.5	57.1	56.2	56.7	56.4	56.5	56.6	57.0	56.9	57.7	56.9	56.6
18	47.5	50.0	46.6	49.1	49.5	49.4	49.4	49.8	49.5	49.7	49.4	49.1
19	49.4	52.5	48.6	43.7	47.5	47.5	47.5	47.4	47.2	47.5	47.1	46.8
20	151.0	215.0	149.7	37.6	151.1	150.8	150.9	150.9	150.4	151.0	150.4	150.1
21	31.0	29.4	30.5	24.6	30.9	31.2	31.3	30.8	30.9	30.9	30.9	30.6
22	37.5	37.7	36.8	37.2	37.3	37.4	37.4	36.9	36.8	37.2	36.7	36.4
23	29.6	210.9	28.7	178.9	179.6	71.9	29.9	181.3	29.8	179.0	71.9	209.7
24	22.7	15.1	22.2	17.8	17.7	18.3	23.0	18.1	22.9	18.0	18.3	14.6
25	18.0	17.2	17.3	16.6	16.5	17.0	16.8	16.8	16.9	16.8	17.1	16.5
26	17.4	17.8	16.2	16.6	16.5	17.7	17.6	16.7	17.7	16.6	17.7	17.4
27	14.4	15.0	14.6	14.5	14.6	14.8	14.8	14.8	14.8	14.8	14.8	14.4
28	179.2	179.4	181.5	179.4	178.6	178.8	178.9	175.0	175.0	175.9	175.0	174.6
29	110.0	30.0	110.2	107.8	109.7	110.0	110.1	110.0	110.2	110.0	110.0	109.9
30	19.2		17.9	16.4	19.2	19.5	19.6	19.4	19.5	19.3	19.5	19.2
Solv.	*a*	*a*	*b*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*

[a] recorded in pyridine-d_5, [b] recorded in methanol-d_4.
Note: NMR data were obtained from **1:** (Srivastava, 1992); **2**, **3** (Park et al., 2010); **4**, **8**: (Kim et al., 2010); **5**, **6**, **10**, **11**: (Chang et al., 1998); **7**, **12:** (Chang et al., 1999)

Table 1. ^{13}C-NMR data of lupane aglycone moieties

Pos.	13	14	15	16	17	18	19	20	21	22	23	24	25
1	33.2	33.1	35.9	35.5	36.5	36.1	36.7	33.3	33.1	34.0	34.2	33.9	34.5
2	26.1	26.7	27.0	26.4	26.6	21.8	19.2	26.1	26.7	26.1	26.1	26.1	26.1
3	72.8	73.0	73.8	73.8	73.5	81.3	82.4	72.7	73.0	73.4	73.3	73.6	73.3
4	51.8	52.5	53.7	53.0	40.3	37.8	38.5	51.7	52.5	52.2	52.1	52.3	52.1
5	45.4	44.0	44.5	45.4	49.9	50.5	51.0	45.6	44.0	46.6	46.9	46.2	47.4
6	21.7	20.9	22.0	22.6	19.2	18.4	22.2	21.7	21.1	22.2	22.2	22.3	22.2
7	34.5	34.1	36.2	36.1	36.3	35.4	36.4	34.6	34.1	35.1	35.5	35.3	35.5
8	41.8	41.8	41.6	43.5	43.4	42.6	43.5	41.7	41.8	42.5	43.0	42.6	43.1
9	51.0	50.6	56.3	56.7	50.2	55.8	56.3	50.6	50.2	51.9	52.0	51.7	52.0
10	37.4	36.9	38.2	40.0	38.3	39.6	40.4	37.4	36.9	38.2	38.1	38.1	38.2
11	21.0	21.0	71.1	71.1	24.2	69.7	71.2	20.9	20.8	22.0	22.4	22.0	22.2
12	27.1	27.0	38.3	39.4	26.5	38.1	39.5	26.9	26.9	28.5	29.8	28.3	26.1
13	38.3	38.3	39.5	38.2	38.2	37.3	38.3	38.2	38.2	38.6	39.5	39.4	39.1
14	42.8	42.8	44.3	44.3	43.8	42.9	44.0	43.0	43.0	43.6	44.5	44.0	44.3
15	30.2	30.1	30.8	30.8	31.6	30.0	30.9	30.0	30.0	30.9	31.2	30.9	30.9
16	32.2	32.1	32.8	32.9	32.8	32.2	32.9	32.0	32.1	32.4	33.1	32.9	34.5
17	57.0	56.9	57.9	57.9	57.9	56.8	58.0	57.0	57.3	58.0	60.1	58.0	59.4
18	50.2	50.2	50.9	50.9	50.1	49.4	51.0	48.5	48.9	50.8	49.5	49.7	50.4
19	43.2	43.2	43.7	43.7	48.2	47.1	43.8	37.3	40.6	52.8	45.7	39.3	85.4
20	156.5	156.5	155.9	155.9	151.3	150.4	156.0	50.1	42.1	215.4	76.1	39.5	36.8
21	32.7	32.7	33.5	33.6	30.6	30.8	33.6	24.6	25.0	29.3	28.8	24.38	34.5
22	36.8	36.7	37.3	37.4	37.5	36.7	37.4	37.4	37.3	37.6	37.5	38.3	37.2
23	181.2	209.8	211.2	180.6	29.5	29.8	29.8	181.8	209.9	183.4	184.2	181.8	185.2
24	18.2	14.6	15.2	18.2	23.0	23.0	23.3	18.3	14.6	18.3	18.4	18.2	18.6
25	16.8	16.4	17.2	17.5	17.8	16.8	17.3	16.8	16.3	17.2	17.4	17.2	17.4
26	16.7	16.5	18.3	18.1	17.0	17.6	18.0	16.6	16.5	16.9	17.2	17.0	17.1
27	14.9	14.8	15.1	15.1	14.9	14.7	15.2	14.7	14.9	15.2	15.6	15.1	15.5
28	175.1	175.0	176.3	176.3	176.2	174.8	176.4	175.0	174.9	176.2	177.0	176.4	176.7
29	106.1	106.1	107.7	107.7	110.8	110.1	107.6	204.6	180.0	29.9	71.2	8.9	21.9
30	64.3	64.3	65.5	65.5	19.6	19.6	65.6	7.0	10.0		19.6	110.3	27.0
Solv.	*a*	*a*	*b*	*b*	*b*	*a*	*b*	*a*	*a*	*b*	*b*	*b*	*b*

[a] recorded in pyridine-d_5, [b] recorded in methanol-d_4.
Note: NMR data were obtained from **13**, **14**, **21**: (Choi et al., 2008); **15**: (Nhiem et al., 2009) (Cai et al., 2004b), **16**, **19**, **25**: (Nhiem et al., 2010b); **17**: (Cai et al., 2004b); **18**: (Chang et al., 1999), **20**: (Park et al., 2005); **21**: (Choi et al., 2008); **22**, **23**, **24**: (Nhiem et al., 2010a).

Table 1. ^{13}C-NMR data of lupane aglycone moieties (continued)

phenylpropanoid, syrinoside (**44**) were isolated from the roots of *A. koreanum* by (Hahn et al., 1985) and then was ariensin (**43**) (Kim et al., 1988a). In study antioxidant activity of chemical components from the leaves of this plant, (Nhiem et al., 2011) isolated one new phenylpropanoid named acanthopanic acid and one known 1,2-O-dicaffeoylcyclopenta-3-ol. These compounds showed significantly antioxidant activity by the intracellular ROS radical scavenging DCF-DA assay with IC_{50} values of 3.8 and 2.9 μM, respectively. Until now, only rutin (**45**), a quercetin glycoside was isolated from this plant with large amount. Rutin is used in many countries as medication for blood vessel protection and are ingredients of numerous multivitamin preparations and herbal remedies. Rutin has various biological activities that are beneficial to human health such as antioxidant effect (Nhiem et al., 2011), protective effect against hepatotoxicity, and anti-inflammatory effect. On the other hand, from the leaves of *A. koreanum*, two monoterpenoids, 4S)-α-terpineol O-β-D glucopyranoside (**46**) (Nhiem et al., 2011) and betulabuside B (**47**) (Park et al., 2010) were isolated. From fruits, citric, maleic succinic, malonic, furmaric, and malic acid were isolated (Shin & Kim, 1985).

3. NMR data of lupane aglycones

Lupane triterpenes are a class of the most compounds isolated from the leaves and roots of *A. koreanum*, which were determined that this type of compounds are main chemical components of this plant.

Structure of lupanes were elucidated with ^{1}H-NMR, ^{13}C-NMR, DEPT (distortionless enhancement by polarization transfer), COSY (^{1}H-^{1}H shift correlation spectroscopy), TOCSY (total correlation spectroscopy), HMBC (heteronuclear multiple bond correlation), HSQC (heteronuclear single quantum coherence), NOESY (nuclear overhauser enhancement spectroscopy, and ROESY (rotating frame overhause effect spectroscopy). Proton coupling networks of sugar moieties were indicated with ^{1}H-NMR, COSY, HMBC and HSQC. Herein, we suggest statistical results of ^{13}C-NMR data of lupane-type triterpene aglycones and their derivatives in comparison with data of references (Table 1).

Observed the isolated compounds from *A. koreanum,* we found that there are four main classes including lupane triterpenoids, pimarane diterpenoids, *ent*-kaurane diterpenoids, and lignans. Among of them, lupane triterpenes were isolated as numerous of compounds with high yield. These lupanes often contain hydroxyl group at C-3, carboxyl at C-28. In some compounds, hydroxyl, aldehydic, carboxylic groups were at C-11, C-23, and C-30, glycoside was at C-28 and rarely at C-3.

From Table 1, we summarized all ^{13}C-NMR characteristics of lupane aglycones as follows:

1. When hydroxyl group at C-3, chemical shift of C-3 was about 73.0 ppm and configuration of hydroxyl group at C-3 is α orientation. When glycosidation is at C-3, chemical shift of C-3 moved to down field with δ_C of 81.0 ppm.
2. Free carboxylic group at C-28 were confirmed by chemical shift about 178.0~180.0 ppm. When sugar moiety was at C-28, chemical shift of C-28 is 174.6~176.3 ppm, decreased about 2.5-3.8 ppm.

3. When 23-methyl group was replaced with aldehydic group, chemical shifts of C-23 and C-4 moved to down field from 28.0-28.8 to 209.0-210.0, 37.5-39.5 to 54.9-56.3 ppm, respectively. When 23-methy group was replaced with carboxylic group, chemical shifts of C-23 and C-4 changed from 28.5 to 178.0, 37.8 to 53.0 respectively, and when 23-methyl group was replaced with CH_2OH, chemical shift of C-23 had a large change from 28.0 to 71.5 ppm; chemical shift of C-4 had small change about 2.0ppm.
4. When 30-methyl group was replaced with CH_2OH, the chemical shifts of C-20 and C-30 downshifted from 151.0 to 156.5, from 19.5 to 64.5 ppm, respectively; chemical shifts of C-19 and C-29 upshifted from 47.5 to 43.0, from 110.0 to 106.0 ppm, respectively.
5. When hydroxyl group was at C-11, chemical shift of C-11 downshifted from 21.1 to 71.0 ppm. Furthermore, configuration of hydroxyl group at this position is α.

4. Conclusion

This chapter is intended to serve as a reference tool for people in all fields of ethnopharmacology and natural products chemistry. The pharmacological studies on *A. koreanum* indicated the immense potential possibility of this plant in the treatment of conditions such as inflammation, rheumatism, diabetes, cardiovascular, and virus. However, the diverse pharmacological activities of solvent extracts and phytochemicals of *A. koreanum* have only been tested in *in vitro* assay using laboratory animals, and obtained too unclearly and ambiguously for the case of human beings to be conducted on enough. However, these gaps in the studies demand to be bridged in order to exploit medicinal potential of the entire plant of *A. koreanum*. It is still clear that *A. koreanum* is massively and widespreadly consumed, and also contiuously studied expecting clinical treatment of various diseases for the future in Korea as well as in the world. From these viewpoints, impressic acid and acanthoic acid, major components of *A. koreanum* are good candidates for further studies in clinical trials, and the development of products derived from *A. koreanum* can be an important part of our biodiversity to respect and sustain for coming generation.

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Polyphenol Antioxidants and Bone Health: A Review

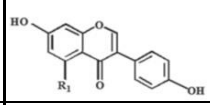
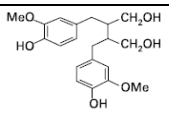
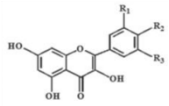
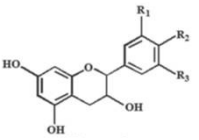
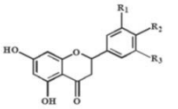
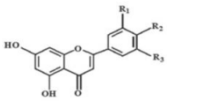
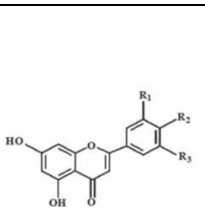
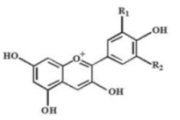
L.G. Rao[1], N. Kang[2] and A.V. Rao[2]
[1]*Department of Medicine, St. Michael's Hospital, University of Toronto,*
[2]*Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, Canada*

1. Introduction

Osteoporosis is a skeletal disease characterized by bone loss and structural deterioration of the bone tissue, leading to an increase in bone fragility and susceptibility to fractures, most frequently in the hip, wrist and spine (Sendur *et al.,* 2009). Bone loss is associated with such factors as age, menopause in women, smoking, alcohol excess, calcium and vitamin D deficiency, low weight and muscle mass, anticonvulsant and corticosteroid use as well as certain co-morbid conditions such as rheumatoid arthritis (Javaid *et al.,* 2008). Worldwide, it has been estimated that fractures caused by osteoporosis account for approximately one in three among women and approximately one in five among men over the age of 50. Although the mechanisms underlying osteoporosis are not fully understood, there is evidence suggesting that oxidative stress caused by reactive oxygen species (ROS) is associated with its pathogenesis (Sahnoun *et al.,* 1997; Basu *et al.,* 2001; Rao *et al.,* 2007).

Oxidative stress is a condition that can be characterized by an imbalance of pro-oxidants and antioxidants with the scale being tipped towards an excess of pro-oxidants, creating abnormally high concentrations of ROS. ROS are a family of highly reactive, oxygen-containing molecules and free radicals, including hydroxyl (OH ·–) and superoxide radicals (O2 ·–), hydrogen peroxide (H_2O_2), singlet oxygen, and lipid peroxides (Juránek and Bezek, 2005). Several recent studies reported the impact of oxidative stress on osteoclast differentiation as well as on its function resulting to an increase in bone resorption (Garrett *et al.,* 1990; Bax *et al.,* 1992; Mody *et al.,* 2001; Lean, 2003). Furthermore, recent *in vitro* studies have shown the important detrimental role of ROS on osteoblast activity (Park *et al.,* 2005; Bai *et al.,* 2004; Bai *et al.,* 2005). In addition to *in vitro* and animal models, there is also increasing clinical evidence that oxidative stress might be involved in the pathogenesis of osteoporosis (Melhus *et al.,* 1999; Sontakke & Tare., 2002; Basu *et al.,* 2001; Maggio *et al.,* 2003).

Antioxidants are known to mitigate the damaging effects of oxidative stress on cells. Epidemiological evidence has indicated a link between dietary intake of antioxidants and bone health. Fruits and vegetables are important sources of antioxidant phytochemicals that have been shown to play an important role in bone metabolism. Higher consumption of fruits and vegetables has been correlated with a reduction in the risk for the development of osteoporosis. (Arikan *et al.,* 2011; Prentice *et al.,* 2006; Macdonald *et al.,* 2004; Macdonald *et al.,* 2008; Palacios *et al.,* 2006; Tucker *et al.,* 1999; Lister *et al.,* 2007; New, 2003; Trzeciakiewicz *et al.,* 2009).

Category	Subclass	Structure	Common Flavonoid	Food Examples
Phenolic acids	Hydroxycinnamic acids		Caffeic acid	coffee beans
	Hydroxybenzoic acids		Gallic acid	gallnuts, sumac, witch hazel, tea leaves, oak bark,
Flavonoids	Anthocyanidins		Cyanidin	berries, purple cabbage, beets, grape seed extract, and red wine
	Flavanols		Catechins	white, green and black teas
			Theaflavins	black teas
			Proanthocyanidins	chocolate, fruits and vegetables, red wine, onion, apple skin
	Flavanones		Hesperidin	citrus fruits
			Narigenin	citrus fruits
			Silybin	blessed milk thistle
	Flavonols		Quercetin	red and yellow onions, tea, wine, apples, cranberries, buckwheat, beans
	Flavones		Apigenin	chamomile, celery, parsley
			Tangeritin	tangerine and other citrus peels
			Luteolin	celery, thyme, green pepers,
	Isoflavones		Genistein	soy, alfalfa sprouts, red clover, chickpeas, peanuts, other legumes.

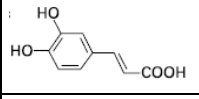
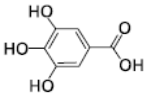
Stilbenes			Resveratrol	gapes skins, red wine
Lignans			Secoisolaiciresinol	flaxseeds

Table 1. The different categories of polyphenols, their chemical structures and sources

Of particular interest among the antioxidant phytochemicals present in fruits and vegetables are the polyphenols. Polyphenols can be sub classified as non-flavonoids and flavonoids. Ellagic acid and stilbenes are among the major non-flavonoid polyphenols. Included in the flavonoid polyphenols are the anthocyanins, catechins, flavones, flavonols and isoflavones. The different categories of polyphenols, their chemical structures and sources are shown in Table 1.

Numerous studies have shown the health-promoting properties of polyphenols, providing additional mechanisms through which they promote skeletal health by reducing resorption caused by high oxidative stress (Trzeciakiewicz *et al.,* 2009; Tucker, 2009; Hunter *et al.,* 2008). The antioxidant properties of polyphenols have been widely studied and reported in the literature (Liu *et al*., 2005; Miyamoto *et al*.,1998; Rassi *et al*., 2002; Viereck *et al*., 2002; Ward *et al*., 2001; Shen *et al*., 2011; Rao *et al*., 2007). They strongly support the role of polyphenols in the delayed onset or reduction in the progression of osteoporosis. The protective effects of polyphenols against diseases, including osteoporosis, have generated new expectations for improvements in health. This review will focus mainly on the role of polyphenols in osteoporosis and present results of studies undertaken in our laboratory.

2. Oxidative stress, antioxidants and osteoporosis

Oxidative stress occurs when the production of free radicals through a number of cellular events exceeds the ability of the cell's antioxidant defense to eliminate these oxidants (Baek *et al.,* 2010). These free radicals have the ability to change the integrity of, and thus, damage several biomolecules, such as DNA, proteins and lipids (Baek *et al.,* 2010). There is increasing evidence that oxidative stress is responsible for the pathophysiology of the aging process and may also be involved in the pathogenesis of atherosclerosis, neurodegenerative diseases, cancer, and diabetes. Recently, ROS were shown to be responsible for the development of osteoporosis (Sahnoun *et al.,* 1997; Basu *et al.,* 2001; Rao *et al.,* 2007; Altindag *et al*., 2008; Becker, 2006; Feng & McDonald, 2011). Several *in vitro* and animal studies have shown that oxidative stress diminishes the level of bone formation by reducing the differentiation and survival of osteoblasts (Baek *et al.,* 2010). Furthermore, it has been reported that ROS activate osteoclasts and thus, enhance bone resorption (Baek *et al.,* 2010). The presence of ROS in osteoclasts was also demonstrated by Rao *et al.* in 2003 Recent evidences from a few clinical studies have also revealed that ROS and/or antioxidant systems might play a role in the pathogenesis of bone loss (Rao *et al.,* 2007; Mackinnon *et al.,* 2010; Abdollahi *et al.,* 2005).

