

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

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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 millennium 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, snuffing 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'ios & 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 *Klebsiella 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

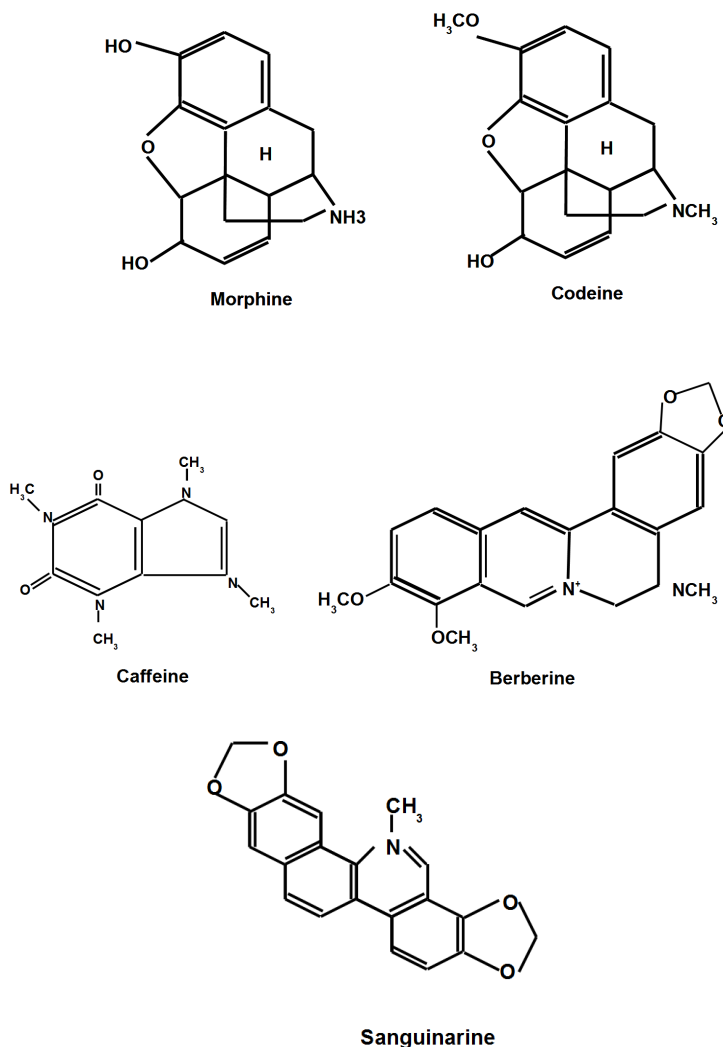


Fig. 1. Basic structures of some pharmacologically important plant derived alkaloids

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 antiarrhythmic 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 | Potassiomeric 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 residue is exposed to ammonia vapour | 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, gentiopicroin, 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/H₂O to hydrolyse the anthraquinone glycosides to respective aglycones, and an aqueous base, e.g. NaOH or NH₄OH solution, is added to it. For C-glycosides, the plant samples are hydrolysed using FeCl₃/HCl, and an aqueous base, e.g. NaOH or NH₄OH 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.

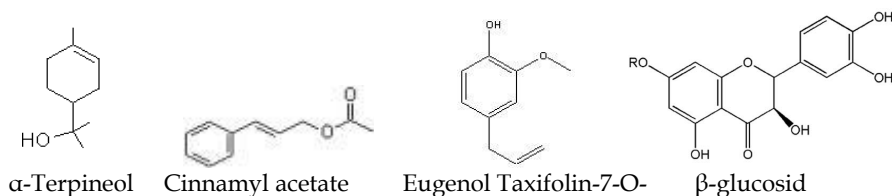


Fig. 2. Basic structures of some pharmacologically important plant derived glycosides

2.3 Flavonoids

Flavonoids are an important group of polyphenols widely distributed among the plant flora. Structurally, 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 groups of flavonoids include flavones, dihydroflavones, flavans, flavonols, anthocyanidins (Fig. 3), proanthocyanidins, chalcones and catechins and leucoanthocyanidins.

