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Review

Phytochemicals in diet and human health with special reference to polyphenols

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Abstract

Plants constitute an important source of natural products which differ widely in their structures, biological properties and mechanism of action. The medicinal value of plants lies in some chemical substances that produce a definite physiological action in the human body. The most important of these bioactive constituents of plants are phenolic compounds, *viz*. tannins, flavonoids which are responsible for their antioxidant activity. The antioxidant effect of phenolic compounds is mainly due to their redox properties and is the result of various possible mechanisms: free-radical scavenging activity, transition metal-chelating activity, and/or singlet oxygen quenching capacity. Antioxidant assays have been developed to measure total antioxidants in plant extracts to provide various biomarkers of oxidative stress. Assays on antioxidant capacity are classified into two types based on their reaction with free radicals, *viz*. assays based on hydrogen atom transfer (HAT) and assays based on electron transfer (ET) reactions. Besides their antioxidant properties, they exhibit a wide range of biological activities, such as anti-inflammatory, antibacterial, antiviral, antiallergic and anticarcinogenic effects. Nevertheless, epidemiological studies are useful tool to study the health effects of polyphenols. By utilizing *in vitro* biological activity assays, polyphenolic extracts and isolates can be tested for their biological response.

Keywords: Phytochemicals, polyphenols, anti-inflammatory, antibacterial, antiviral, antiallergic and anticarcinogenic effects.

1. Introduction

'Ayurveda' supposed to be the oldest medical system in the world, provides potential leads to find active and therapeutically useful compounds in plants. The World Health Organisation (WHO) estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs. Also, modern pharmacopoeia still contains at least 25% drugs derived from plants and many others which are synthetic analogues built on prototype compounds isolated from plants (WHO, 2000). Traditional herbal medicines form an important part of the health care system in India. The World's one fourth population is dependent on traditional medicines, particularly plant drugs for curing various ailments, proving that there is much more to get from herbs and spices than mere culinary function as seasonings, used to improve sensory properties of food (Iqbal, 2013). A wide array of plant derived active principles, representing numerous chemical compounds, has demonstrated consistent medicinal activity with their possible use in the treatment of several degenerative diseases (Tiwari *et al*., 2013). Medicinal plants have therapeutic potential due to the presence of natural antioxidants functioning as reducing agents, free radical scavengers and quenchers of singlet oxygen. Majority of their antioxidant activity is due to bioactive compounds, *viz*. flavones, isoflavones, flavonoids, anthocyanins, coumarins, lignans, catechins and isocatechins (Rajeshwari and Andallu, 2011). Currently there has been increasing interest globally to identify the antioxidant compounds that are pharmacologically potent and have low or no side effects for use in preventive medicine and in the food industry. As plants produce significant amounts of antioxidants to prevent the oxidative stress induced by the free radicals, they represent a potential source of new compounds with antioxidant activity (Ali *et al*., 2008).

Phytochemicals/bioactive compounds are non-nutritive plant chemicals, naturally occurring biologically active compounds in plants. The prefix 'phyto' is from a Greek word meaning plant. In plants, phytochemicals act as a natural defense system for host plants and provide color, aroma and flavor. More than 4000 of these compounds have been discovered to date and it is expected that scientists will discover many more. In fact, some people claim that many of the diseases afflicting human beings are the result of lack of phytonutrients in their diet. Phytonutrients have various health benefits, for example, they may have antimicrobial, anti-inflammatory, cancer preventive, antidiabetic and antihypertensive effects to mention but a few. The phytochemical constituent of a plant will often determine the physiological action on the human body (Awoyinka *et al.*, 2007).

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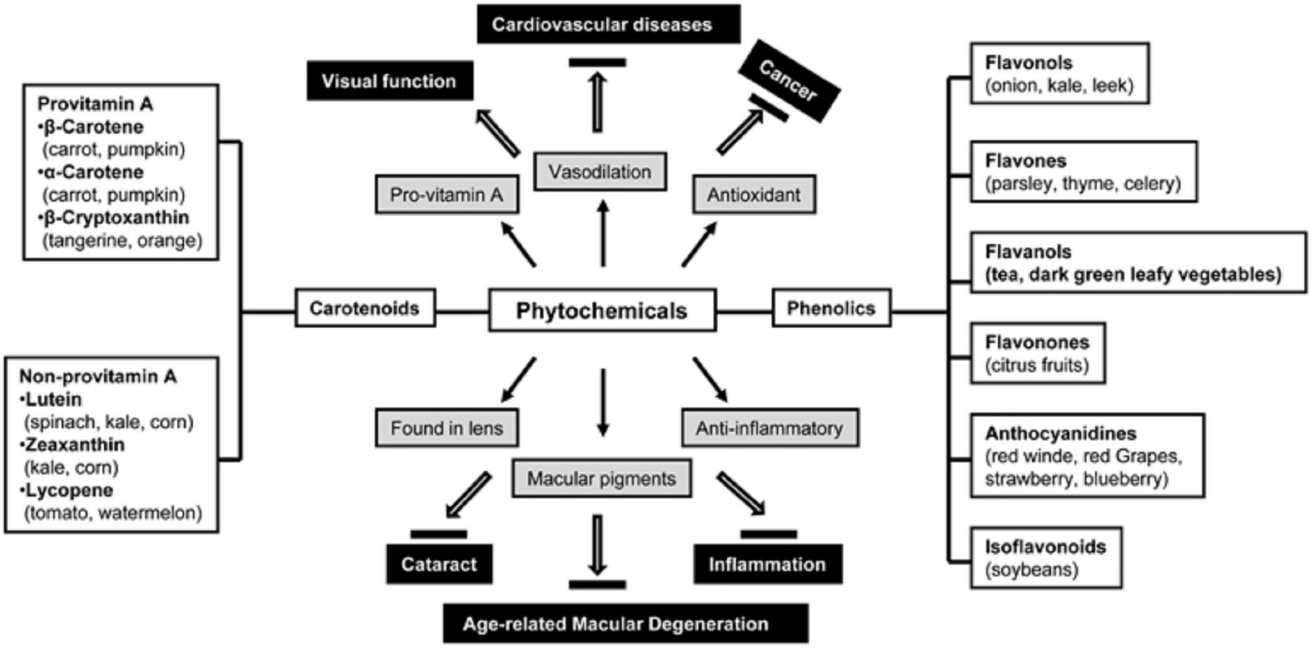