A number of studies have shown that antioxidants have a fundamental role in preventing postmenopausal osteoporosis. For instance, estrogens, whose antioxidant activity is essential in protecting women of reproductive age from cardiovascular disease, stimulate osteoblastic activity through specific receptors, thus favouring bone growth (Banfi *et al.,* 2008). Antioxidant deficiency has been shown to have adverse effect on bone mass (Maggio *et al.* 2003).

Antioxidant enzymes are regarded as the markers of antioxidant defense mechanism against bone resorption. Several studies have investigated the relationship between antioxidant enzymes such as glutathione peroxidase (GP_x) and catalase (CAT) and osteoporosis (MacKinnon *et al.,* 2011; Hahn *et al.,* 2008; Maggio *et al., 2003;* Sontakke & Tare, *2002*).

Recently, many dietary antioxidant nutrients have also been reported to decrease the oxidative stress that takes part in bone-resorptive processes (Rao *et al.*, 2007; Weber, 2001; Peters & Martini, 2010; Macdonald *et al.,* 2004). In addition to the antioxidant enzymes and nutrients, studies have also been directed towards the role of antioxidant phytochemicals such as the carotenoids in osteoporosis which will not be covered here, but has previously been reviewed (Rao & Rao, 2007; Sahni *et al., 2009;* Tucker, *2009*).

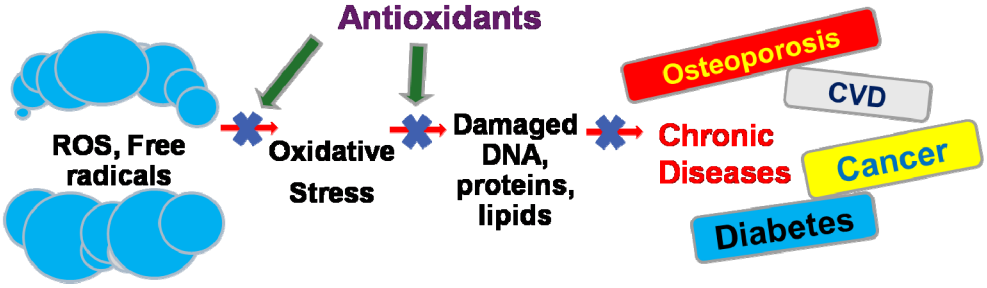


Fig. 1. The role of oxidative stress in osteoporosis and how/where antioxidants play a role in mitigating ROS

3. Natural phytochemical antioxidants

Within the last decade, there has been an increased interest on polyphenols as a result of the *in vitro* evidence demonstrating that they may have numerous benefits to human health, mainly due to their antioxidative and free radical quenching properties (Hendrich, 2006; Lotito & Frei 2006; Heinonen, 2007; Stevenson & Hurst 2007; Aron & Kennedy 2008; Lopez-Lazaro, 2009; Saura-Calixto *et al.* 2007). It is therefore hypothesized that polyphenols may aid in the prevention of aging-associated diseases, particularly cardiovascular diseases, cancers, and osteoporosis.

Polyphenolic compounds are the products of the secondary metabolism of plant and are an essential part of human diet (Goldberg, 2003; Stevenson & Hurst 2007; D'Archivio *et al.*, 2007; Saura-Calixto *et al.* 2007). To date, more than 8,000 polyphenols that have one common structural feature have been identified, a phenol, which is an aromatic ring possessing at least one hydroxyl substituent (Hendrich, 2006; Scalbert & Williamson, 2000; Harborne, 1993). The main classes of polyphenols include phenolic acids, flavonoids, stilbene, and lignans (Spencer *et al.*, 2008; D'Archivio *et al.*, 2007). Figure 1 illustrates the different groups of polyphenols, the chemical structures and food sources. Their total dietary intake can range up to 1 gram/day, which is considerably higher than that of all other classes of phytochemicals (Velioglu *et al.*, 1998). There is much evidence demonstrating that polyphenols improve the status of different oxidative stress biomarkers. However, there is uncertainty regarding both the relevance of these biomarkers as predictors of disease risk and the appropriateness of the different methods used.

Polyphenol Class	Reference	Principal polyphenol	Model	Main findings
Phenolic Acids	Papoutsi et al., (2008)	Ellagic acid (10-100nM)	KS483	↑ nodule formation
	Ayoub et al., (2009)	3-methoxyellagic acid (25ug/ml)	HOS58 & SaOS-2	↑ mineralization of bone cell
Flavonoids	Zhang et al. (2009)	Naringin	bone mesenchymal stem cells (BMSCs)	Dose-specific (1–100 μg/ml) of the naringin solution may enhance the proliferation and osteogenic differentiation of human BMSCs
	Choi (2007)	Apigenin	MC3T3-E1 cells	Apigenin (0.01 mM) increased the growth of MC3T3-E1 cells and caused a significant elevation of alkaline phosphatase (ALP) activity and collagen content in the cells
	Kim et al. (2011)	Luteolin	Bone marrow cells were prepared by removing from the femora and tibiae of ICR mice	luteolin decreased differentiation of both bone marrow mononuclear cells and Raw264.7 cells into osteoclasts, inhibited the bone resorptive activity of differentiated osteoclasts.
	Choi (2011)	Kaempferol	MC3T3-E1 cells	induced the activation of PI3K (phosphoinositide 3-kinase), Akt (protein kinase B), and CREB (cAMP-response element-binding protein). This may prevent or reduce degerneration of osteoblasts
	Wattel et al. (2004)	Quercetin	RAW 264.7 cells, peripheral blood monocytic cells (PBMC)	Quercetin (0.1–10 mM) decreased osteoclastogenesis in a dose dependent manner in both models with significant effects observed at low concentrations, from 1 to 5 mM
Isoflavones	Sugimoto & Yamaguchi (2000)	Daidzein	MC3T3-E1 cells	increase alkaline phosphatase activity
	Rassi et al. (2002)	Daidzein	osteoclasts from young female piglets	inhibits development of osteoclasts from cultures of porcine bone marrow and reduces bone resorption
	Viereck et al. (2002)	Genistein	mature human osteoblasts (hOB)	up-regulated OPG production 2–6-fold in a time- and dose-dependent manner, neutralizing RANKL
Lignans	Hasegawa et al. (2010)	Honokiol	bone marrow cells of 6wk old mice	Inhibits osteoclast differentiation by suppressing the activation of MAPKs (p38 MAPK, ERK and JNK)
Stilbenes	Chang et al. (2006)	Piceatannol	immortalized fetal osteoblasts (hFOB), and osteosarcoma cells (MG-63)	piceatannol increased BMP-2 synthesis, induced osteoblasts maturation and differentiation
	Kupisiewicz et al. (2010)	Modified resveratrol analogues	Myeloma cell lines U266 and OPM-2	Resveratrol analogues showed an up to 5,000-fold increased potency to inhibit osteoclast differentiation and promoted osteoblast maturation compared to resveratrol.

Table 2. Polyphenols- *In vitro* studies

4. Polyphenols and osteoporosis

There has been an increase interest in the field of bone health and nutrients, and within the last decade, it has been well recognized that some polyphenols, whether ingested as supplements or with food, do in fact improve bone health status. Currently, most of the research on polyphenols and their effects has emerged from *in vitro* and *in vivo* studies with only a few clinical studies available. Compounds present in fruits and vegetables influence bone health as shown with *in vitro* osteoblast cell culture. On the other hand, epidemiologic studies tend to have mixed results with regards to the protective effects of polyphenol consumption against osteoporosis. Tables 2, 3, and 4 illustrate some of the recent *in vitro, in vivo* and clinical studies that have been reported in the literature, respectively.

Polyphenol Class	Reference	Substance given	Principal polyphenol	Model	Dose per day	Main findings
Phenolic Acids	Chen (2010)	Blueberries	Phenolic acid mixture	Sprague-Dawley rats		Increase serum osteoblast progenitors, increased osteoblast differentiation, reduced osteoclastogenesis, increase bone mass
	Zych et al. (2010)		Ferulic,caffeic, *p*-coumaric, chlorogenic, clohexanecarboxylic acid	Wistar Cmd:(WI)WU rats	10 mg/kg p.o.	caffeic acid worsened bone mechanical properties
	Folwarczna et al. (2010)		Curcumin	Wistar Cmd:(WI)WU rats	10 mg/kg, po	no sig. improvement of bone mineralizasation or mechanical properties
	Folwarczna et al. (2009)		Caffeic, *p*-coumaric, chlorogenic acid	Wistar Cmd:(WI)WU rats	10 mg/kg p.o.	caffeic acid ↓ bone mass, p-coumaric acid ↑ bone mass/body mass ratio and bone mineral mass/body mass ratio in long bones
Flavonoids	Devareddy et al. (2008)	Blueberries	Variety of phenolic acids and flavonols	OVX rat	5% w/w	Ovx resulted in loss of whole-body, tibial, femoral, and 4th lumbar BMD by approximately 6%. Blueberry treatment was able to prevent the loss of whole-body BMD and had an intermediary effect on prevention of tibial and femoral BMD
	Arjmandi et al. (2010)	(1) 2% Fructooligosaccharides (FOS); 5% FOS+7.5% DP; 2% FOS+5% DP; 2% FOS+2% DP	Variety	OVX rat		diet of 5% FOS + 7.5% dried plum was most effective in reversing both right femur and fourth lumbar BMD and fourth lumbar

	polyphenol (equivalent to 7.5% DP powder); (5) 2% FOS+7.5% DP juice; (6) 2% FOS+7.5% DP puree; (7) 2% FOS+7.5% DP pulp skins; (8) 2% FOS+7.5% raisin; (9) 2% FOS+7.5% fig; (10) 2% FOS+7.5% date; (11) 2% FOS+7.5% blueberry; (12) 2% FOS+0.25% HMB; and (13) 0.25% HMB.				calcium loss while significantly decreasing trabecular separation. No significant effects of treatment on serum or urine measures of bone turnover.
Shen et al. (2008)	Green tea polyphenols (GTP)	(-)Epigallocatechin gallate	OVX rat	0.1% or 0.5% concentration of GTP in drinking water	GTP supplementation increased urinary epigallocatechin and epicatechin concentrations, femur BMD, decreased urinary 8-hydroxy-2'-deoxyguanosine and urinary calcium levels; no effect on serum estradiol
Shen et al. (2010)	Green tea polyphenols (GTP)	(-)Epigallocatechin gallate	40 female CD rats	0.5% concentration of GTP in drinking water	GTP supplementation increased urinary epigallocatechin and epicatechin concentrations and showed higher values for femur BMC, BMD and serum OC, but lower values for serum TRAP, urinary 8-OHdG and spleen mRNA expression of TNF-α and COX-2 levels.
Shen et al. (2011)	Green tea polyphenols (GTP)	(-)Epigallocatechin gallate	50 OVX	0.5% concentration of GTP in drinking water	GTP supplementation resulted in increased serum osteocalcin concentrations, bone mineral density, and trabecular volume, number, and strength of femur; increased trabecular volume and thickness and bone formation in both the proximal tibia and periosteal tibial shaft
Das et al. (2005)	Black tea extract	Theaflavin	Bilaterally oophorecto	2.5% aqueous	BTE increase serum estradiol level

				mized rats	BTE at a single dose of 1 ml /100 g body weight	
	Chiba et al. (2003)	hesperidin & α-glucosylhesperidin	Hesperidin & α-glucosylhesperidin	OVX mice	0.5 g/100 g hesperidin, 0.7 g/100 g α-glucosylhesperidin	hesperidin or α-glucosylhesperidin restored BMD caused by OVX, α-glucosylhesperidin significantly prevented loss of trabecular bone volume and trabecular thickness in the femoral distal metaphysis
	Park et al. (2008)	apigenin	Apigenin	OVX rats	10 mg/kg	apigenin increased the mineral content and density of the trabecular bone at the neck of the left femur, decreased body weight and dietary consumption
	Kim et al. (2011)	luteolin	Luteolin	OVX mice	5 and 20 mg/kg	luteolin increased bone mineral density and bone mineral content of trabecular and cortical bones in the femur as compared to those of OVX controls
	Do et al. (2008)	Rubus coreanus	Anthocyanin	OVX rats	100 & 200 mg/kg	RCM increased femur trabecular bone area in a dose-dependent manner in ovariectomized rats, increased osteoblast differentiation and osteoclast apoptosis.
	Horcajada-Molteni et al. (2000)	Rutin	Rutin	OVX rats	2.5 g/kg	Rutin prevented decrease in both total and distal metaphyseal femoral mineral density by slowing down resorption and increasing osteoblastic activity caused by OVX,
Isoflavones	Arjmandi et al. (1998)	Soy protein	Genistein	72 OVX rats	1462 mg/kg genistein, 25.1 mg/kg daidzin, 11.3 mg/kg daidzein	no effect on BMC
	Lee et al. (2004)	Soybean	Glycitein	24 OVX rats	6.25 g/kg	soybean isoflavone appear to prevent bone loss in femur and lumber vertebrae via a

						different mechanism of estrogen
	Miyamoto et al (1998)	8-isopentenylnaringenin	8-isopentenylnaringenin	OVX rats	30 mg/day	8-isopentenyl naringenin prevented decrease in BMD and bone turnover markers
Lignans	Xiao et al. (2011)	Sambucus williamsii HANCE (SWH)	Lignans	56 OVX/6J specific-pathogen-free (SPF) female mice	17b-oestradiol (3·2 mg/kg), SWH (60% ethanol crude extract; 1·0 g/kg), SWA (water eluate; 0·570 g/kg), SWB (30% ethanol eluate; 0·128 g/kg) or SWC (50 and 95% ethanol eluates; 0·189 g/kg)	SWC significantly restored bone mineral density and improved bone size and bone content in femur and tibia
	El-Shitany et al. (2010)	Silymarin	Silymarin	OVX rats	50 mg/kg	protected trabecula thickness, decreased serum levels of ALP and increased serum levels of both calcium and phosphorus
	Ward et al. (2001)	Flaxseed	Secoisolariciresinol diglucoside	20 Sprague-Dawley male rats	293 µmol SDG/kg	exposure to a diet with flaxseed during lactation through to early adolescence can reduce bone strength, but lignan does is not the mediator, no sig. change in BMD and BMC those fed flaxseed
Stilbenes	Pearson et al. (2008)	Resveratrol	Resveratrol	Male C57BL/6NIA mice	100 mg/kg or 400 mg/kg	Both diets improved distal trabecular tissue mineral density (TMD) and bone volume to total volume ratio over the entire femur compared to control
	Liu et al. (2005)	*trans-Resveratrol*	Resveratrol	OVX rat	0.7 mg/kg	epiphysis BMD and bone calcium content was significantly greater with resveratrol treatment than that in the OVX group, no differences in femoral midpoint BMD

Table 3. Polyphenols- *In vivo* Studies

Polyphenol Class	Reference	Substance given	Principal polyphenol	Model	Dose per day	Main findings
Flavonoids	Hardcastle et al. (2011)	None	Catechin	perimenopausal Scottish women		flavanones were negatively associated with bone-resorption markers, association between energy-adjusted total flavonoid intakes and BMD at the femoreal neck and lumbar spine, annual percent change in BMD was associated with intakes of procyanidins and catechins
Isoflavones	Chen et al. (2004)	Soy isoflavone	Daizein	203 postmenopausal women	placebo: 0 mg isoflavones + 500 mg calcium, mid-dose:40 mg isoflavones+ 500 mg calcium, high-dose:80 mg isoflavones + 500 mg calcium	no effect on BMD in all groups, effect of soy isoflavones on BMC at the total hip and trochanter was less strong in women in early menopause or in those with higher body weight, nonsignificantin BMC in those with a high level of dietary calcium intake
	Arjmandi et al. (2005)	Soy protein	Daizein	87 postmenopausal women	25 g protein and 60 mg isoflavones	Whole body and lumbar BMD and BMC significantly decreased, and BSAP and osteocalcin increasedin control and soy groups
	Kenny et al. (2009)	Soy protein + isoflavone tablets	Isoflavones	131 postmenopausal women >65 years old	18 g soy protein and 105 mg isoflavone tablets	no differences in BMD
Lignans	Cornish et al. (2009)	Flaxseed	Secoisolariciresinol diglucoside	50 men, 50 postmenopausal women		no effect on BMD
	Dodin et al. (2005)	Flaxseed	Secoisolariciresinol diglucoside	199 menopausal women		no sig. change in BMD

Table 4. Polyphenols - Clinical Studies

There have been several results suggesting that the combination of polyphenolic compounds found naturally in fruits and vegetables may reduce the risk of osteoporosis via increasing bone mineral density (Wu *et al.*, 2002; Morton *et al.*, 2001; Melhus *et al.*, 1999; Leveille *et al.*, 1997; Singh, 1992). In 1992, Singh was able to show that polyphenols afford protection against oxidative stress-induced bone damage during strenuous exercise. Similarly, Melhus was able to show its counteractive effect of polyphenols among smokers (Melhus *et al.*, 1999).

5. Research results on the role of polyphenols in osteoporosis from the author's laboratory

Previous *in vitro* results from our laboratory have shown that a supplement rich in a variety of polyphenols commercially known as greens+^{TM}, is more effective in stimulating

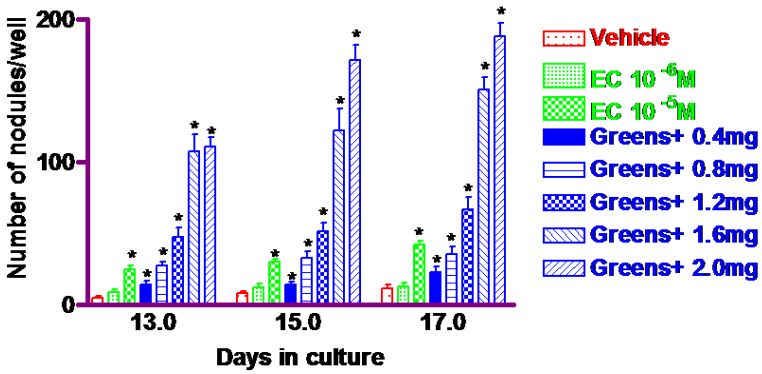


Fig. 2. Dose dependent effect of greens+^{TM} (g+) and epicatechin (EC) compared to vehicle. ($p<0.05$)

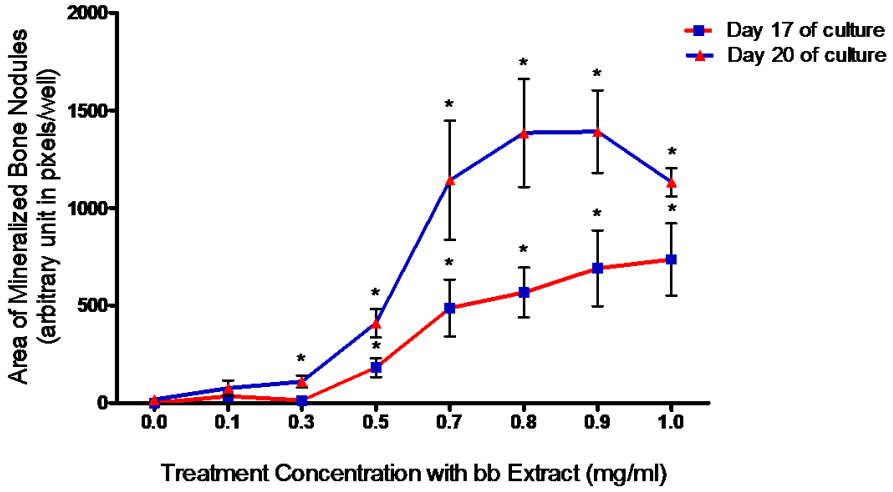


Fig. 3. Time and dose-dependent effects of bone builder^{TM} on mineralized bone nodule area in Sa0S-2 cells ($p< 0.05$).

osteoblasts to form more bone nhodules in a dose-dependant manner than epicatechin, the main polyphenol found in green tea (Fig. 2). Our laboratory also studied the effects of a second supplement, bone builder^{TM}, which is rich in minerals, vitamins and nutrients. Similarly to the greens+^{TM}, the water-soluble bone-builder extract had a significant dose-dependent stimulatory effect on bone nodules formation (Fig. 3). Figure 4 shows that when the two supplements, greens+^{TM} and bone builder^{TM}, were tested as combination, the effects were six times more effective than either one alone. This led us to believe that synergistic effects of greens+^{TM} and bone builder^{TM} may have a beneficial effect on osteoporosis. We then conducted a clinical evaluation of this nutritional supplement greens+ bone builder^{TM} Results have shown that there was an increase in total antioxidant capacity after 8 weeks of treatment compared to placebo (Fig 4). as well as a decrease in both lipid and protein oxidation over a 4 and 8-weeks of intervention with greens+ bone builder^{TM} compared to placebo (Fig. 6 & 7). This suggests that the nutritional supplement may have a beneficial effect on bone health by mitigating the effects of oxidative stress.