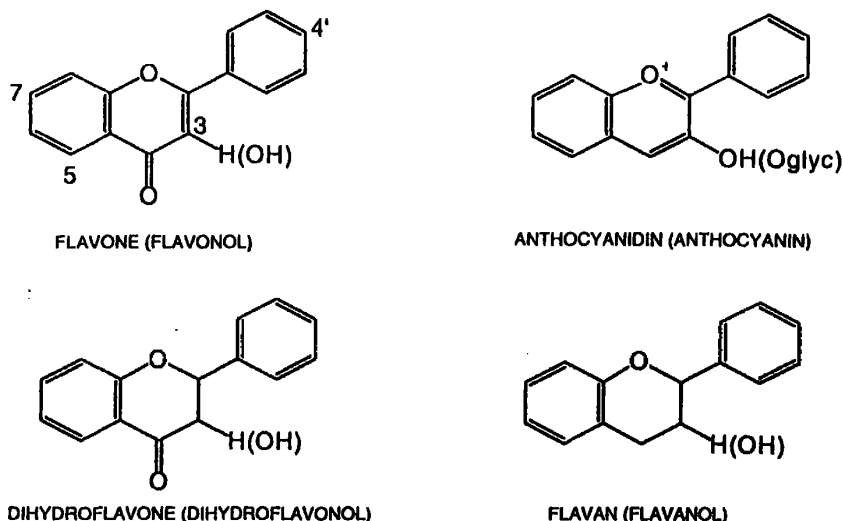


Fig. 3. Basic structures of some pharmacologically important plant derived flavonoids

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 polyphenolics. 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

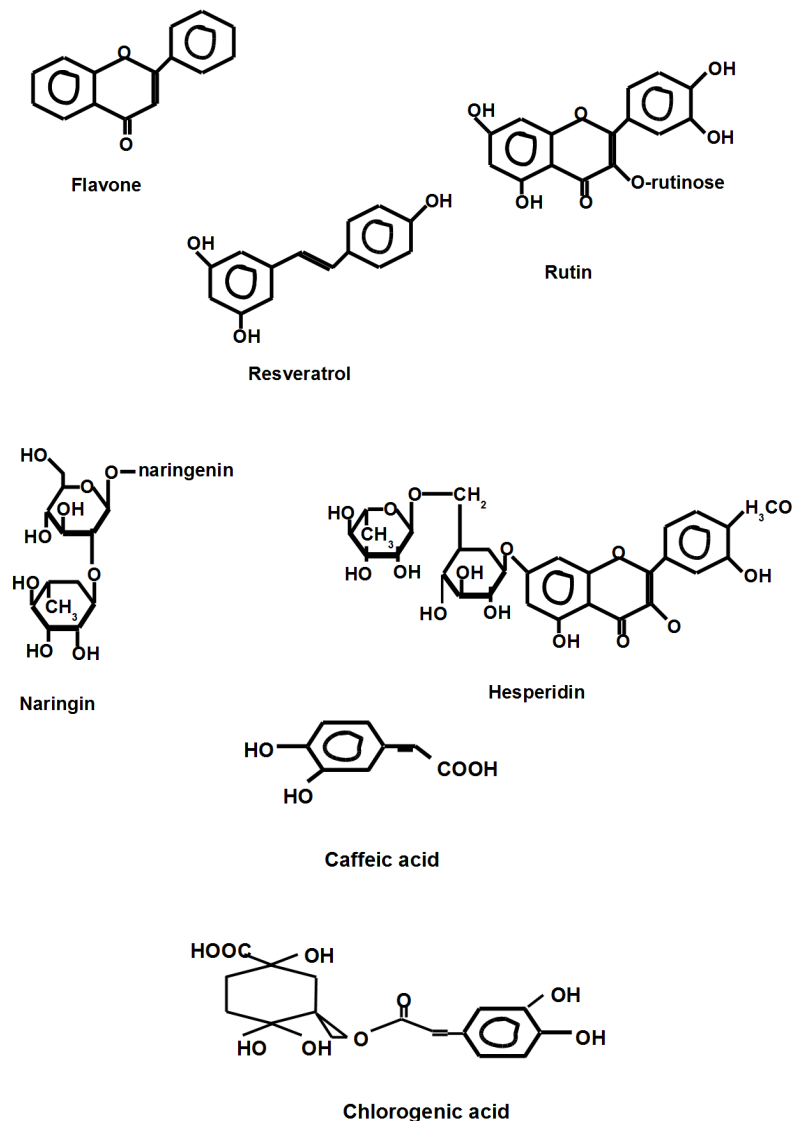


Fig. 4. Basic structures of some pharmacologically important plant derived phenolics