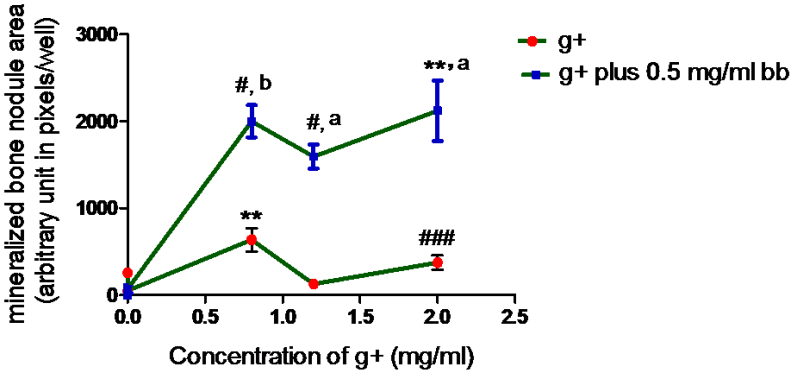


Fig. 4. Dose Dependent Effect of greens + (g+) with and without 0.5 mg/ml of bone builder (bb) on the area of mineralized bone nodules in osteoblasts Sa0S-2 Cells. * p<0.0005, **p<0.005; ***p<0.05; # p<0.0001; ## p<0.001; ### p<0.01; significance differences were found when treatment with g+ plus 0.5 mg/ml bb was compared to treatment with g+ alone as follows: $^{a\backslash b}$< 0.0001; ^{b}p< 0.005

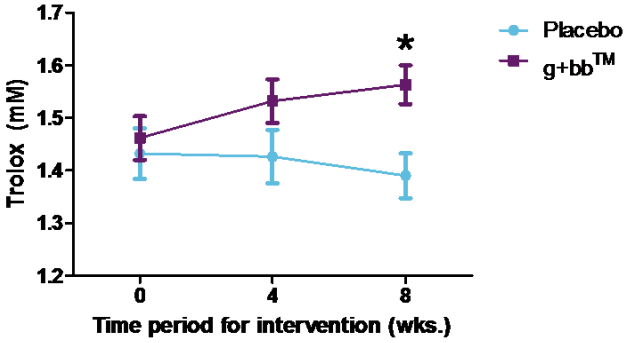


Fig. 5. The effect of nutritional intervention with g+bb^{TM} compared to placebo on serum total antioxidant capacity (p<0.05).

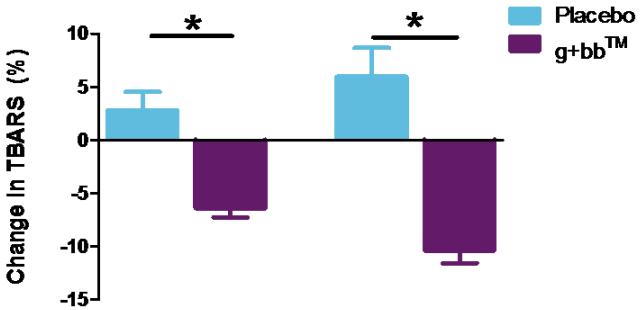


Fig. 6. Change in TBARS over 4 and 8-weeks of nutritional intervention with g+bb™ compared to placebo ($p<0.001$).

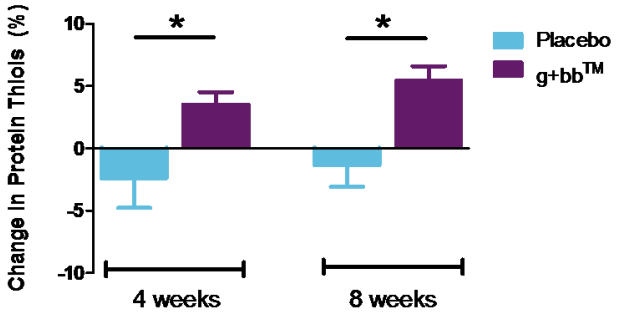


Fig. 7. Change in protein oxidation over 4 and 8-weeks of nutritional intervention with g+bb™ compared to placebo ($p<0.05$).

6. Conclusions

Although epidemiologic studies are practical for the evaluation of human health effects on the physiologic concentrations of polyphenols, reliable data on polyphenol contents of foods are limited. This review has shown that polyphenols or polyphenol-rich diets can provide significant protection or treatment for the development and progression of osteoporosis. Keeping in mind that many nutrients are co-dependent, and they may interact among themselves and others. The complexity of these interactions may possibly be the reason why many studies show controversial or inconsistent results regarding the effects of a single nutrient or groups of nutrients in bone health. Based on current knowledge, polyphenols offer a platform for the prevention of many human chronic diseases involved with oxidative stress, including osteoporosis.

To value the actual significance of food phenolics, it is necessary to investigate not only their bioavailability, but also their mechanisms of action and their possible synergism with other constituents either in the diet or within the human body, as well as the polyphenolic content and composition of foods. We have attained this goal by studying the nutritional supplement greens+™, which is rich in polyphenols and their interactions with minerals, vitamins and nutrients that were present in the nutritional supplement bone builder™.

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The Pentacyclic Triterpenes α, β-amyrins: A Review of Sources and Biological Activities

Liliana Hernández Vázquez[1], Javier Palazon[2] and Arturo Navarro-Ocaña[3,*]
[1]*Universidad Autónoma Metropolitana, Unidad Xochimilco, Depto. Sistemas Biológicos,*
[2]*Laboratorio de Fisiología Vegetal, Facultat of Farmacia, Universitat de Barcelona,*
[3]*Departamento de Alimentos y Biotecnología, Facultad de Química "E" – UNAM,*
[1,3]México
[2]Spain

1. Introduction

Pentacyclic triterpenes are ubiquitously distributed throughout the plant kingdom, in a free form as aglycones or in combined forms, and have long been known to have a number of biological effects. The compounds α-amyrin and β-amyrin are commonly found in medicinal plants and oleo-resin obtained by bark incision of several species of *Bursera or Protium* of the Burseraceae family. Both *in vitro* and in *vivo* studies have shown that β-amyrin also has important biological functions.

In light of the considerable interest recently generated in the chemistry and pharmacological properties of amyrins and their analogs, we have undertaken this review in an effort to summarize the available literature on these promising bioactive natural products. The review will detail the recent studies on the chemistry and bioactivity of α, β-amyrins, which is presented in the following sections: the isolation and distribution of α-amyrin and β-amyrin, giving a brief introduction to amyrins as natural products and the methods used in their isolation; the biological activities of amyrins, examining the biological properties associated with these compounds with a focus on their potential chemotherapeutic applications.

2.1 Chemical structure, detection, analysis and sources

2.1.1 Structure

The chemical structure of α-amyrin (3β-hydroxy-urs-12-en-3-ol) is shown in Fig. 1. The chemical formula of α-amyrin is $C_{30}H_{50}O$, its melting point is 184-186 ^{0}C (Sirat, et al., 2010), and it presents an MS ion Peak at m/z 426 (M^+) (Dias et al., 2011). The infra-red spectrum of α-amyrin is IR u_{max} (KBr) cm^{-1}: 3450, 2895 and 2895. The chemical structure of β-amyrin (3β-hydroxy-olean-12-en-3-ol) is also depicted in (Fig. 1) and its formula is $C_{30}H_{50}O$. The infra-red spectrum of β-amyrin shows the presence of a hydroxyl function and the olefinic moiety at a spectrum of 3360 and 1650 cm^{-1} and MS studies of β-amyrin confirm a parent ion peak at m/z 426 (M^+) (Dias et al., 2011), other work of HR-EI-MS m/z: 426.2975 (calcd. for $C_{30}H_{50}O$, 426.3861) (Jabeen et al., 2011). The melting point of β-amyrin is 189-191 ^{o}C (Lin et al., 2011).

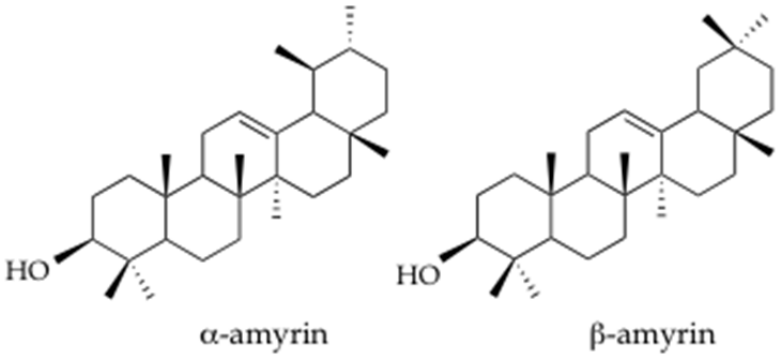

Fig. 1. Estructure of amyrins

NMR methods have indisputably become the single most important spectroscopic techniques for the identification and structure elucidation of amyrins. Several ID and 2D NMR methods are now commonly used for the characterization of pentaclyclic triterpenes. These methods incluye ^{1}H and ^{13}C-NMR, APT, DEPT, COSY, HMQC, HMBC and TOCSY. The ^{1}H and ^{13}C-NMR assignments of α- β-amyrin are presented in Table 1, (Dias et al., 2011).

2.1.2 Detection

Amyrins are found in various plants and plant materials such as leaves, bark, wood, and resins. This material has to be pre-treated prior to isolation of the target compounds. First, the plant material is usually dried, then ground into a power and sieved. Second, extractions are carried out with dichloromethane, chloroformo, *n*-hexane, and methanol. The samples can be subjected to alkaline hydrolysis, derivatization and separation by thin layer chromatography, and the resulting material can be directly subjected to analysis. Gas chromatography (CG) and high performance thin layer chromatography (HPTLC) techniques are the most commonly employed methods to quantitate α-, β-amyrin in plants.

TLC provided an easy and rapid way to study plant extract profiles and partially identify compounds. The first step for the identification of α-amyrin, β-amyrin and 3-*epi*-lupeol was to compare R_F values of reference standards with those of sample extracts. TLC on silica gel revealed that α-amyrin on tracks 6 and 15, β-amyrin on track 14 and the α-, β-amyrin mixture on track 16, as well as two standards, all had the same R_F (Fig. 2). The α-amyrin band was observed as brown, while the β-amyrin band appeared violet, as did the band for the α-, β-amyrin mixture. TLC analysis revealed the presence of α-amyrin, β-amyrin and 3-*epi*-lupeol by a comparison of the position and color of the triterpene spots with those of authentic compounds (Fig. 2). The bands of α- and β-amyrin or their mixture were observed in all commercial resin tracks 1-5 and medicinal plant tracks 8-13, while 3-*epi*-lupeol track 7 was detected only in the commercial Mexican Copal resins tracks 1-4. Attempts were made to separate the α-, β-amyrin mixture, which had appeared homogenous on TLC, but without success. These results showed that TLC can be used as a simple method for a preliminary analysis of these triterpenes in extracts of commercial resins and plants, but cannot be employed for the analysis of the α-, β-amyrin mixture. (Hernández-Vázquez et al., 2010)

Position	α-amyrin		β-amyrin	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1		38.7		38.7
2		28.7		27.2
3	3.16 (*dd*, *J* = 5.1; 11.2)	79.6	3.15 (*dd*, *J* = 4.4; 10.8)	79.3
4		38.7		38.5
5	0.67 (*d*, *J* = 11.6)	55.1	0.68 (*d*, *J* = 11.0)	55.1
6		18.4		18.6
7		32.2		32.4
8		40.7		39.8
9		47.7		47.6
10		36.6		36.9
11		23.3		23.6
12	5.06 (*t*, *J* = 3.2)	124.4	5.12 (*t*, *J* = 3.2)	121.7
13		139.5		145.2
14		42.0		41.7
15	1.94 (*td*, *J* = 4.5; 13.5 Hβ)	27.2	1.89 (*td*, *J* = 4.0; 14.0 Hβ)	26.2
16	1.76 (*td*, *J* = 5.0; 13.5 Hβ)	26.6	1.70 (*td*, *J* = 4.3; 13.5 Hβ)	26.1
17		33.7		32.6
18		59.0		47.2
19		39.6	1.93 (*dd*, *J* = 4.0; 13.7 Hβ)	46.8
20		39.6		31.0
21		31.2		34.7
22	1.85 (*dt*, *J* = 3.0; 7.0)	41.5	1.80*m*	37.1
23	0.93*s*	28.1	0.77*s*	28.0
24	0.74*s*	15.6	0.90*s*	15.4
25	0.73*s*	15.6	0.73*s*	15.4
26	0.89*s*	16.8	0.93*s*	16.8
27	1.01*s*	23.2	1.19*s*	25.9
28	0.94*s*	28.1	1.07*s*	28.4
29	0.85 (*d*, *J* = 6.0)	17.4	0.87*s*	33.8
30	0.73 (*d*, *J* = 7.0)	21.4	0.80*s*	23.7

Table 1. The ^{1}H and ^{13}C-NMR Spectral Data of α- and β-amyrin

α-Amyrin, β-amyrin and other triterpenes were analysed by TLC and HPLC, the chromatographic techniques including silica gel and reversed-phase (C18RP) TLC and C18 RP-HPLC using UV and mass spectrometric (MS) detection with APCI (Martelanc et al., 2009). HPTLC combined with densitometry has been used to analyse the triterpenoids α-, β-amyrin and the oleanolic acid content of acetone and ethyl acetate extracts of the leaves of *Jovibara sobolifera* (Sims) (Szewczyk et al., 2009). The detection and/or quantitation of α-, β-amyrin either in plants or plant products using GC methods requires pre-derivatization of the samples, for example, acetylation or trimethylsilylation. Sometimes a sample clean-up employing silica gel columns or liquid-liquid partition is also necessary.

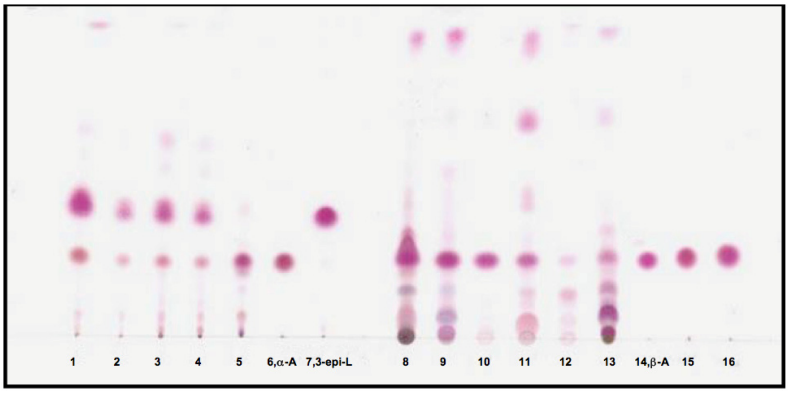

Fig. 2. TLC plate. Tracks: 1=MCT; 2=MCS; 3=MCN; 4=MCP; 5=MER; 6 and 15=α-amyrin; 7=3-*epi*-lupeol; 8=Dandelion; 9=Olive; 10=Cancerina; 11=Nance wastes; 12=Bearberry; 13=Pot marigold; 14=β-amyrin; 15=α-amyrin and 16=mixture α-, β-amyrin.

2.1.3 Analysis

Gas chromatography (CG) is applied to determine the concentration of α-, β-amyrin and α-, β-amyrin mixtures. The chemical composition of the essential oil of Lemon Catnip (*Nepeta cataria* L. var. citriodora Balbis) was determined by CG-MS, and α-amyrin was detected in the hydrodistilled volatile of Lemon Catnip (Wesolowska et al., 2011). α-amyrin has also been determined in the kernel fats of the shea tree (*Vitellaria paradoxa*; Sapotaceae) of sub-Saharan countries (Akihisa et al., 2010). CG of the apolar extract from *Clusia Minor* L. leaves led to the identification of 25 compounds, lupeol and α-amyrin being the most abundant triterpenoids (Mangas-Marin et al., 2008). CG-MS fingerprints for cerumen from the stingless bee *Tetragonula carbonaria* in South East Queensland, Australia, showed trace quantities of TMS ethers of β-amyrins (Massaro et al., 2010). Studies on the constituents of yellow Cuban propolis by CG-MS revealed the presence of large amounts of triterpenic alcohols including β-amyrin, (Márquez-Hernández et al., 2010). Solid-phase extraction and GC-MS were developed to separate and enrich only sterols from unsaponifiables of vegetable, hazelnut and olive oils, detecting sterols, lupeol and δ-, β-amyrin (Azadmard-Damirchi et al., 2010). Epicuticular and intracuticular waxes from both adaxial and abaxial surfaces of *Kalanchoe daigremontiana* leaves (Hamet et Perr. De la bathie) were analyzed by CG. All wax mixtures were found to contain triterpenoids and fatty acids, the triterpenoid fraction containing small amounts of β-amyrin (van Maarseveen & Jetter., 2009). Fatty acid, phytosterol, and polyamine conjugate profiles of corn edible oils were analyzed by GC-MS and HPLC, and a few minor sterols and β-amyrin were identified and quantified using GC-FID (Moreau et al., 2009). Fatty acids, phytosterols and tocopherols of Milk thistle (*Silybum marianum*) seeds were determined in four varieties grown in Ardebil-Iran. In this study using TLC-GC, dimethylsterols were predominant followed by cycloartenol and β-amyrin, (Fathi-Achachlouei, Azadmard-Damirchi., 2009). CG proved an effective method for quantitative measurement of the β-sitosterol content of white mulberry (*Morus alba*) leaves and bark without derivatization. Sterols, lupeol and α–, β-amyrin were identified in leaves and bark by GC-FID analysis (Böszörményi et al., 2009). GC and CG-MS were used to

analyse hexane extracts from seven oleoresins of *Protium* species. High concentrations of α- and β-amyrin were identified in *P. strumosum* (64%) and *P. tenuifolium* (66.7%) (Silva et al., 2009). Finally, the analytical performances of three atmospheric-pressure sources, electrospray (ESI), atmospheric-pressure chemical ionization (APCI) and atmospheric-pressure photoionization (APPI), were evaluated for the analysis of pentacyclic triterpenes in liquid chromatography-mass spectrometry (LC-MS) (Zarrouk et al., 2010). The developed LC-MS method was used to characterize pentacyclic triterpenes in tree plant extracts. The main component of birch bark was betulin and the extracts of Okume resin exhibited high amounts of α- and β-amyrin (Rhourri-Frih et al., 2008). Other technique used to quantitate and determine amyrins is Reversed-Phase High Performance Liquid Chromatography (RP-HPLC). HPLC was used for analysis of some isomeric plant triterpenoids (α-amyrin and β-amyrin δ-amyrin, lupeol, lupenon, lupeol acetate, cycloartenol acetate, ursolic acid oleanolic acid and two sterols) (Martelanc et al., 2009), other studies was for analysis of medicinal plants and Mexican Copal resins (Hernández-Vázquez et al., 2010) and resin obtained from species of the genus *Protium* (Burseraceae) (Dias et al., 2011).

2.1.4 Sources of α-, β-amyrins

α-amyrin is a triterpene of natural origin isolated from various sources, most notably plant resins. Considerable amounts (up to g/kg) of this triterpene are available in the resins of *Bursera* and *Protium* species of the *Burseraceae* family. Other known sources of α-amyrin include Mexican copal (5 g/kg) (Hernández-Vázquez, et al., 2010), *Cassia obtusifolia* (140 mg/kg) (Sob et al., 2010) and the resin of *Commiphora holtziana* (syn. *Commiphora erythraea*) (200 mg/kg) (Manguro, et al., 2009). The most important sources of β-amyrin include lotus (*Nelumbo nucifera* Gaertn) bee pollen (3 g/kg) (Xu. et al., 2011), bark of "cuachalalate" (*Amphipterygium adstringens*) (2.4 g/kg) (Rosas-Acevedo et al., 2011), semi-preparative isolation from resin of *Protium* (α-amyrin 1g /kg and β-amyrin 1.7 g/Kg) (Dias et al., 2011), *Eucalyptus globulus* biomass residues from the pulping industry (326 mg/kg) (Domingues et al., 2010), *Ficus carica* latex (1.2 g/kg) (Oliveira et al., 2010), root bark of *Ficus cordata* (20 mg/kg), steam bark *Ficus cordata* (200 mg/kg) (Kuete et al., 2008) and leaves and bark of *Byrsonima crassa* Niedenzu (IK) (1.3 g/kg) (Higuchi et al., 2008). Mixtures of α and β–amyrin were obtained from steam bark residues of *Byrsonima crassifolia* (Nance) (9 g/kg) (Hernández-Vázquez et al., 2010), leaves of *Byrsonima fagifolia* Niedenzu (2.3 g/Kg) (Higuchi et al., 2008) and leaves of *Pouteria gardnerii* (Mart & Miq) Bahemi gave α-, β-amyrin and other triterpenes (Silva et al., 2009).

Plants reported since 2008 to possess α-amyrin, β-amyrin and a α, β-amyrin mixture in minor amounts (detected and isolated) are listed here. α-Amyrin has been isolated from the resin of *Boswellia carterii* Birdw (Wang et al., 2011), detected in stemwood and bark from *Populus x euramericana* (Xu et al., 2010), isolated (65 mg/kg) from the *n*-hexane extract of the leaves of *Melastoma malabathricum* L (Sirat et al., 2010), identified in the methanol extract of the stem bark of *Poncirus trifoliate* (Feng et al., 2010), isolated (1 mg/kg) from the methanol extract of the stem bark of the African tree *Antiaris Africana* Engler (Vouffo et al., 2010), detected in seed oil of Saskatoon berries (*Amelanchier alnifolia* Nutt.) (Bakowska-Barczak et al., 2009), isolated (23 mg/kg) from the methanol extract of stem bark and leaves of *Ficus pandurata* Hance (Ramadan et al., 2009), dried rhizomes of *Nelumbo nucifera* (Chaudhuri et al., 2009), and detected in bread wheat (Nurmi et al., 2008).

Plant	α-amyrin	β-amyrin	α/β-amyrin	Ref
Mexican copal	5g/Kg			Hernandez-Vazquez et al., 2010
Cassia obtusifolia	0.14g/kg			Sob et al., 2010
Commiphora holtziana (syn. *Commiphora erythraea*)				Manguro, et al., 2009.
Nelumbo nucifera Gaertn		3g/kg		(Xu. et al., 2011)
Amphipterygium adstringens		2.4g/kg		Rosas-Acevedo et al., 2011
Protium sp	3.1g/kg	1.7g/Kg		Dias et al., 2011
Eucalyptus globulus		0.3g/kg		Domingues et al., 2010
Ficus carica		1.2g/Kg		Olivera et al 2010
Ficus cordata		0.2g/Kg		Kuete et al., 2008
Byrsonima crassa Niedenzu (IK)		1.3g/kg		(Higuchi et al., 2008.
Byrsonima crassifolia (Nance)			9g/kg	Hernández-Vázquez et al., 2010
Byrsonima fagifolia			2.3g/Kg	Higuchi et al., 2008
Pouteria gardnerii (Mart & Miq)			X	Silva et al., 2009

Table 2. List of selected materials containing α-, β-amyrin and α/β-amyrins

β-Amyrin has been isolated and detected in various materials: an ethanolic fraction of oleo-gum-resin from *Ferula gummosa* (Jalali et al., 2011), the plant *Carpobrotus edulis*, (Martins et al., 2011), the leaves of *Clerodendrum inerme* (L.) (22.5 mg/kg) (Parveen et al., 2010), the chloroform and ethyl acetate fractions of the methanolic extract of *Carpobrutus edulis* (Martins et al., 2010), chloroform extract of aerial parts of the plants or calli of *Euphorbia tirucalli* L. (Uchida et al., 2010), seed oil of *Capparis spinosa* (Tlili et al., 2011), chloroform extract of the leaves of *Ficus benjamina* (var. camosa) (Moraceae) (Simo et al., 2009), leaves (2 mg/kg) of *Pyrenacantha staudii*, (Falodun et al., 2009), ethanol extract of leaves of *Olea europea* L. (Wang et al., 2009), ethyl acetate extract of apple peels of the Red Delicious variety (*Malus domestica* Borkh) (He et al., 2008), air-dried leaves of *Tectona philippinensis*, an endemic and endangered Philippine medicinal plant (Ragasa et al., 2008), a mixed benzene and chloroform extract of leaves of *Rhus alata* (Parveena et al., 2008), and an extract of stem bark of *Piptadenia Africana*, a western Cameroonian plant (Mbouangouere et al., 2008).

An α- and β-amyrin mixture has been detected in the following plants: *n*-hexane and chloroform extracts of the epicuticular wax layer of *Mandevilla guanabarica* and *Mandevilla moricandiana*, (Cordeiro et al., 2011), an ethanolic extract of roots of *Salacia amplifolia*, (Wang et al., 2011), and chloroform extracts of Blue Honeysuckle (*Lonicera caerulea* L.) (Palíkova et al., 2008).

A multitude of extraction and isolation schemes have been used for the procurement of α-amyrin, β-amyrin and an α/β amyrin mixture. Typically, dry material (resins, leafs and stem barks) is extracted with hexane or another non-polar solvent, (Fig. 3), and the resulting extract is directly subjected to column or thin layer chromatography. An alternative procedure is sequential fractionation by silica gel columns using various solvents. The

amyrins are not readily visible on TLC plates UV (λ = 254 and 365 nm) but are easily detected following exposure to iodine vapors, anisaldehyde-H_2SO_4 or vanillin-H_2SO_4 spray reagents.

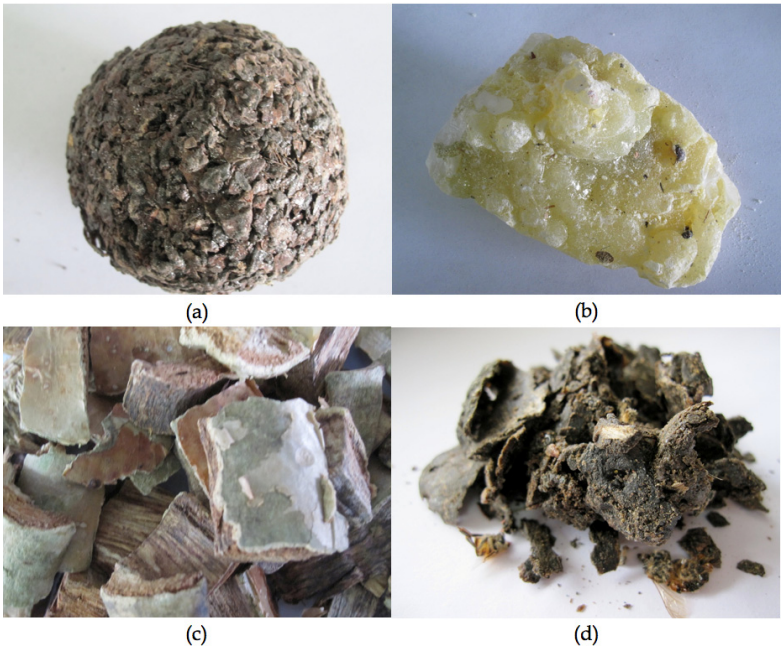

Fig. 3. Sources of amyrins; a) Copal Piedra, b) White Copal, c) Bursera bark, d) Propolis

2.2 An overview of pharmacological activities of α, β-amyrins

α, β-amyrins have been shown to exhibit various pharmacological actitivies *in vitro* and *in vivo* conditions against various health-related conditions, including conditions such as inflammation, microbial, fungal, and viral infections and cancer cells.

2.2.1 Anti-microbial and anti-fungal

The antimicrobial properties of *n*-hexane and methanol extracts of *Bombax malabaricum* flowers were examined against different bacterial, fungal and yeast strains. The methanol extract was highly active against *Staphylococcus aureus*, *Bacillus subtilis*, *Stretoccocus faecalis*, *Neisseria gonorrehea*, *Pseudomonas aeruginosa* and *Candida albicans*, whereas the *n*-hexane extract displayed moderate-to-weak activities against the same test microorganisms. An *n*-hexane extract afforded sterols including α-amyrin (El-Hagrassi et al., 2011). A bioassay-guided fractionation of *n*-hexane extracts of *Bursera simaruba* (L) Sarg. leaves resulted in the isolation and identification of five sterols and β-amyrin. Additionally, *n*-hexane extracts have displayed anti-inflammatory activity on adjuvant-carrageenan-induced inflammation in rats (Carretero et al., 2008). α-Amyrin and other compounds have been proposed as possible biomarkers for the fungal resistance of grape-vine leaves (*Vitis vinifera*) (Batovska et al., 2008).

β-Amyrin has been found to exhibit antifungal and antimicrobial activity against some microbes. The antifungal activity of *Melia azedarach* L. leaves was investigated against *Ascochyta rabiei* (Pass.) Lab., the cause of destructive blight disease of chickpea (*Cicer arietinum* L.). Bioassay-guided fractionation revealed that the chloroform fraction of the methanolic extract of *M. azedarach* leaves was highly effective against *A. rabiei*. Six compounds, namely β-sitosterol (1), β-amyrin (2), ursolic acid (3), benzoic acid (4), 3,5-dimethoxybenzoic acid (5) and maesol (6), were isolated from this fraction. All compounds showed antifungal activity, β-amyrin being the most effective, with an MIC value of 0.0156 mg mL^{-1} (Jabeen et al., 2011).

In a recent study on the leaves of *Siraitia grosvenorii*, β-amyrin and other bioactive compounds were obtained, and their activities against the growth of oral bacterial species *Streptococcus mutans*, *Actinobacillus actinomycetemcomitans*, and *Fusobacterium nucleatum* and the yeast *C. albicans* were evaluated *in vitro*. β-amyrin only exhibited a slight inhibition of *Streptococcus mutans* and *Fusobacterium nucleatum* (Zheng et al., 2011). Bioassay-guided fractionation of the methanol extract of the stem bark of *Klainedoxa gabonensis* Pierre ex Engl. (Irvingiaceae) afforded 12 compounds: four flavonoids and eight (including β-amyrin) triterpenes. Antimicrobial activities in the triterpenoids ranged from low to non-existent (Wansi et al., 2010). In this study, the *in vitro* antibacterial activity of the methanolic extract and isolated compounds from the bark of *Byrsonima Crassifolia* against twelve bacteria and the yeast *C. albicans* was investigated. Eight known compounds, β-amyrin, betulin, betulinic and oleanolic acid, quercetin, epicatechin, gallic acid and β-sitosterol, were isolated and evaluated for their antimicrobial activity. Bacterial growth was inhibited by β-amyrin, olenolic and gallic acid at concentrations ranging from 64 to 1088 $\mu g.mL^{-1}$ (Rivero-Cruz et al., 2008).

2.2.2 Anti-inflammatory activity

Hexane extracts of *Bursera simaruba* (L.) Sarg. leaves display an anti-inflammatory effect on adjuvant-carrageenan-induced inflammation in rats. In order to isolate and identify the active compounds of the hexane extract, we performed a preliminary phytochemical study and a bioassay-directed fractionation using the carrageenan-induced paw oedema test in mice. From the nine fractions (A–I) obtained, A and E showed the strongest anti-inflammatory activity, comparable to that of the reference drug phenylbutazone. Sterols and α-amyrin have been isolated and characterized from these fractions, the evidence suggesting that these bioactive compounds may play a key role in the anti-inflammatory effects of *B. Simaruba* extracts (Carretero et al., 2008).

Ligustrum (privet) plants are used by Chinese physicians to prevent and cure hepatitis and chronic bronchitis. Three common *Ligustrum* plant spp., namely *Ligustrum lucidum* Ait. (LL), *L. pricei* Hayata (LP) and *L. sinensis* Lour. (LS) were collected to assess their analgesic/anti-inflammatory effects on chemical-induced nociception and carrageenan-induced inflammation in rodents. The methanol extracts from *Ligustrum* plant leaves effectively inhibited nociceptive responses induced by 1% acetic acid and 1% formalin. LP and LL reduced the edema induced by 1% carrageenan. The most potent *Ligustrum* plant was LP, which also reduced abdominal Evans blue extravasations caused by lipopolysaccharide, lipoteichoic acid, autocrines and sodium nitroprusside. The triterpenoid content of the three

Ligustrum spp. was measured by HPLC, the highest content of β-amyrin, betulinic acid and lupeol being found in LP. This work suggested that these three triterpenoids are responsible for the anti-inflammatory potency of LP (Wu et al., 2011).

A recent report describes that the roots of *Calotropis gigantea* (Linn.) R.Br, traditionally used in India to treat asthma, possess anti-lipoxygenase activity, it was found that intraperitoneal administration of indomethacin did not block edema formation, but edema was inhibited by montelukast and methanolic extracts of *C. gigantea* roots. This result indicates that the extract from *C. gigantea* was responsible for the inhibition of the lipoxygenase pathway in the arachidonate metabolism. Therefore, it can be concluded that *C. gigantea* may have a similar mechanism of action as dexamethasone as well as antioxidant and anti-lipoxygenase effects, possibly due to the presence of α-amyrin and β-amyrin (Bulani et al., 2011).

Aqueous and organic extracts of *Acacia visco* Lor. Ap Griseb (Fabaceae) were tested for anti-inflammatory activity in experimental rat models. The extracts revealed an anti-inflammatory effect against carrageenan-induced oedema, phospholipaseA-induced oedema, and cotton pellet-induced granuloma without any acute toxic effects. Among the class of compounds characterized from *A. visco* leaves, the triterpenoids lupeol, α-amyrin and β-amyrin may be mainly responsible for these anti-anflammatory properties (Padernera et al., 2010). α-, β-Amyrin ameliorates L-arginine-induced acute pancreatitis in rats. It has been demonstrated that the crude resin of *Protium heptaphyllum* (March.) has an α- and β-amyrin ratio of 63:37. The mixture of both compounds and methylprednisolone treatments significantly ($P < 0.05$) attenuated the L-arginine-induced increases in pancreatic wet weight/body weight ratio, and decreased the serum levels of amylase and lipase, and TNF-α and IL-6, in comparison with the vehicle control. Also, pancreatic levels of MPO activity, TBARS, and nitrate/nitrite were significantly lower. The conclusion of this study is that α, β-amyrin has the potential to combat acute pancreatitis by acting as an anti-inflammatory and antioxidant agent (Melo et al., 2010).

Another study has shown the systemic preventive or therapeutic anti-inflammatory action of the triterpenes α- and β-amyrin in TNBS-induced colitis in mice. It was found that α–, β-amyrin is as efficacious as dexamethasone in reversing the macroscopic and microscopic outcomes of TNBS-induced colitis, including the restoration of cytokine balance. Furthermore, the results also indicate that inhibition of NF-κB and CREB activation is certainly the main mechanism through which these triterpenes exert their anti-inflammatory action (Vitor et al., 2009). Another report demonstrated for the first time that α–, β-amyrin isolated from *Protium heptaphyllum* modulates acute periodontal inflammation in rats by reducing neutrophil infiltration, oxidative stress and the production of proinflammatory cytokine TNF-a, and suggests that these triterpenes might be useful as a therapeutic agent for the treatment of gingivitis and to retard the progression of periodontitis (Holanda-Pinto et al., 2008).

2.2.3 Other pharmacological activities

α- and β-Amyrin have been tested for a variety of other biological activities. An anti-ulcer effect of *Cytocarpa procera* and *Amphipterygium adstringens* was assayed on experimental gastric injury in rats and phytochemical analysis allowed the identification of β-amyrin and β-sitosterol in *A. adstringens* (Rosas-Acevedo et al., 2011). The triterpenoids β-amyrin,

cohulupone and garcinielliptone were isolated from the pericarp, heartwood and seed of *Garcinia subelliptica*, respectively, and the three compounds showed an inhibitory effect on xanthine oxidase. Treatment of NTUB1, a human bladder cancer cell, with β-amyrin or β-amyrin in cotreatment with cisplatin for 24 h resulted in a reduced viability of cells. This work suggested that β-amyrin exhibited weak cytotoxic activities against NTUB1 cells (Lin et al., 2011). The antiproliferative effects of *n*-hexane, chloroform and aqueous methanol extracts prepared from the whole plant of *Centaurea arenaria* M.B. ex Willd. were investigated against cervix adenocarcinoma (HeLa), breast adenocarcinoma (MCF7) and skin epidermoid carcinoma (A431) cells, using the MTT assay. Only the flavonoids and lignans showed moderate activity against these cell lines and β-amyrin was inactive (Csapi et al., 2010). From the ethyl acetate fraction of the stem bark of *Camellia japonica*, three new triterpenoids, 3-β-O-acetyl-16b-hydroxy-12-oxoolean, 3β-O-acetyl-16β-hydroxy-11-oxoolean-12-ene, and 3-β-O-acetyl-16β-hydroxyolean-12-ene, along with seven known compounds, 3-α-hydroxy-1-oxofriedelan, friedelin, 3-β-friedelanol, canophyllol, 3-oxofriedelan-1(2)-ene, β-amyrin, camellenodiol, and camelledionol, were isolated. Their structures were established on the basis of spectroscopic analysis and chemical evidence. The isolated compounds were tested *in vitro* for their cytotoxic activities against the A549, LLC, HL-60 and MCF-7 cancer cell lines. Among them, β-amyrin exhibited weak cytotoxicity against A549 and HL-60 cancer cell lines with IC (50) values of 46.2 and 38.6 μM, respectively (Thao et al., 2010). Another report showed that the methanol extract obtained from soxhlet extraction of leaves of *Ardisia elliptica* Thunberg (Myrsinaceae) contained α- and β-amyrin, determined by GS-MS. The leaf extract inhibited platelet aggregation with an IC_{50} value of 167 μg/mL, using bioassay guided fractionation. β-Amyrin was isolated and purified showing an IC_{50} value of 4.5 μg/mL, while that of aspirin was found to be 11 μg/mL, indicating that β-amyrin is more potent that aspirin in inhibiting collagen-induced platelet aggregation (Ching et al., 2010). Two triterpenes, β-amyrin and 12-oleanene 3β, 21β-diol, were isolated as a mixture from the chloroform soluble fraction of an ethanolic extract of *Duranta repens* (Verbanaceae) stem. The mixture was highly effective against the larvae of *Culex quinquefasciatus* Say (Diptera: Culicidae) as a mosquitocide. *C. quinquefasciatus* is a potential vector of *Wuchereria bancrofti* (Filarioidae), the causative agent of human lymphatic filariasis (Nikkon et al., 2010). One study has examined the potential trypanocidal activity of different plant species growing in the Brazilian Cerrado, after *in vitro* screening of 20 extracts obtained from 10 plants. The phytochemical analysis of the most active extracts (hexane extracts) allowed the identification of β-amyrin, α-amyrin, lupeol and other triterpenes and sterols. The results showed that pure amyrins are inactive whereas the *n*-hexane leaf extract of *Tibouchina stenocarpa* cogn. Melastomataceae was active. The trypanocidal activity of the extract may be due to the presence of other compounds (Cunha et al., 2009).