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 hemolysis 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 saponin 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 ellagitannins. On heating, they form pyrogallallic 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), daidzein, 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, rhinorrhoea and diarrhea.

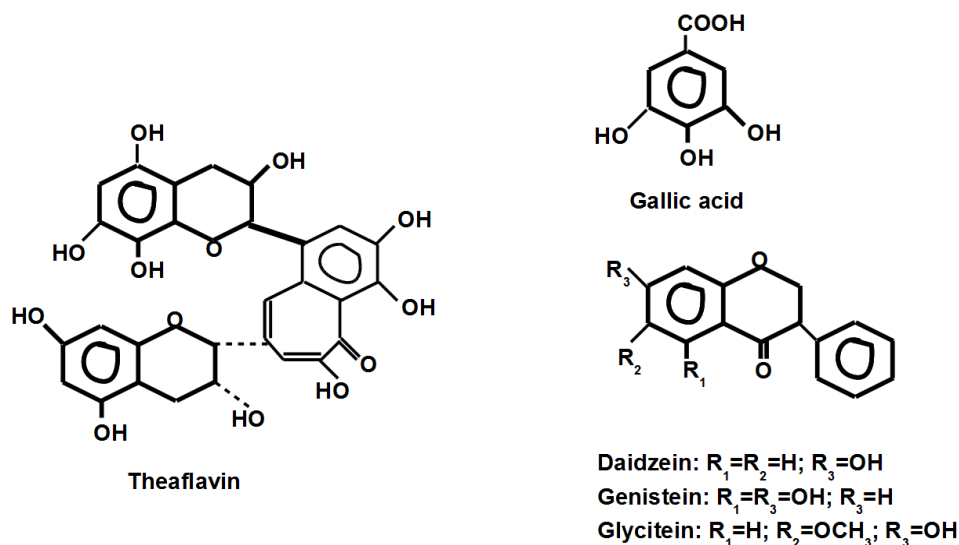


Fig. 5. Basic structures of some pharmacologically important plant derived tannins

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 (Firm, 2010). Terpenoids includes hydrocarbons of plant origin of general formula $(C_5H_8)_n$ and are classified as mono-, di-, tri- and sesquiterpenoids depending on the number of carbon atoms. Examples of commonly important monoterpenes 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 *triterpenes* (C₃₀) include steroids, sterols, and cardiac glycosides with anti-inflammatory, sedative, insecticidal or cytotoxic activity. Common triterpenes: amyrins, ursolic acid and oleanic acid *sesquiterpene* (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.

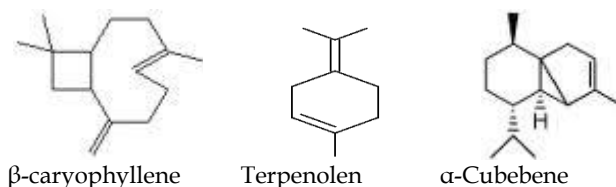


Fig. 6. Basic structures of some pharmacologically important plant derived terpenes

| 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 isoprene 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; Firm, 2010). Other derivatives such as chrysophanol, aloe-emodin, rhein, salinosporamide, 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).

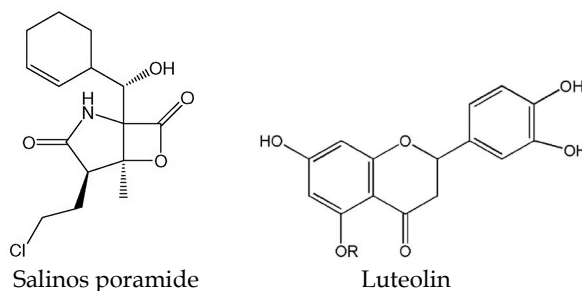


Fig. 7. Basic structures of some pharmacologically important plant derived anthraquinones

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 lysigenous 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. citrus 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 secretory 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).

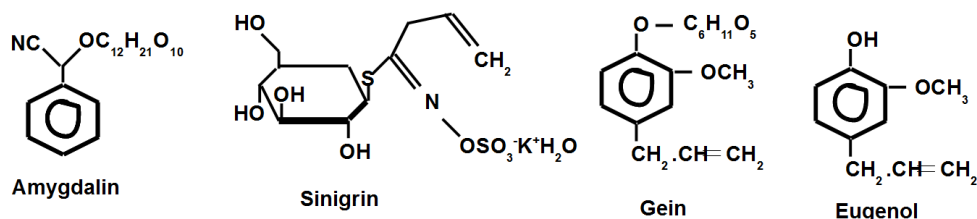


Fig. 8. Basic structures of some pharmacologically important plant derived essential oils

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

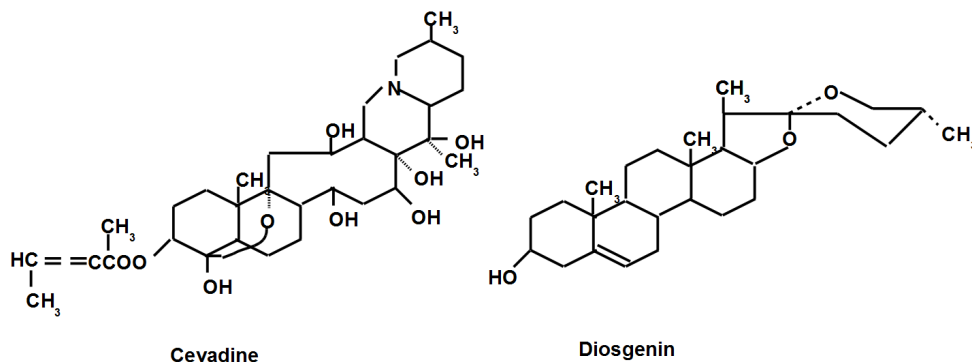


Fig. 9. Basic structures of some pharmacologically important plant derived steroids

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, peroxy 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 ischemia, 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 that 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, carotenoids, 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 Anticarcinogenesis

Polyphenols particularly are among the diverse phytochemicals that have the potential in the inhibition of carcinogenesis (Liu, 2004). Phenolic 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 metabolite 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 CO₂ (40%) and low concentration of O₂ (<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, IR β , 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 CO₂, 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 CO₂ 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 (CO₂ 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 then 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 (R_f) values, the colour of the separated bands, their absorption spectra, λ_{max} 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 quadrupole 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 violet (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'ios & 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'-diphenyl-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:

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

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 ($\text{Fe}^{2+}/2, 4, 6$, 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 (10-100 μl) in phosphate buffer (0.2 M, pH 6.6) and equal amounts of 1% (w/v) potassium ferricyanide are incubated at 50°C for

20 minutes and then the reaction terminated by the addition of equal volumes of 10% (w/v) tricarboxylic 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 LD₅₀ 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 vernacular names, the World Health Organization published standards for herbal safety to minimize adulteration 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 (*Syzygium aromaticum*), garlic (*Allium sativum*) lemon grass (*Cymbopogon citratus*) ginger (*Zingiber officinale*) cargueja (*Baccharis trimera*), and mint (*Mentha piperita*) 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 verocytotoxic 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