3. Conclusion

α- and β-Amyrin are bioactive compounds commonly found in leaves, barks and resins. Such plant material is an interesting source of these triterpenoids, as it allows for easy extraction. Extensive research over the last four years has identified α- and β-amyrin in several plants and the pure compounds have shown anti-microbial, anti-inflammatory and other interesting biological activities. Amyrins are also involved in the biosynthetic

pathways of other biologically active compounds such as avenacine, centellosides, glycyrrhizin or ginsenosides. The development of biotransformation systems to convert amyrins into these or other compounds would open new ways for using α- and β-amyrins as a source of bioactive plant secondary metabolites more scarcely distributed in the plant kingdom. In this context, the bioconversion of α-amyrin into centellosides in *Centella asiatica* cell cultures has been recently reported (Hernandez-Vazquez et al., 2010).

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5. References

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24

Phytochemical Studies of Fractions and Compounds Present in Vernonanthura Patens with Antifungal Bioactivity and Potential as Antineoplastic

Patricia Isabel Manzano Santana[1], Mario Silva Osorio[2], Olov Sterner[3] and Esther Lilia Peralta Garcìa[1]
[1]Laboratorio Bioproductos, Centro de Investigaciones Biotecnológicas del Ecuador, CIBE-ESPOL,
[2]Laboratorio de Productos Naturales de la Facultad de Ciencias Naturales y Oceanográficas de la Universidad de Concepción,
[3]Centre for Analysis and Synthesis, Lund University,
[1]Ecuador
[2]Chile
[3]Sweden

1. Introduction

Phytochemical research is closely related to the needs of finding new and effective pharmaceuticals. Searching for plant substances that are capable forbeing used to develop new therapeutic drugs against catastrophic recognized illnesses such as cancer, diabetes and AIDS is one of the main topics that researchers around the world have been focusing.

The wonderful plant diversity of South America and more specifically from the Amazon region has around 30-50% of the worlds biodiversity therafor it is an important source for this type of study. Beside the significant undiscovered resources from these regions, ancestral knowledge of indigenous peoples is another relevant and complementary source for biodiscovery programs. Traditional healers guard centuries of accumulated knowledge about natural medicinal resources of this region. These ancient "physicians" hold the key to discovering new drugs that could benefit millions of people around the world. The Amazon forest has contributed dozens of substances to western medicine. Among the best known are the "curare"; a key component of modern anesthetics and quinine, the first contribution of "natural medicine" to treat malaria[1].

Study of new plant species and the structural elucidation of its bioactive molecules are the most important aims of phytochemical research which is in constant technological development.

[1] Fundación Icaro. La medicina tradicional de los pueblos indígenas amazónicos: Descubriendo la Amazonía europea. Disponible en el sitio: http://www.fundacion-icaros.org/index.php/component/content/article/8-descubriendo-la-amazonia-europea

Initial phytochemical screening and further isolation, purification and identification of molecules structure have made a major breakthrough with the development of new methods of chromatography and spectroscopy. The establishment of new and more effective bioassays is also one of the essential aspects that support biodiscovery programs today.

This chapter contains the main results on the phytochemical study of *Vernonanthura patens* leaves which according to ancestral knowledge, have been used to treat different diseases in humans.

2. Botanical classification, general characteristics and ethnobotanical knowledge on *Vernonanthura patens*

Vernonanthura patens is a wild plant broadly distributed throughout America. It grows from 0 to 2200 meters above sea level in the Ecuadorian coastal region. Folk medicine uses its leaves cooked to combat malaria, postpartum treatment and for healing infected wounds of animals by washing with a plant mixture which includes *V. patens* leaves (Blair, 2005).

It is also used against headaches, to clean and heal wounds (Kvist *et al.,* 2006); treatment of leishmaniasis (Gachet *et al*., 2010); preparation of antivenom (Tene *et al*., 2007) and as a poultice of leaves to combat athlete's foot (Valadeau *et al*., 2009). Its usefulness for treating certain types of cancer has also been referred by indigenous healers. There is however there are few chemical studies about this species

2.1 *Vernonanthura patens* (Kunth) H. Rob. botanical classification and general characteristics

Species *V. patens* belongs to the *Asteraceae* family, quoting 60 synonyms and one basionym (*Vernonia patens* Kunth) (ARS-GRIN, 2009). Referred to *Vernonia patens* HBK in the list of lignocellulose species investigated in Ecuador, it is a source of raw material for pulping and papermaking (Acuña, 2000). It is also commercially important in the beekeeping industry, and is ranked as one of the most important honeybee plants from Tundo, Olmedo and Loja (Camacho, 2001) for its excellent production and availability of nectar and pollen (Ramirez *et al*., 2001).

In the Ecuadorian province of Zamora it is one of four ecologically important species belonging to the typical families of disturbed forests that are been regenerated (Camacho, 2001; REMACH, 2004). It is now registered as representative tree species of secondary forests in Ecuadorian coastal zone (Aguirre, 2001).

The species has the following synonyms (Blair, 2005):

Cacalia patens (Kunth), Kuntze
C. aschenborniana (Schauer) Kuntze
C. baccharoides (Kunth) Kuntze
C. haenkeana (DC.) Kuntze
C. lanceolaris (DC.) Kuntze
C. suaveolens (H.B.K.) Kuntze
Vernonanthura patens (Kunth) H. Rob
Vernonia ascherbotniana Schauer
V. lanceolaris D.C.

V. micradenia DC.
V. monsonensis Hieron
V. pacchensis Benth
V. salamana Gleason
V. suaveolens Kunt
V. treberbaneri Hieron

2.1.1 Taxonomy

Taxon	*Vernonanthura patens* (Kunth) H. Rob
Genus	*Vernonanthura*
Family	*Asteraceae* (alt. Compositae)
Number	415138
Synonyms	*Vernonia patens* Kunth (basionym)
Place of publication	Phytologia 73:72, 1992
Verified name	02-Jun-2008 by systematic botanists of ARS. Last update: 02-Jun-2008 (ARS-GRIN, 2009)

2.1.2 Vernacular names

Table 1 presents a list of vernacular names that are assigned to *V. patens* according to the countries it is grown.

Country/Location		Vernacular name	References
Colombia	Tulua, Valle del Cauca-Valle del Cauca	Yasmiande, varejón Pebetero	Blair, 2005 Terreros *et al*, proyecto ECOFONDO-ACDI 2004-2009
Costa Rica		Cusuco Tuete, tuete blanco	Chavarría *et al*., 1998 Rodríguez, 2005
Ecuador	Prov. Loja and El Oro	Laritaco	Tobías, 1996
	Prov. Guayas	Chilco Blanco	León, 2006
	Quinindé, Bilsa, Viche, Esmeraldas, Muisne and Salina Prov. Esmeralda	Chilca	REMACH, 2004
El Salvador	Chalatenango	Sukunang	PROMABOS a, 2006
Guatemala		Xuqunán Xuquinái	PROMABOS, 2006
Panamá		Salvia blanca, Sanalego	Diéguez et al, 2006

Table 1. List of vernacular names assigned to *V. patens*

2.1.3 Geographical distribution

V. patens is native from America and can be found in Belize, Costa Rica, Brazil, Venezuela, Panama, Bolivia, Mexico and Ecuador according to the data reported by Missouri Botanical Garden[2].

[2] Tropicos.org. Missouri Botanical Garden. 23 Jun 2011.http://www.tropicos.org/Name/2740044.

2.1.4 Habitat

V.patens grows wild in the inter-Andean forest located in the south of Ecuador; its maximum height is 3-6 meters and its altitudinal distribution is between 0 and 2000 meters above sea level (Tobías, 1996; León, 2006). This species has been identified in the vegetal community of dry forests at the south-west of Ecuador[3].

This species is sometimes grown or kept in farms after its spontaneous appearance. Generally it can be found near the forest trail and on the edge of the rivers. Flowering and fruiting occurs between May and October.

2.1.5 Botanical information

V. patens (Figure 1), is a small branched shrub, growins up to six meters high with furrowed stems and ferruginous trichomes. Alternate leaves are petiolate, narrowly lanceolate, petiole tomentose with ferruginous trichomes, 4-11mm long; the leaves are entirely or weakly serrate, rounded base with a sharp or acuminate apex leaves are 7-15 cm long and 1.3 - 1.2 cm wide, the adaxial surface is bright and the abaxial is pubescent or puberulent, subcoriaceous, penninerved. Inflorescence is paniculate, terminal, extended branched with the endings scorpioid, provided with leaves and bracts, capitates sessile and very shortly pedicellate, with numerous bell-shaped flowers, 8 mm long, 4-5 sets bracts imbricated, tomentose and of dark brown color, corolla glabrous, about 5 mm long, weakly pubescent achenes, pappus hairs-layered irregular shaped edges that are about 7 mm long. A detailed description of the botanical characteristics of this species has been published by Blair (2005).

Fig. 1. *Vernonanthura patens* (laritaco). It grows wild in different Ecuadorian areas belonging to the provinces of Loja, El Oro, Guayas, Manabí and Los Ríos**.**

2.2 Ethnomedical information

In Ecuador the inhabitants of the south-west of Loja and the Marcabelí region of El Oro province recognize both its healing power and analgesic action. They use the leaves of *V. patens* to wash wounds and to relieve headaches. It is also employed as anti-inflammatory to soothe coughs and against certain types of cancers. In addition, a veterinary practice is described as it can heal infected wounds by washing with a mixture of plants that includes leaves from this species (Blair, 2005)**.** Other interesting uses have been also reported.

[3] http://www.darwinnet.org/index.php?option=com_content&view=article&id=153%3Aarticulos-cientificos-y-reportes-&catid=25%3Acontenido&Itemid=1

Gacheta *et al*., (2010) informed its usefulness for leishmanianis treatment; Tene *et al* (2007) indicating its use in the preparation of antivenon and the use of "laritaco" leaves in poultices to combat athlete's foot is referred by Valadeau *et al*., (2009).

Different uses of *V. patens* have been registered in other South American countries. In the Bolivian community of Tacama, the juice of the plant stem is applied against conjunctivitis (Tacana, 1999) and in Colombia the watery brews of the aerial parts mixed with "panela"[4], white wine and rosemary are used against malaria. It is also used to relieve pain due to labor and to purge (Blair, 2005).

2.3 Biological and chemical activity

There are very few biological and chemical studies of the specie *V. patens*. The only results published so far refer to the antimalarial activity against *Plasmodium falciparum*, Itg2 strain (Blair, 2005) ,anti-*Leishmania* activity (Valadeau *et al.,* 2009) of the leaves of this species and no antiprotozoal activity against different strains of Leishmania (Fournet, 1994). On the chemical composition of the species, reports lack of sesquiterpene lactones and sesquiterpenes present in the aerial parts (Mabry, 1975; Jakupovic, 1986). There are some references on genus *Vernonanthura* that show the presence of diterpenes compounds (Portillo *et al.,* 2005; Valadeau *et al.,* 2009), flavonoids (Borkosky *et al*., 2009; Mendonça *et al.,* 2009), triterpenes (Tolstikova *et al.,* 2006, Gallo *et al.,* 2009), saponins (Borkosky *et al.,* 2009) and sesquiterpene lactones. In addition, different biological activities have been described assuming that certain chemical groups could be responsible for the therapeutic properties attributed to species of this genus (Pollora *et al.,* 2003, 2004; Portillo *et al.,* 2005; Bardon *et al.,* 2007).

These were the main factors that led to the Laboratorio Bioproductos Centro de Investigaciones Biotecnológicas del Ecuador to undertake a chemical-pharmacological study of *Vernonanthura patens* leaves from plants growing in Ecuadorian areas. Such investigations are part of the Biodiscovery Program developed by this center.

3. Phytochemical screening

As an initial step of thephytochemical screening research allows to determine qualitatively the main groups of chemical constituents present in a plant. This screening can guide the subsequent extraction and / or fractionation of extracts for the isolation of groups of interest. The phytochemical screening routine is performed by extraction with suitable solvents of increasing polarity and the application of color reactions (Miranda & Cuellar, 2001).

These reactions are characterized by their selectivity to types or groups of compounds, their simplicity, short time consuming and capacity to detect small amount of compounds using a minimum requirement of laboratory equipment. The results are recorded by the presence (+) or absence (-) of the color reactions.

[4] "Panela" is a unrefined sugarcane product obtained from the boiling and evaporation of sugarcane juice. It contains sucrose and fructose and is a typical product of Latin America, but can be finding in certain Asian countries.

The general outline of steps followed for performing the phytochemical screening of *V. patens'* leaves is presented in Figure 2, while the analysis of the extracts obtained at different polarities is schematically shown in Figure 3. This methodology has been referred previously (Miranda & Cuellar, 2000; Manzano *et al.*, 2009).

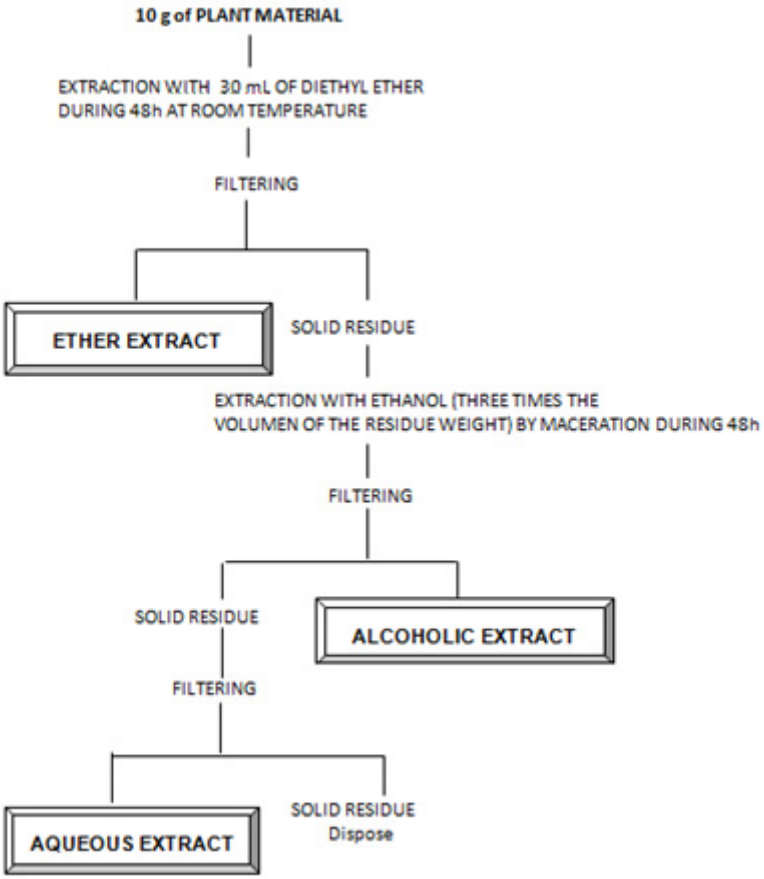


Fig. 2. General procedure used for performing the phytochemical screening of *V. patens* leaves.

The plant material of adult leaves of *Vernonanthura patens* (laritaco) were used from plants at the vegetative state which were growing in the citadels "July 25", "Imbabura" and "June 24" and all belonget to the Canton Marcabelí, province El Oro, Ecuador. Leaves were collected at early morning at different dates during the months of December to February in 2009 and 2010.

Botanical identification was performed and voucher specimens of the herbs were prepared and deposited at the National Herbarium of Ecuador (QCNE) and a duplicated sample (CIBE37) was kept as herbal witness in the laboratory of the CIBE-ESPOL Bioproducts. Prior

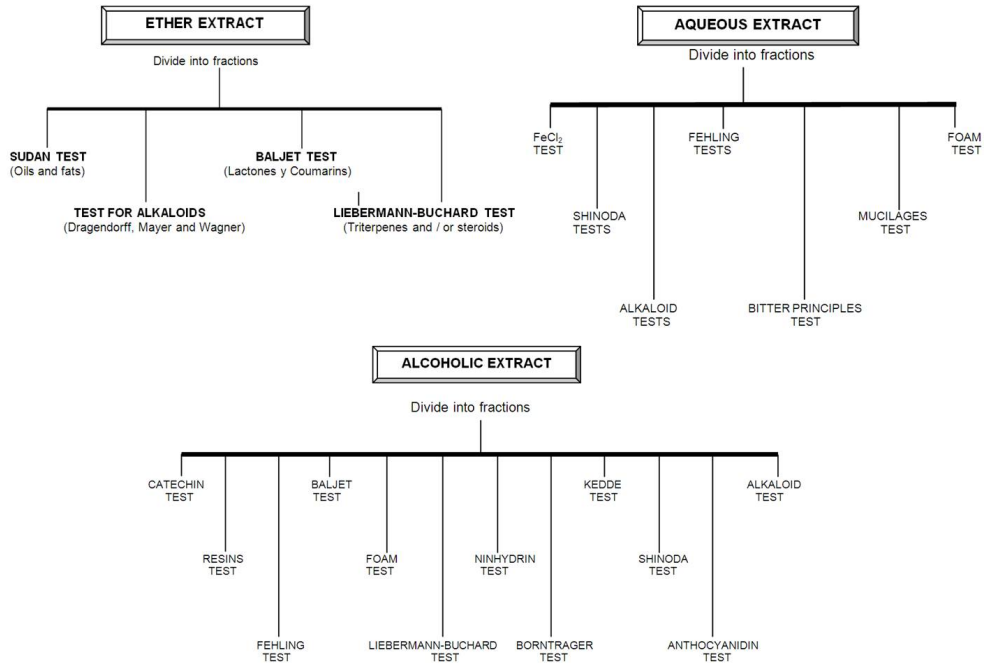


Fig. 3. Chemical reactions carried out in each type of V. *patens'* leaf extracts obtained from using solvents of different polarity.

consent was obtained and authorized by the corresponding agencies of the government. The fieldwork and data collection were conducted in accordance with the institutional, national and international principles and guidelines for using and conserving plant biodiversity.

For conducting the phytochemical screening, extraction and fractionation, leaves samples were dried using an automatic dryer (45 °C, 8 hours) and then pulverized in a blender and screened. The fraction that remained in the sieve of 2 mm in diameter was collected and kept in polyethylene bags of low density at 24 ˚C.

The result of phytochemical screening is presented in Table 2. This reveals moderate to low concentration of essentials oils, alkaloids, reducing compounds, phenols, tannins, flavonoids, quinones, saponins, triterpenes and steroids. Some of these chemical compounds have been associated to antibacterial, antifungal, antiprotozoal and citotoxicity properties and thus have a potential therapeutic use (Nweze *et al.,* 2004; Reuben *et al.,* 2008; Vital *et al.*, 2010).

4. Plant extracts, fractions and compounds

The dry plant material (67 g of leaves of *V. patens*) was subjected to successive extractions with HPLC grade methanol by maceration in a closed container and in the absence of light. The extraction time was eight days and was conducted until total depletion of plant material; agitator and a rotary evaporator were used for solvent recovery.

Chemical groups	Essays	Extracts		
		Ether	Alcoholic	Aqueous
Essential oils, fatty compounds	Sudan	+		
Alkaloids	Dragendorff Mayer		+	+
Aminoacids	Ninhidrine		-	-
Antocianidine	Antocianidine		-	-
Cardiotonic	Kedde		-	-
Reducing compounds	Fehling		-	+
Phenols and tannins	Ferric chloride		+	+
Flavonoids	Shinoda		-	+
Lactones	Baljet		-	-
Mucilages	Mucilages		-	-
Bitter principles	Bitter principles		-	-
Quinones	Börntrager		+	-
Resins	Resins		-	-
Saponins	Foam		+	+
Triterpenes and steroids	Lieberman-Buchard	+	+	-

Table 2. Chemical groups detected in *V. patens* leaves through the phytochemical screening.

The extract was evaporated to dryness, yielding 7g (10.44%) of methanol extract. The methanol residue was subjected to fractionation by successive column chromatography (CC) packed with activated silica from 60 to 200 mesh; elution was performed with solvents of increasing polarity using mixtures of hexane and ethyl acetate (10, 9:1 , 8:2, 3:7, 10) (Table 3). The extracts were analyzed by thin layer chromatography (TLC) on 60 F254 silica gel cromatofolios (Merck) with fluorescent indicator and a solvent system hexane / ethyl acetate (9:1). Plates were observed under UV light at 254 and 366 nm wavelengths.

Solvent	Proportion (%)
Hexane	100
Hexane/ethyl acetate	90:10
Hexane/ethyl acetate	80:20
Hexane/ethyl acetate	30:70
Ethyl acetate	100
Ethyl acetate/methanol	70:30

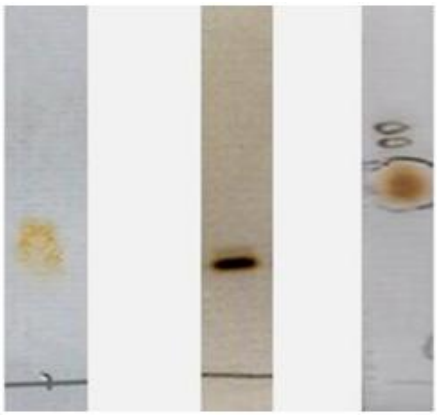
Table 3. Solvents and proportions used in the chromatographic column fractionation of *V. patens*.

Six fractions were obtained (Figure 6): Fr 1 hexane (79mg), Fr 2 Hex / EtOAc 90:10 (1370mg), Fr 3 Hex / EtOAc 80:20 (0.60 mg), Fr 4 Hex / EtOAc 30:70 (0.41mg), Fr 5 EtOAc (0.21 mg), fraction 6 EtOAc / MeOH 70:30 (1760m g) and three pure compounds of the EtOAc fraction 10 and 20% (Figure 7): 57 mg of the compound [1] , 20 mg of the compound [2] and 90 mg of the compound [3].

The isolated fractions with different solvents from methanol extract of leaves of *V. patens* by column chromatography, have not been referred to this species, resulting in a high mass in the hexane fraction (79mg) compared with other extracted fractions. Nevertheless, methanol, ethyl acetate and hexane extracts from other plant species had showed a relevant antimicrobial activity (Ramya *et al.,* 2008).

Fraction 1 Hexane	Fraction 2 Hex/EtoAc: 90:10	Fraction 3 Hex/EtoAc: 80:20	Fraction 4 Hex/EtoAc: 30:70	Fraction 5 EtoAc 100%	Fraction 6 EtoAc/MeOH 70:30

Fig. 6. Isolated fractions from methanol extract of *V. patens*



Compound 1 Compound 2 Compound 3

Fig. 7. Chromatographic plate (TLC) showing the three pure compounds isolated from *V. patens*. Pure compounds were isolated from Fr 2 Hex / EtOAc 90:10 (1370mg).

5. Bioassays

Assays for screening the bioactivity of natural products has had an impressive history of development and is one of the keys for discovering new natural bioactive compounds.

In this study, a qualitative preliminary evaluation of the antifungal capacity of fractions and pure compounds isolated were conducted in order to select the most active. Those selected were re-evaluated to quantify their ability to inhibit fungal growth.

The diffusion method (Avello *et al.,* 2009) in potato dextrose agar (PDA) was used to determine the antifungal activity of fractions and pure compounds isolated from *V. patens* leaves at 100 and 200 μg mL^{-1}. Dilutions were made with dimethylsulfoxide (DMSO) 10%.

Strains of *Fusarium oxysporum* and *Penicillium notatum*, isolated from infected *Pinus radiata* and *Citrus sinense* fruits and maintained in the Collection of Fungi at University of Concepcion were used.

Holes of 5 mm Ø were made in the agar with a sterile cork borer and filled with 20 µL of each concentration of fractions and pure compounds. DMSO 10% was used as negative control in each plate. A disc (5 mm Ø) of already grown fungus was placed in the center of Petri dishes and incubated at 22 °C. Evaluations were made during two weeks.

Experimental design was completely randomized and each assay was performed in triplicate. Descriptive statistics of the experimental data was made in order to represent and point out its most important features.

Most relevant antifungal activity was observed in fraction 1 (100% hexane) and pure compounds 1 and 3 at the both concentrations tested.

The hexane fraction inhibited the growth of both fungal species tested. Highest inhibition exerted against *Penicillium notatum* (80.2%) and *Fusarium oxysporum* (81.5%) occurred when using 200 µg mL^{-1} of this fraction. Statistical differences ($P \leq 0,05$) with negative controls indicated that DMSO did not influence the results of biological evaluation.

Pure compounds showed selective inhibition properties and a certain concentration dependence in its antifungal activity. Compound 1 showed a rate of inhibition of 50 and 90% (100 and 200 µg mL^{1} respectively) against *Penicillium notatum* while compound 3 was capable to inhibit 80 and 100% of the *Fusarium oxysporum* growth for each assayed concentrations.

Screening for antifungal activity of fractions and pure compounds of *V. patens* has been conducted for the first time. The potential of these results is relevant.

6. Structural identification and quantitative analysis of the fractions and isolated compounds

6.1 Chemical characterization of the fraction with antifungal activity

The isolated fraction with antifungal activity were analyzed for structural identification by gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890A gas chromatograph with an Agilent 5975 detector (Avondale, PA.USA) equipped with a column HP-5MS of 5m long (0.25 mm in diameter and 0.25 cm inside diameter). Helium was used as the carrier gas; the analytical conditions were: initial temperature: 100 ° C (increasing 8 ° C per minute to a final temperature of 250 ° C); inlet temperature and mass detector: 250 °C and 300 °C respectively. The mass detector was used in scan mode ("scan") with a range of 100 to 400 amu.

According to this technique and the analytical conditions described, this chromatogram was obtained and is as shown in Figure 8.

Using the library computer and taking into consideration those compounds that exceeded the 90% of confidence, structures of 33 components could be assigned (Table 4).

The compounds identified are mostly hydrocarbons, a logical result given the solvent used. There was a relative abundance of possible bicyclical sesquiterpenos (peaks 1-5) and of the acyclic triterpeno squalene (peak 30). For the sesquiterpenos exist antecedents of antimicrobial activity (Gregori *et al .*, 2005) and for the escualeno reports of activity antioxidant, antitumor

and antimicrobial activities, in addition to its beneficial effect for preventing cardiovascular diseases by reducing cholesterol and triglycerides (Garcia *et al.,* 2010).

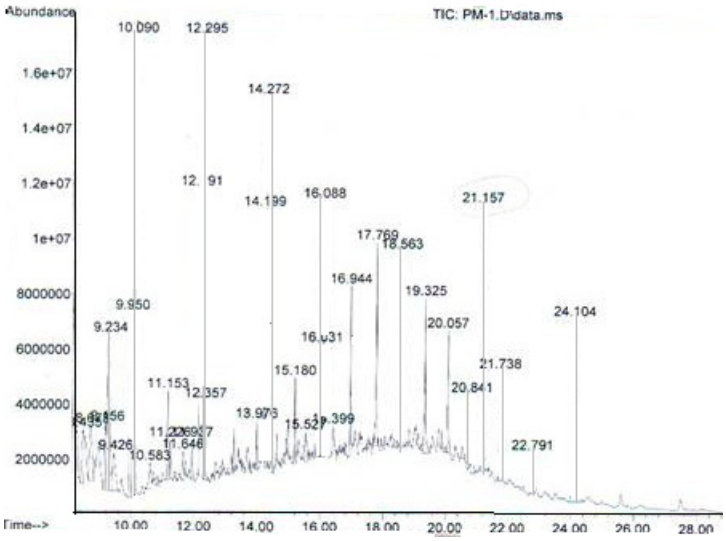


Fig. 8. Analytical gas chromatogram of the hexane fraction of *Vernonanthura patens*.

For this reason, it is possible to hypothesis that antifungal activity of *V. patens* against *F. oxysporum* and *P. notatum* which has been determined could be directed related to the squalene presence despite not being the main component of the fraction tested. The remaining compounds, individually or collectively, could also be involved in the bioactivity demonstrated. The results described here have not been reported previously for *V. patens*.

6.2 Structural identification of isolated compounds

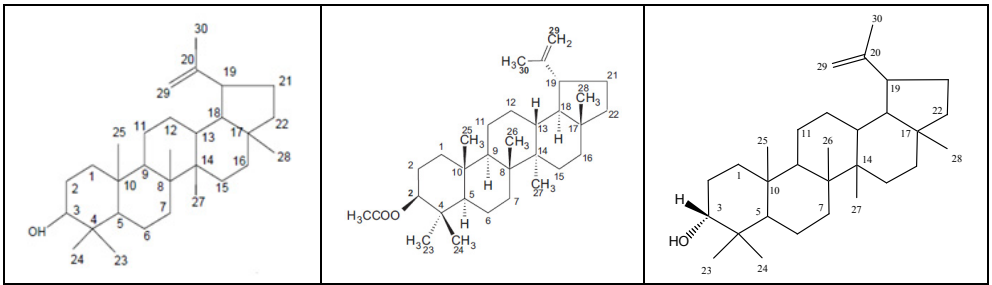
The structures of the three compounds isolated from the hexane soluble fraction by column chromatography were identified by their spectroscopic patterns as compared with references. These pure compounds were identified as Lupeol (compound 1), Acetyl Lupeol (compound 2) and Epi Lupeol (compound 3) (Figure 9).

Spectroscopy was performed in the Laboratory of Organic Chemistry at the University of Lund. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probe head equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (*J*) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^{1}J_{CH}$=145 Hz and $^{n}J_{CH}$=10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101).

The results that are shown in this chapter are unpublished and have not been previously registered for the species *V. patens*. Even though, the elucidated structures of the pure compounds have been found in other vegetal species, and recognize their diverse biological activity which includes antineoplastic action against certain types of cancer (Gallo & Sarachine, 2009).

Peak	Time retention	Name
1	8.435	α-caryophyllene (sesquiterpene)
2	8.678	Napthalene, 1, 2, 3, 4, 4a, 5, 6, 8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl) - (1 α., 4a. α, 8a. α). (bicyclic sesquiterpene)
3	9.156	Naphthalene, 1, 2, 4a, 5, 6, 8a-hexahydro-4, 7-dimethyl-1-(1-methylethyl) (bicyclic sesquiterpene)
4	9.234	Naphthalene, 1, 2, 3, 5, 6, 8a-hexahydro-4, 7-dimethyl-1-(1-methylethyl) (bicyclic sesquiterpene)
5	9.426	Naphthalene, 1, 2, 4a, 5, 6, 8a-hexahydro-4, 7-dimethyl-1-(1-methylethyl) - [1S-(1. α, 4a. β., 8a. α)] (bicyclic sesquiterpene)
6	9.950	2 - tetradecene (E) -
7	10.090	Hexadecane
8	10.583	2, 6, 10, - trimethyl-pentadecane,
9	11.153	2,6,11-trimetil-dodecano,
10	11.226	2,6,11-trimethyl-dodecane,
11	11.646	Tritetracontano
12	11.937	Heptadecane, 3-methyl-
13	12.191	3 - octadecane, (E) -
14	12.295	Heptadecane
15	12.357	4-methyl-heptadecane,
16	13.976	Octadecane
17	14.199	(E) -3 - eicosane,
18	14.272	Eicosane
19	15.180	Heneicosano
20	15.527	Octadeciloxy –2-Ethanol
21	16.031	Docosenoic
22	16.088	2 - Bromo dodecane
23	16.399	1 - bromo-octadecane
24	16.944	1-iodo-Hexadecane
25	17.769	Tetracosanoic
26	18.563	11-decyl-tetracosanoic
27	19.325	1-chloro-Heptadecosano,
28	20.057	5,14-dibutyl-octadecane
29	20.841	Nonadecane
30	21.157	Squalene
31	21.738	Eicosane
32	22.791	9-octyl-Heptadecane
33	24.104	Hentriacontane

Table 4. Identified compounds in hexane fraction of *V. patens* with antifungal activity.



Lupeol	Acetyl Lupeol	Epi Lupeol

Fig. 9. Structure of compounds identified in *V. patens*.

7. Concluding remarks

Phytochemical screening of *V. patens* has showed the presence of essentials oils, alkaloids, reducing compounds, phenols, tannins, flavonoids, quinones, saponins, triterpenes and steroids, of which some have been previously associated to important biological activities.

Fractions and pure compounds of this species were screened for the first time for antifungal activity. Hexane fraction and two pure compounds further identified as Lupeol and Epilupeol, were active against two important fungal pathogens at high rate (80-100%). Hexane fraction reduced the growth of *Fusarium oxysporum* in 80% and Epilupeol completely inhibited the *Fusarium oxysporum* growth.

Thirty-three chemical compounds in the hexane fraction from *V. patens* leaves were determined, Of which must are hydrocarbons. Antifungal activity of this fraction can be related to presence of squalene and/or combined activity of others identified compounds. Further research must be done for determining specific bioactivity of identified compounds.

Chemical structures of three isolated compounds were elucidated, corresponding to Lupeol, Acetyl Lupeol and Epi Lupeol. These compounds are recognized for their significant and diverse biological activities, including antimicrobial and antineoplastic actions.

Results of this study show that *V. patens* can be considered as important potential candidate for further chemical and biological researches and justify its inclusion in the biodiscovery program of CIBE.

8. Acknowledgements

This study was supported by grants from SENESCYT and ESPOL (Ecuador)

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25

The Inhibitory Effect of Natural Stilbenes and Their Analogues on Catalytic Activity of Cytochromes P450 Family 1 in Comparison with Other Phenols – Structure and Activity Relationship

Renata Mikstacka[1], Zbigniew Dutkiewicz[1],
Stanisław Sobiak[1] and Wanda Baer-Dubowska[2]
[1]*Departament of Chemical Technology of Drugs, Poznań University of Medical Sciences*
[2]*Department of Pharmaceutical Biochemistry, Poznań University of Medical Sciences*
Poland

1. Introduction

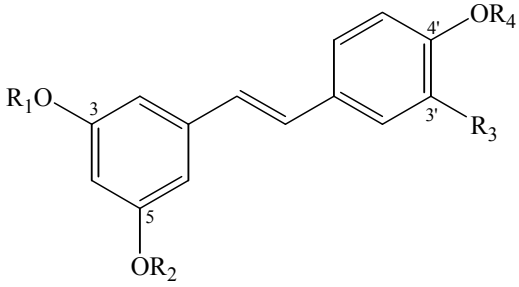
In the last decade, increasing interest in the role of nutrition in disease prevention has been observed. The World Health Organization (WHO) reported that one-third of all cancer deaths could be prevented, and that diet plays a key role in prevention (Bode & Dong, 2009). The term *chemoprevention* introduced and developed by Sporn (2005) and Wattenberg (1985) refers in general to multi-targeted pharmacological and nutritional intervention with the use of naturally occurring or chemically synthesized compounds. For this purpose, dietary phytochemicals believed to be safe for human use seem to be very promising. The importance of natural chemopreventive agents relies on their non-toxicity when given in small amounts for longer periods of time. Moreover, using a combination of phytochemicals provides synergistic or additive preventive effects.

Cancer cell growth arises through a complex multistep process by which cancer cells acquire characteristics of unlimited proliferation potential, lack of response to growth signals, and resistance to cell death. Thus, preventive/therapeutic action of phytochemicals may be directed towards numerous molecular targets that are proteins involved in procarcinogen metabolism, cell transformation and proliferation, and signaling pathways leading to apoptosis of damaged or transformed cells (William et al., 2009). Targeting enzymes of the P450 superfamily may provide one of the strategies for enhancing the efficacy of chemopreventive and therapeutic agents (Swanson et al., 2010).

Mechanistic studies of natural compounds are of great value regarding their characteristics of bioactivity, efficacy, selectivity and potential adverse side effects. Targeted inhibition of metabolic activation of carcinogens and induction of detoxifying enzymes has been considered a fundamental strategy for blocking the early stage of carcinogenesis. For example, inhibition of CYP1 enzymes was one test in the battery of assays employed in

screening of potential cancer chemopreventive agents (Gerhauser et al., 2003). Variable dietary exposure to phytochemicals may contribute to some of the inter-individual variation in the pharmacokinetics and pharmacological responses that are observed for drugs such as phenacetin, caffeine, and theophylline, which are substrates for CYP1A2 (Rendic & Di Carlo, 1997). Further research is needed to determine the extent to which the effect of dietary exposure may be modified by genetic polymorphism of xenobiotic metabolizing enzymes.

Phenolics are a diverse group of aromatic compounds broadly distributed in plants. Among this group, stilbenoids are compounds displaying multiple activities of interest with regard to cancer prevention and therapy, and their anticancer properties have been proven in various animal models (Szekeres et al., 2010). In this review, we summarize the results of studies on inhibitory activity of *trans*-resveratrol (3,4′,5-trimethoxy-*trans*-stilbene), the best recognized *trans*-stilbene (Figure 1), and its natural and synthetic analogues toward expression and activity of CYPs responsible for procarcinogen activation. We discuss the role of cytochrome family 1 inhibitors in cancer chemoprevention and chemotherapy. Additionally, we compare their effect with other natural phenols occurring in plant foods in relatively high amount and exerting significant bioactivity. Finally, we analyze the use of computational methods for biomolecular docking in structure and activity relationship studies of CYP1 inhibitors.



		R_1	R_2	R_3	R_4
1.	*trans*-Resveratrol	H	H	H	H
2.	Piceatannol	H	H	OH	H
3.	Rhapontigenin	H	H	OH	CH_3
4.	Desoxyrhapontigenin	H	H	H	CH_3
5.	Pinostilbene	H	CH_3	H	H
6.	Pterostilbene	CH_3	CH_3	H	H

Fig. 1. Structure of *trans*-resveratrol and its natural analogues

2. Potential strategies targeting CYPs for cancer therapy and prevention

One of the strategies of cancer chemoprevention is directed at drug-metabolizing enzymes such as cytochromes P450 (CYPs), a superfamily which metabolizes a wide spectrum of endogenous and exogenous substrates. Cytochrome P450 family 1 comprises three important isoforms: CYP1A1, CYP1A2 and CYP1B1 that catalyze the activation of procarcinogens such as polycyclic aromatic hydrocarbons, and aromatic and heterocyclic

amines. Additionally, CYP1B1 metabolizes 17β-estradiol (E2) to 4-hydroxyestradiol (4-OH-E2), which is further oxidized by peroxidase to estradiol-3,4-quinone to form a quinone-DNA adducts responsible for estrogen-related carcinogenesis (Liehr et al., 1996). This pathway of metabolism is extensively studied with respect to polymorphism of CYP1 enzymes and its association with carcinogenic metabolite formation (Kisselev et al., 2005).

All members of the human CYP1 family are expressed in extrahepatic tissues. However, CYP1A2 is the only constitutive form of liver enzyme, and as such takes part in metabolism of xenobiotics, including numerous drugs (caffeine, theophylline, methadone, verapamil, propranolol, warfarin, tamoxifen). On the other hand, it is worth mentioning that microbial CYPs are considered as drug targets and may be used as biocatalysts in drug biosynthesis (Lamb et al, 2007).

In humans, CYP1B1 is overexpressed in tumor cells, and this has important implications for tumor development and progression (Castro et al., 2008). It was found that CYP1B1 knockout mice were highly resistant to 7,12-dimethylbenz[a]anthracene induced tumor formation (Gonzalez, 2002). Thus, regulators of the expression and catalytic activity of family 1 cytochromes appear to play an important role in cancer chemoprevention by blocking the initial stages of tumorigenesis. With respect to cancer chemotherapy, CYP1A1 and CYP1B1 have the ability to metabolize cytostatics, diminishing their toxic effect on cancer cells (McFadyen & Murray, 2001). Considering this, the inhibition of CYP1B1, an enzyme up-regulated in many cancers, would be a strategy to prevent the loss of cytostatics effectiveness. On the other hand, the development of anticancer prodrugs specifically activated by CYP1B1 to cytotoxic compounds might be a promising novel strategy in cancer chemotherapy (Bruno & Njar, 2007).