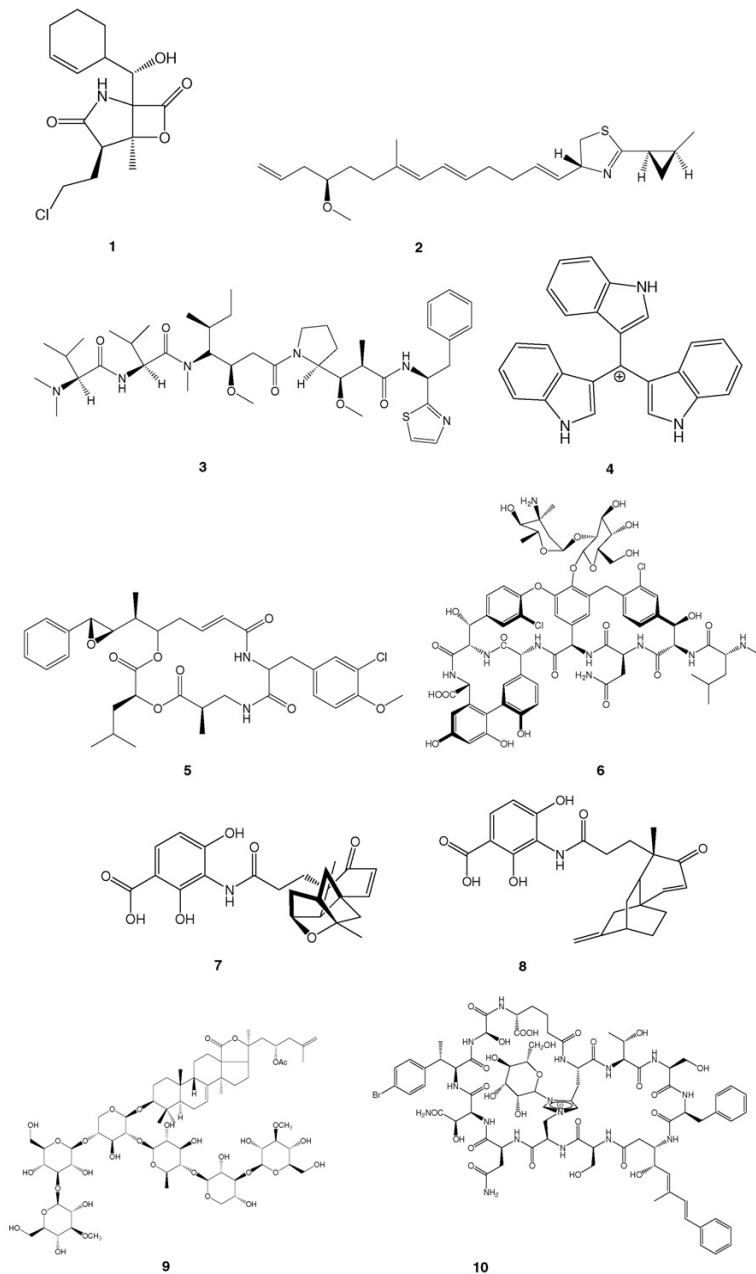


Fig. 10. Natural products - recently discovered and/or in development. (1) Salinosporamide A; (2) curacin A; (3) dolastatin 10; (4) turbomycin A; (5) cryptophycin; (6) vancomycin; (7) platensimycin; (8) platencin; (9) stichloroside; (10) theopalauamide (Source; Doughari *et al.*, 2009).

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 including 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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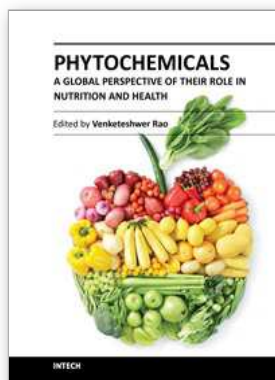
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Phytochemicals are biologically active compounds present in plants used for food and medicine. A great deal of interest has been generated recently in the isolation, characterization and biological activity of these phytochemicals. This book is in response to the need for more current and global scope of phytochemicals. It contains chapters written by internationally recognized authors. The topics covered in the book range from their occurrence, chemical and physical characteristics, analytical procedures, biological activity, safety and industrial applications. The book has been planned to meet the needs of the researchers, health professionals, government regulatory agencies and industries. This book will serve as a standard reference book in this important and fast growing area of phytochemicals, human nutrition and health.

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