3. Mechanism of the expression of CYP1 genes – AHR as a target for effective chemopreventive approach

Members of the CYP1 family are under the transcriptional control of the aryl hydrocarbon receptor (AHR) localized in cytosol that is activated by polyhalogenated aromatic hydrocarbons, among them 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). AHR agonists are well known environmental pollutants. As a result of activation AHR translocates into the nucleus and forms a dimer with ARNT (aryl hydrocarbon nuclear translocator). The AHR/ARNT complex is characterized by a high affinity to specific DNA recognition sites termed DREs (dioxin response elements) or AHREs (aryl hydrocarbon response element) which upregulate a battery of target genes, including those involved in metabolism of chemical carcinogens such as CYP1A1, CYP1A2 and CYP1B1 (Fig. 2). In this way, agonists induce the expression of xenobiotic metabolizing enzymes (XMEs) that activate procarcinogens to genotoxic forms. Thus, the treatment with AHR antagonists by preventing this undesirable effect might be a chemopreventive strategy.

There are phytochemicals that possess the ability to block agonist interaction with the ligand-binding site of the AHR and agonist induction of the AHR-signaling pathways. In that respect, resveratrol is the best recognized stilbene derivative. Moreover, it is one of the best-characterized chemopreventive phytochemicals (Goswami and Das, 2009). It occurs mainly in small fruits like berries and grapes, peanuts and red wine. Its chemopreventive properties found in studies on animals *in vivo* were described for the first time by Jang and

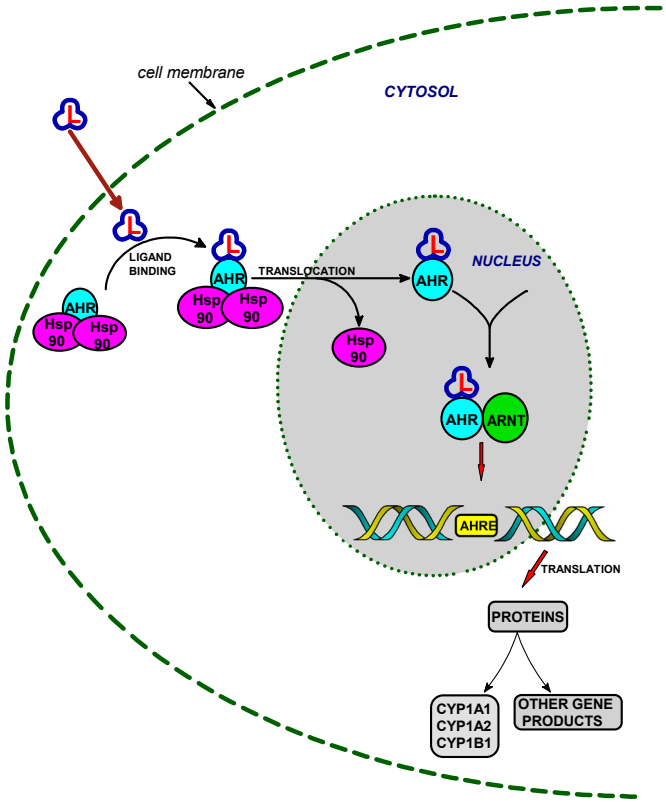


Fig. 2. AHR signaling pathway; L – ligand; Hsp90 – heat shock protein 90; ARNT – aryl hydrocarbon nuclear receptor; AHRE – aryl hydrocarbon response element. AHR/ARNT complexes bind to the DNA recognition sequence AHRE located in regulatory regions of phase I (CYP1A1, CYP1A2, CYP1B1) and phase II drug metabolizing enzymes.

coworkers (Jang et al., 1997). Chen and collaborators have reported that resveratrol strongly inhibited TCDD-induced AHR binding activity in human mammary epithelial (MCF-1-A) cells (Chen et al., 2004). The inhibition of CYP1A1 expression by resveratrol was observed in rat primary hepatocytes (Andrieux et al., 2004). In human HepG2 hepatoma cells, resveratrol inhibited the increase in CYP1A1 mRNA caused by TCDD in a concentration-dependent manner. The induction of transcription of an aryl hydrocarbon-responsive reporter vector containing the CYP1A1 promoter by TCDD was likewise inhibited by resveratrol. Resveratrol also inhibited the constitutive level of CYP1A1 mRNA and reporter vector transcription in human hepatoma HepG2 cells (Ciolino et al., 1998). Resveratrol was also effective in inhibiting CYP1A1 transcription induced by the aryl hydrocarbon dimethylbenz[a]anthracene in human mammary carcinoma MCF-7 cells and B[a]P-treated HepG2 cells (Ciolino et al., 1999). These data demonstrate that resveratrol inhibits aryl hydrocarbon-induced CYP1A activity *in vitro* by directly inhibiting CYP1A1/1A2 enzyme activity, and by inhibiting the signal transduction pathway that up-regulates the expression of carcinogen activating enzymes. The antagonistic action of resveratrol was supported by *in*

vivo experiments on phytochemicals with four different structures, where only resveratrol given topically on mouse epidermis inhibited aryl hydrocarbon hydroxylase (AHH) activity in a dose dependent manner (Table 1) (Szaefer et al., 2004). Moreover, resveratrol has been shown to prevent genotoxicity of B[a]P by inhibiting B[a]P-induced CYP1A1 expression and BPDE-DNA adduct formation in the lungs of mice (Revel et al., 2003).

Treatment	Dose	Activity [pmol/min/mg protein]
Acetone	0.2 ml	65.3 ± 4.6
5,6-Benzoflavone	8 μM	336 ± 17.2
Protocatechuic acid	8 μM	75.3 ± 2.1
	16 μM	83.4 ± 6.4
Chlorogenic acid	8 μM	71.7 ± 3.2
	16 μM	83.9 ± 2.8
trans-Resveratrol	8 μM	18.9 ± 2.6
	16 μM	0.08 ± 0.01

Table 1. Effect of phenolic compounds on mouse epidermal AHH activity

Summarising, resveratrol inhibits AHR-dependent transcription by preventing AHR/ARNT binding to the AHRE. The activity of preventing the conversion of ligand-bound cytosolic AHR into its nuclear DNA-binding form and/or the interaction between the AHR and the transcription initiation complex at the CYP1A1 gene promoter may be an important part of the chemopreventive activity of resveratrol. However, the action of resveratrol is not specific because this natural stilbene as a phytoestrogen is also a potent ER (estrogen receptor) agonist. Recently, experiments on human breast cancer cells revealed that the estrogenic properties of resveratrol and its influence on the ER expression are independent of its ability to inhibit the expression of genes controlled by AHR (MacPherson & Matthews, 2010). New stilbene derivatives of resveratrol that were synthesized appeared to be selective for AHR and devoid of affinity for ER. Among the *trans*-stilbenes synthesized, all displayed a significantly higher affinity than resveratrol for AHR. Substitution of 3- and/or 5-hydroxy groups with chlorine atoms coupled with replacement of 4'-hydroxy with chlorine or a methoxy group yielded selective TCDD antagonists with high affinity for the AHR that was much higher than resveratrol. Interestingly, one of the studied compounds, 3-hydroxy-5-chloro-4'-trifluoromethyl-*trans*-stilbene, was a selective AHR agonist exerting extremely high-affinity to AHR with a K_i of 0.2 nM. None of the compounds studied showed any detectable affinity for the ER that should eliminate estrogen-related risks, such as the increased risk of ER-related cancers (de Medina et al. 2005).

In the Table 2 we summarized the studies on the effects of resveratrol and its derivatives on AHR related expression of CYP1 enzymes. However, the results of *in vivo* experiments on animals are highly dependent on the dose of a studied compound, as well as the duration and manner of its administration. Further, the effect of a studied substance may also be tissue-dependent. The expression of CYP1A1 and CYP1A2-related monooxygenases in hepatic subcellular preparations from resveratrol treated male mice did not differ from the control; while in pulmonary subcellular preparations significantly lower expression of CYP1A1/2 –dependent enzymes was observed (Canistro et al., 2009).

Effect	Compound	Experimental Model	References
AHR translocation ↑ AHRE transactivation ↓	resveratrol	47DRE reporter cell line	Casper et al., 1999
AHR DNA binding ↓, expression and activity of CYP 1A1/1B1 ↓	resveratrol	TCDD-treated MCF-10A cells	Chen et al, 2004
Expression and activity of CYP1A1/1A2 ↓	resveratrol	B[a]P-treated HepG2 cells and DMBA-treated MCF-7 cells	Ciolino et al., 1999
CYP1A1 expression ↓ Induction of transcription of AHR reporter vector containing the CYP1A1 promoter by TCDD ↓ constitutive level of CYP1A1 mRNA and reporter vector transcription ↓	resveratrol	TCDD treated human HepG2 cells	Ciolino et al., 1998
CYP1A1 expression ↓ BPDE-DNA adduct formation ↓	resveratrol	lung tissue from BP-treated mice	Revel et al., 2003
CYP1A1 expression by resveratrol ↓	resveratrol	rat primary hepatocytes	Andrieux et al., 2004
AHR binding ability of resveratrol and its derivatives ↓	resveratrol and 24 other stilbenes	47DRE reporter cell line	de Medina et al., 2005
Expression of human CYP1A1 and CYP1B1 ↓ Recruitment of the AHR complex and RNA polymerase II to the regulatory regions ↓	resveratrol	TCDD-induced human breast cancer cell line MCF-7, and human hepatocellular carcinoma cell line, HepG2	Beedanagari et al., 2009
AHR-dependent transcription of CYP1A1 and CYP1B1 ↓	resveratrol	TCDD-induced human breast cancer cells T-47D	MacPherson and Matthews, 2010
CYP1A1 and CYP1B1 expression ↓ Recruitment of AHR and ARNT to CYP1A1 and CYP1B1 enhancer regions ↓	piceatannol	TCDD-induced human breast cancer cells T-47D	MacPherson and Matthews, 2010

Table 2. AHR as a molecular target for chemopreventive action of resveratrol and its derivatives

4. Inhibitory effect of stilbene derivatives on CYP1A enzymes

4.1 *Trans*-resveratrol

The studies of the inhibitory effect of phytochemicals on cytochrome P450 dependent enzymes are mainly conducted with the use of *in vitro* techniques on cDNA-expressed enzymes. Recombinant biscistronic supersomes express particular CYP activity and cytochrome c reductase activity. It was reported that resveratrol inhibited human recombinant P450 1A1 activity in a competitive manner (Chun et al., 1999), but the IC_{50} value (the concentration that causes 50% inhibition of enzyme activity) of 23 μM was much higher than the IC_{50} value of 1.4 μM obtained for CYP1B1 inhibition (Chang et al., 2000). Interestingly, resveratrol inactivated human recombinant CYP1A2 indirectly in a mechanism-based manner (Chang et al, 2001).

Mechanism-based inhibition was not observed in rat liver microsomes; EROD (7-ethoxyresorufin-O-deethylase) activity as an indicator of both CYP1A1 and CYP1A2 was inhibited by resveratrol and piceatannol (3,3′,4,5′-tetrahydroxy-*trans*-stilbene) with K_i value of 0.4 μM for both compounds and a mixed type of inhibition (Chang et al., 2007). It was found that resveratrol is metabolized to piceatannol in the reaction of hydroxylation catalyzed by CYP1A2 (Piver et al. 2004) and CYP1B1 (Potter et al. 2002). Poor bioavailability of resveratrol caused by its fast metabolism to glucuronides and sulphates limits the use of this stilbene as a potent chemopreventive / chemotherapeutic agent (Walle et al., 2004). To explain the bioactivity of resveratrol, its accumulation to active levels in target organs or synergistic / additive effects with other food components are taken into account.

4.2 Natural resveratrol analogues

During the last decade, other naturally occurring stilbenoid compounds with potential health benefit were found and examined. Piceatannol and pterostilbene (3,5-dimethoxy-4′-hydroxy-*trans*-stilbene) occur mainly in grapes and blueberries, with their amount depending on plant variety (Rimando et al., 2004). Pterostilbene that was shown to have cancer chemopreventive activity similar to resveratrol (Rimando et al., 2002) occurs also in some medicinal plants used in traditional medicine. Beneficial bioactivity of natural resveratrol analogues have been demonstrated in numerous *in vitro* experiments and in preclinical animal models (Rimando and Suh, 2008). Resveratrol analogues exert multiple bioactivities involved in cancer chemoprevention; for example, they are efficient inhibitors of family 1 cytochromes. The inhibitory action of natural stilbenes appears to be highly selective depending on the cytochrome isoform. Moreover, the extent of CYP inhibition changes according to the stilbene structure; the types and positioning of functional groups linked to the stilbene scaffold significantly influence inhibitory activity of stilbene derivatives. Rhapontigenin (3,5,3′-trihydroxy-4′-methoxystilbene) was found to be a very selective and potent inactivator of CYP1A1 activity with IC_{50} value 0.4 μM and K_i value of 0.09 μM (Chun et al., 2001a). Pinostilbene (3,4′-dihydroxy-5-methoxy-*trans*-stilbene), pterostilbene and desoxyrhapontigenin (3,5-dihydroxy-4′-methoxy-*trans*-stilbene) were more efficient inhibitors of CYP1A1 and CYPA2 in comparison to the parent compound, while they inhibited CYP1B1 to the same extent as resveratrol (Guengerich et al., 2003; Mikstacka et al., 2006, 2007). The data on the inhibition of CYP1 enzymes by natural stilbenes are summarized in Table 3.

4.3 Resveratrol methyl ethers and other synthetic stilbenes

In the last decade, new stilbene derivatives have been designed and synthesized in order to find more potent chemopreventive agents (Szekeres et al., 2010). The additional aim of this approach was to find resveratrol derivatives demonstrating better bioavailability in comparison to the parent compound. The bioactivity of resveratrol analogues could be altered due to the presence and positioning of methoxy groups on the basic resveratrol backbone that prevent the conjugation reaction with sulphuric and glucuronic acids. Synthesized derivatives are tested with regard to their inhibitory activity toward CYP 1 enzymes in order to find more efficient and selective inhibitors. A series of *trans*-stilbene derivatives containing a 3,5-dimethoxyphenyl moiety were prepared and evaluated on human recombinant CYP1A, CYP1A2 and CYP1B1 to find a potent and selective CYP1B1 inhibitor. It was shown that substitution at the 2-position of the stilbene skeleton plays a very important role in discriminating between CYP1A1/2 and CYP1B1. Chun and his group found 3,5,2′,4′-tetramethoxy-*trans*-stilbene as a new selective and very potent inhibitor of human CYP1B1 (Chun et al., 2001b). Among the whole series of compounds tested, 3,5,2′,4′-tetramethoxy-*trans*-stilbene exerted the most potent inhibitory activity toward CYP1B1 with an IC_{50} value of 2 nM. 2-[2-(3,5-dimethoxyphenyl)vinyl]tiophene showed comparable inhibitory activities, but its selectivity toward CYP 1B1 was lower (Kim et al. 2002).

Another series of stilbenes with 4-methylthiophenyl moiety were synthesized and their inhibitory potency toward human recombinant CYPs: CYP1A1, CYP1A2 and CYP1B1 was evaluated. Among compounds tested, 2-methoxy-4′-methylthio-*trans*-stilbene and 3-methoxy-4′-methylthio-*trans*-stilbene demonstrated the most potent and selective inhibitory effect on CYP1A1 and CYP1B1 activities (Mikstacka et al, 2008).

Compound	CYP1A1		CYP1A2		CYP1B1	
	K_i [μM]	Mode of inhibition	K_i [μM]	Mode of inhibition	K_i [μM]	Mode of inhibition
Resveratrol	1.2[a]	mixed type	15.5[a] 5.33[c]	mixed type	0.75[a]	mixed type
Piceatannol	3.01	competitive	9.67[c]	mixed type	0.27	competitive
Desoxyrhapontigenin	0.16	competitive	1.04	mixed type	2.06	competitive
Pinostilbene	0.13	mixed type	0.94	mixed type	0.90	competitive
Pterostilbene	0.57	competitive	0.39[c]	mixed type	0.91	competitive
Rhapontigenin	0.21[b] 0.4 (IC_{50})	competitive	 160 (IC_{50})	n.d.	 9 (IC_{50})	n.d.

[a] Chang et al., 2001; [b] Chun et al., 2001; [c] in mouse liver microsomes (Mikstacka et al., 2006); n.d. not determined

Table 3. Effect of natural *trans*-stilbenes on human recombinant CYP1A1, CYP1A2 and CYP1B1 activities

4.4 Other natural phenols

The influence of other phenolic phytochemicals on CYP1 activities is worth presenting in the context of possible additive or synergistic effects of the micro-components of human diet. The properties of plant extracts rich in numerous bioactive substances are particularly interesting in terms of herb-drug interaction, which could be a subject of independent review. At the beginning of the last decade, Piver and collaborators (2003) discovered that non-volatile components of red wine or various Cognac beverages exert stronger inhibitory effect on CYP1A1, CYP1A2, and CYP1B1 than resveratrol and its dimer ε-viniferin. Another extract, prepared from the most widely used herbal medicine *Ginkgo biloba,* was tested for its ability to inhibit the major human cytochrome P450 enzymes (Gaudineau et al., 2004). It was demonstrated that the flavonoidic fraction of standardized extract inhibits human CYP1A2 and other cytochromes (CYP2C9, CYP2E1, and CYP3A4), wheras its terpenoidic fraction significantly inhibits only CYP2C9. *In vivo* CYP1A2 induction was observed as a result of herbal dietary supplementation (Rye et al., 2003). Effects of Cuban and Mexican herbal extracts used in traditional medicine (obtained from *Heliopsis longipes*, *Mangifera indica* L. and *Thalassia testudinum*) on CYP1A1/2 and other cytochromes involved in drug metabolism of CYP3A4 and CYP2D6 were studied with the use of human liver microsomes and compared with the pure constituents isolated from the extracts of affinin (an alkamide isolated from the *H. longipes* extract), N-*iso*-butyl-decanamide, and mangiferin. The extracts significantly inhibited CYP1A1/2 activities, which reflects the high content of flavonoids with recognized CYP1A1/2 inhibitory properties (Rodeiro et al., 2009).

Numerous natural phenols demonstrate inhibitory activity toward CYP1 enzymes. Phytochemicals that exert inhibitory effects on CYP1A enzymes comparable to natural stilbenes comprise: flavonoids, isothiocyanates, coumarin and its derivatives.

Flavonoids represent a large class of phenolic phytochemicals. They are ubiquitously present in plant-derived foods and are important microcomponents of the human diet. Humans ingest approximately 0.6-1 g of these bioactive compounds daily (Kuhnau, 1976). The effects of flavonoids on CYP1 activities have been explored since the early nineties, including the effects of flavone and five hydroxylated derivatives on the methoxyresorufin O-demethylase activity catalyzed by human recombinant CYP1A1 and CYP1A2 (Zhai et al., 1998). The authors found galangin (3,5,7-trihydroxyflavone) as the most potent inhibitor of CYP1A2 with K_i value of 8 nM. It should be mentioned that no stilbene derivative with a comparable inhibitory potency toward CYP1A2 was found. Furthermore, galangin showed almost 5-fold selectivity for CYP1A2 over CYP1A1; while, 7-hydroxyflavone exhibited 6-fold greater selectivity for CYP1A1 over CYP1A2. The other hydroxylated flavone derivatives: 3-hydroxy; 5-hydroxy; 7-hydroxy- and 3,7-dihydroxyflavone were also potent inhibitors of CYP1A1 (IC_{50} < 0.1 μM) and CYP1A2 (IC_{50} < 0.3 μM).

In experiments with the use of human recombinant CYPs, seven flavonoids (myricetin, apigenin, kaempferol, quercetin, amentoflavone, quercitrin, and rutin) occurring in St. John's Wort were tested. They were found to be slightly more selective for CYP1B1 activity compared to CYP1A1. Apigenin and amentoflavone were competitive inhibitors of CYP1B1, while quercetin showed a mixed type of inhibition. The most potent CYP1B1 inhibitor was apigenin with K_i of 60 nM. The same authors investigated CYP1 inhibition in cell system. Myricetin, apigenin, kaempferol and quercetin inhibited TCDD-induced EROD activity in

intact 22Rv1 human prostate cancer cells. Because flavonoids were added 30 minutes prior to the EROD assay, the inhibition did not reflect down regulation of CYP1 mRNA or protein level (Chaudhary et al., 2006). The influence of flavonoid constituents of St. John's Wort were also studied by Schwarz's group. They demonstrated the differentiated inhibition of CYP1A1-catalyzed estradiol 2-hydroxylation according to CYP1A1 genotype. The variant CYP1A1.2 (Ile462Val) was significantly inhibited by quercetin, hypericin and pseudohypericin (naphthodiantrones), with IC_{50} values for 2-hydroxylation being more than two times lower than the wild-type enzyme. Additionally, the wild-type enzyme was efficiently inhibited by kaempferol, myricetin and resveratrol (Schwarz et al., 2011).

The synthesis of structures differentiated by type and positions of substituents leads to a continuation of structure and activity relationship (SAR) studies. Recently, Takemura and coworkers (2010) evaluated the structure–property relationship of 18 major flavonoids on inhibiting enzymatic activity of CYP1A1, 1A2 and 1B1 by using an ethoxyresorufin O-deethylation assay. Flavones and flavonols indicated relatively strong inhibitory effects on CYP1s compared with flavanone that does not have the double bond between C-positions 2 and 3 on the C-ring. Flavonoids used in this study selectively inhibited CYP1B1 activity.

Special attention is paid to methoxy derivatives of flavone, which have inhibitory potency exceeding that of the parent compound (Walle & Walle, 2007). In particular, methoxy types of flavones and flavonols such as chrysoeriol and isorhamnetin showed strong and selective inhibition against CYP1B1 (Takemura et al., 2010). The most potent inhibitors of CYP1 catalyzed ethoxyresorufin O-deethylation were the methoxylated flavones acacetin, diosmetin, eupatorin and the dihydroxylated flavone chrysin, indicating that the 4'-OCH_3 group at the B ring and the 5,7-dihydroxy motif at the A ring play a prominent role in EROD inhibition (Androutsopoulos et al., 2011). It was observed that high metabolic turnover of methoxylated flavonoids may result in enhanced antiproliferative activity. Several flavonoid metabolites produced in reactions catalyzed by CYP1A1 or CYP1B1 have been shown to inhibit cancer cell cycle progression. The authors observed CYP1A1-catalyzed biotransformation of acacetin to luteolin, apigenin and scutellarein. The chemopreventive ability of these metabolites was previously established. Generally, it is suggested that dietary flavonoids exhibit three distinct modes of action with CYP1 enzymes: (1) inhibitors of CYP1 enzymatic activity, (2) CYP1 substrates and (3) substrates and inhibitors of CYP1 enzymes.

Coumarin (1,2-benzopyrone) and its derivatives occur naturally in several plant families. They are components of essential oils, and are often used as fragrance ingredients in human diet. Their effect on CYP1 activities have been studied since the early nineties. The naturally occurring coumarins: bergamotin, coriandrin, isoimperatorin, imperatorin, ostruthin are potent inhibitors of the metabolic activation of benzo(a)pyrene and dimethylbenzanthracene in the cell culture model system of mouse epidermis (Cai et al., 1997). In experiments *in vitro*, mechanism-based inactivation of hepatic EROD activity by natural coumarin coriandrin was observed (Cai et al., 1996). These results demonstrate that certain coumarins to which humans are exposed in their diet are bioactivated by CYP1A1 to reactive intermediates that subsequently form covalent adducts with the apoprotein, effectively destroying enzyme activity.

Curcumin is a natural plant food additive obtained from turmeric used in spices and traditional Indian medicine. Its chemopreventive anticancer potential is well documented (Aggarwal et al., 2003). It belongs to hydroxycinnamic acid derivatives observed ubiquitously in plants. Earlier reports on the inhibition of rat liver microsomal CYPs by curcumin showed that curcumin is a strong inhibitor of CYP1A enzymes and CYP2B as well (Oetari et al., 1996; Thapliyal and Maru, 2001, 2003). However, these data were not confirmed in studies with human recombinant cytochrome P450s, where curcumin appeared to be a moderate inhibitor of CYP1A2 with IC_{50} value 40 μM (Appiah-Opong et al., 2007). Appiah-Opong and coworkers synthesized curcumin derivatives that exhibited about 10- to 40-fold greater potency towards inhibition of CYP1A2 than curcumin itself (Appiah-Opong et al., 2008).

Other natural phenols studied more recently with respect to CYP1 inhibition include **phytocannabinoids,** constituents of marijuana, and **chromene amides** from *Amyris plumieri*, a plant grown in the Caribbean, Central America and Venezuela used in folk medicine. Three major constituents in marijuana; Δ^9-tetrahydrocannabinol, cannabidiol and cannabinol inhibited activities of human recombinant CYP1s: CYP1A1, CYP1A2 and CYP1B1 in a competitive manner (Yamaori et al., 2010). One of the amides (chromene acetamide) tested appeared to inhibit potently CYP1A1 activity *in vitro* with IC_{50} and K_i values 1.547 μM and 0.37 μM, respectively (Badal et al., 2011).

Interestingly, in the studies on different natural phenols Schwarz and Roots demonstrated that the inhibitory effect depends not only on the structure of the inhibitor, but also the substrate of the reaction catalyzed by CYPs used in the assay. They found flavonoids like myricetin, apigenin, quercetin, and kaempferol, as well as tea polyphenol (-)epigallo catechin gallate, strongly inhibited the formation of benzo(a)pyrene diolepoxide, the ultimate carcinogenic product of benzo(a)pyrene activation. Furthermore, resveratrol, an inhibitor of CYP1A1-catalyzed ethoxyresorufin deethylation, exhibited only slightly inhibitory effect on CYP1A1-mediated epoxidation of 7,8-diol-B(a)P (Schwarz & Roots, 2003).

5. Docking studies – The new approach to CYPs-phytochemical interaction

Mechanistic studies of the inhibitory effect of stilbenes on enzyme activities are mainly conducted *in vitro* with the use of human recombinant cytochromes. However, the affinity of compounds to cytochromes may be determined by computational analysis of inhibitor/substrate docking in the enzyme active site. Molecular modeling is presumed to be helpful in predicting inhibitory potential of CYP regulators by characteristics of ligand-enzyme interactions. We review *in silico* research on elucidating the mechanism of inhibitory action of phytochemicals by analysis of structure and activity relationship. Potential phytochemical candidates can be selected by *in silico* virtual screening, based on natural compound libraries (www.bioscreening.com). When active chemicals are selected, they may be "docked" into the target protein by using available programs, enabling detailed protein-ligand interactions to be obtained and the best fit of a candidate compound to be identified. The main objective of molecular docking is to determine the binding interactions between protein and ligand.

Computational procedures of molecular modeling have been employed since the nineties. Studies of Lewis and coworkers (1997) on CYP1 family enzymes structure and ligand docking in enzyme cavities have been a great contribution to the development of this field. Lewis formulated the general characteristic of CYP1 ligands as planar and polar polycyclic molecules. Substituents linked to the polycyclic hydrocarbon core influence the ligand binding responsible for molecular interactions: hydrogen bonds; π-π stacking; and hydrophobic interactions. The effect of structural modification on the inhibitory selectivity of phytochemical derivatives on CYP1A1, CYP1A2, and CYP1B1 help to elucidate which interactions determine the inhibitory ability of the compounds. There are similarities between the active sites of CYP1A2 and CYP1A1 which are in accordance with the overlapping substrate specificities of the two enzymes. However, the CYP1A1 substrates are generally of higher lipophilicity than those of CYP1A2. The reason lies in the more hydrophobic character of the CYP1A1 active site region (including the access channel) in comparison to CYP1A2 active site (Lewis et al., 1999). The differences in the structure of enzyme binding sites may determine the metabolism pathways of a substrate. With the use of computational docking the mechanism of E2 2-hydroxylation and 4-hydroxylation catalyzed by CYP1A1/2 and CYP1B1, respectively, were elucidated. CYP1A1 and CYP1A2 produced 2-OH-E2 and 4-OH-E2 in a ratio of 10 : 1; whereas CYP1B1 produces 2-OH-E2 and 4-OH-E2 in a ratio of 1 : 3 (Lee et al., 2003). The docking study suggests that CYP1A1 and CYP1A2 generate 2-OH-E2 rather than 4-OH-E2, and that CYP1B1 generates both 2-OH-E2 and 4-OH-E2. Particular amino acids residues for each CYP were identified as playing an important role in estradiol recognition (Itoh et al., 2010).

Several groups of phytochemicals were tested for affinity to active sites of CYP1 members. The first studied compounds were rutaecarpine derivatives. An alkaloid rutaecarpine preferentially inhibited CYP1A2 activity with IC_{50} value of 22 nM. However, 1-methoxyrutaecarpine and 1,2-dimethoxyrutaecarpine were the most selective CYP1A2 inhibitors. Molecular modeling showed a good fitting of rutaecarpine and the active site of CYP1A2. Two hydrogen bonds between the keto- and N14-groups of rutaecarpine and the Thr^{208} and Thr^{473} residues of CYP1A2, respectively, were visualized with molecular modeling procedures. The C-ring moiety of rutaecarpine formed π-π stacking interaction with the aromatic ring of Phe^{205} residue (Don et al., 2003).

Coumarin was shown to be a substrate of human CYPs, specifically: CYP1A1 and CYP1A2. Molecular modeling led to recognition and localization of the amino acid residues which interact with coumarin molecules resulting in the orientation of coumarin with 3,4 bond directly above the heme moiety. Coumarin 3,4-epoxide is produced and then rearranged to hydroxyphenylacetaldehyde, which can be further metabolized to toxic products. In the CYP1A1 active site, Ser^{113} forms a hydrogen bond with coumarin, while Phe^{205} and Phe^{358} are responsible for aromatic π-π stacking. In CYP1A2, Thr^{113} forms hydrogen bonds with coumarin, and Phe^{205} is responsible for π-π stacking (Lewis et al., 2006). However the different key residues take part in the interactions with coumarin, they determine the same site of metabolism, and in consequence, the pathway of coumarin metabolism is the same for both CYP1A1 and CYP1A2 .

7,8-benzoflavone (α-naphthoflavone) is a prototype flavonoid which has been used to examine the mechanism of action on P450 enzymes. Molecular modeling studies revealed that 7,8-naphthoflavone is positioned in a hydrophobic cavity of CYP1A2 next to the

active site where it may cause a direct effect on substrate binding (Cho et al., 2003). Further studies with the use of molecular docking were aimed at methoxyflavonoids with a 2–3 double bond, which exerted strong inhibitory effect on CYP1 activities, particularly CYP1B1 (Takemura et al., 2010). The authors observed that the binding specificity of methoxyflavonoids is based on the interactions between the methoxy groups and specific CYP1s residues. For example, chrysoeriol and isorhamnetin fit well into the active site of CYP1B1, but do not fit into the active site of CYP1A2 and 1A1 because of steric collisions between the methoxy substituent of these methoxyflavonoids and Ser^{122} in CYP1A1 and Thr^{124} in CYP1A2. Androutsopoulos's group described molecular docking of several flavonoids with regard to their metabolism and inhibitory activity. The simulated binding orientation of the compounds tested was in accordance with the study of Takemura and coworkers (2010). Diosmetin and eupatorin are predicted to be oriented with ring-B over the prosthetic group so that 4'-methoxy group is at ~4.5 Å from the heme iron. The less substituted chrysin and acacetin also were shown to bind CYP1A1 with ring-B over the iron-heme group. However, a lower number of interactions were found within the active site of CYP1A1 (Androutsopoulos et al., 2011).

To better characterize stilbenes as ligands of CYPs, we performed molecular docking by simulation of resveratrol and pterostilbene binding in active sites of CYP1A2 and CYP1B. Resveratrol and pterostilbene molecules were docked into the cavities of CYP1A2 (PDB code: 2hi4) and CYP1B1 (PDB code: 3pm0) with the use of the CDOCKER procedure implemented in Accelrys Discovery Studio 2.5.5. CDOCKER uses a CHARMm-based molecular dynamics (MD) scheme to dock ligands into a receptor binding site. For assigning receptor and ligand atom partial charges, we applied the charging rules used in the MMFF94 forcefield. Docked poses were scored by the negative value of CDOCKER energy for the –CDOCKER_ENERGY function, which include interaction energy and internal ligand energy: the higher positive value of –CDOCKER_ENERGY, the stronger affinity of a ligand to the binding site.

Our docking experiment showed that in the CYP1A2 active site, all possible poses of resveratrol can be grouped into two sets. This observation indicated that two binding modes are possible for resveratrol molecule. In mode A, represented by the pose with highest score, a resveratrol molecule is directed with 4'-OH group toward a heme (Fig. 3a). In mode B, the second ring with 3-OH and 5-OH substituents is situated in the vicinity of a prosthetic group (Fig. 3b). In both orientations, resveratrol binding is stabilized by π–π stacking interactions, with phenyl ring of Phe^{226} (mode A), and with Phe^{226} and Phe^{260} (mode B). Contrary to resveratrol, a pterostilbene molecule was docked in the CYP1A2 active site only in one orientation with 4'-OH group directed toward a heme (Fig. 3c). Pterostilbene binding was stabilized by π–π interaction with an aromatic ring of Phe^{226} . For a resveratrol molecule docked in the active site of CYP1B1, we also distinguished two binding modes. In contrast to CYP1A2, the highest scored pose corresponded to binding mode B. In both orientations (A and B), resveratrol was stabilized by two π–π interactions between both of its rings and a phenyl ring of Phe^{231}, and additionally by two hydrogen bonds with Asn^{265} and Asp^{333} in mode B, or Asn^{265} and Asn^{228} in mode A (Fig. 3d and 3e).

Similar to interaction with CYP1A2, a pterostilbene molecule represented only one type of orientation in the CYP1B1 cavity (Fig. 2f). The binding conformation with 4'-OH group close to a heme was stabilized by two π–π stacking interactions with Phe^{231}. In the case of

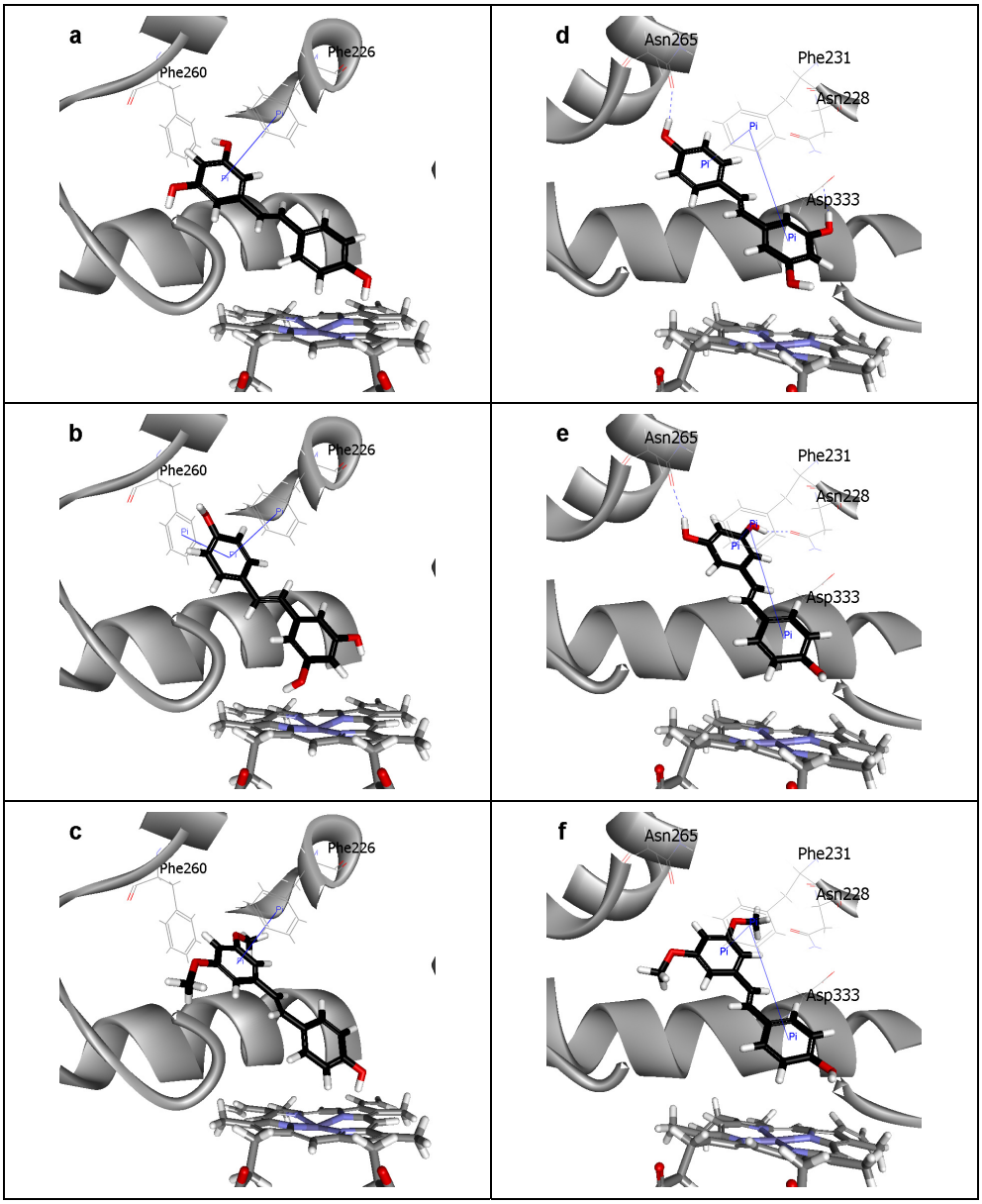


Fig. 3. Putative binding modes of resveratrol and pterostilbene in active sites of CYP1A2 (a – c) and CYP1B1 (d – f) with key residues involved in π–π stacking interactions and hydrogen bonds represented by solid blue lines and dashed blue lines, respectively. Heme molecule is at the bottom. CYP1A2 active site in complex with: (a) resveratrol in binding mode A, (b) resveratrol in mode B, (c) pterostilbene. CYP1B1 active site in complex with: (d) resveratrol in binding mode B, (e) resveratrol in mode A, (f) pterostilbene.

pterostilbene, which is a dimethoxy analogue of resveratrol, it is suggested that hydrophobic interactions might play a key role determining and stabilizing its docking orientation.

In studies of *trans*-resveratrol metabolism by human microsomal CYP1B1 enzyme (Potter et al., 2002), the authors observed formation of two metabolites, M1 and M2. The major metabolite M2 has been identified as piceatannol (3,4,3',5'-tetrahydroxystilbene), while 3,4,5,4'-tetrahydroxystilbene was proposed as the M1 product. More recent work (Piver et al., 2004) provided evidence that CYP1A2 is also engaged in the metabolism of *trans*-resveratrol to piceatannol and tetrahydroxystilbene M1. Our studies confirmed the possibility of two pathways of metabolism on the grounds of molecular docking analysis.

6. Conclusion

The finding of high affinity ligands among natural compounds for each of the CYP1 family enzymes will help to reveal more about enzyme specificity, providing a starting point for more extensive studies and improved predictive capabilities. Particularly, a selective inhibition against CYP1B1 that influences the chemopreventive properties of phytochemicals for E2 related breast cancer seems to be promising. There is a need for better characterization of potential chemopreventive/therapeutic agents in order to understand their abilities and limits to influencing numerous pathways leading to cancer development. Novel classes of anti-cancer drugs including those of plant origin are being developed that can target both drug-metabolizing enzymes and disease modifying pathways. Recently, interest in the combinatory effect of different phytochemicals is growing, with respect to the multi-targeted action of numerous components of a food matrix. Wenzel and co-workers found that metabolism of resveratrol present in beverages such as wine or grape juice is inhibited by other polyphenols due to competitive reactions with Phase -II enzymes, resulting in an increased concentration of the free form (Wenzel et al., 2005). It is suggested that an efficient chemoprevention strategy lies in the use of combinations of several chemopreventive and/or therapeutic agents which may exert multi-targeted action. In conclusion, the search for potent and selective CYP1A inhibitors appears to hold promise and should be continued with the use of novel computational techniques.

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