Figure 1: Multiple health benefits of phytochemicals (Rajeshwari *et al.*, 2013a)

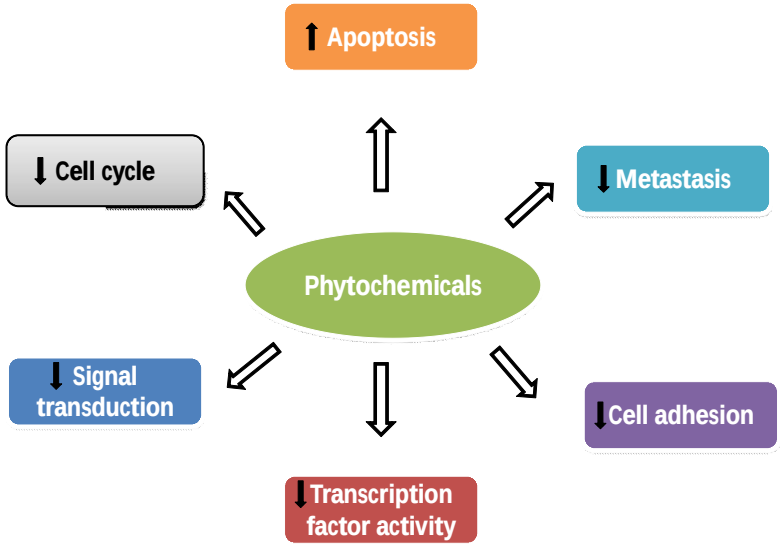

Figure 2: Therapeutic role of phytochemicals (Rajeshwari *et al.*, 2013a)

Overwhelming evidences from epidemiological studies indicate that diets rich in phytoconstituents are associated with a lower risk of several degenerative diseases. Phytochemicals provide protection against stress-induced diseases as they adopt multimodal therapeutic approach against multifactorial pathogenecity of diseases, *viz.* diabetes (controlling blood glucose and lipids), cancer (inhibition of one or more of the stages of cancer process) and inflammatory diseases [inhibition of proinflammatory enzymes such as lipoxygenase (LPO), cyclo-oxygenases (COX-1 and COX-2)] (Rajeshwari *et al.*, 2013a) (Figures 1 and 2), targets and drugs for effective disease management. Herbal products or plant products rich in phenolic compounds, flavonoids, terpenoids, coumarins, and other constituents show reduction in blood glucose levels. Therefore, to combat the ever-increasing oxidative stress-induced diseases, increased consumption of diet derived antioxidants may be particularly helpful as antioxidants, in diminishing cumulative

oxidative damage (Rajeshwari *et al*., 2013). In contrast to current combination therapies, however, plant based drugs contain a mixture of multiple components thereby saving considerable time and expense (Agarwal *et al*., 2009). However, the health promoting capacity of bioactive compounds in the foods strictly depends on their processing history (Agarwal *et al*., 2009).

The phytochemicals may also be specifically found in certain taxa of plants and vary in presence among different parts of plant tissues (Gottlieb, 1990). A complete understanding of medicinal plants involves a number of disciplines including commerce, botany, horticulture, chemistry, enzymology, genetics, pharmacology and quality control. There is a great wealth of knowledge concerning the medicinal properties of plants that continues to be transmitted from generation to generation. The use of modern isolation techniques and pharmacological testing make way for new plant drug as purified substances (Trease and Evans, 2008).

Secondary metabolites are a group of compounds that do not get involved in primary metabolism of the plant. But these compounds are now recognized to be involved in adaptional and survival mechanism. They are produced in specially designed secondary metabolic pathways. These compounds help the plants to face adversities, enemies and competition. Studies on the dynamics of secondary metabolism indicated that there is a definite turnover of these compounds evidenced by diurnal variation, seasonal variation and different stages of development. Alkaloids are defensive agents due to their bitter taste; volatile oils act as pheromone for pollination by insects and protect the plant from microbes, competition, *etc*; diterpenes or triterpenes perform wound healing and antimicrobial functions. Phenols act as antioxidant and protect cellular membranes and tissues containing lipids against oxidation. Anthrocyanins and flavonoids act as pollinator guide for insects as they are responsible for attractive coloration in flowers. All sulfur containing compounds are antimicrobial in nature. Cardiac glycosides are used by plants to protect from herbivore can be used in heart treatment. There are three major classes of secondary metabolites, the largest group being that of alkaloids followed by terpenoids and phenolics. Gums and mucilages are polysaccharides but considered as secondary metabolite due to their function (Daniel, 2006). There is growing interest in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity. Screening active compounds from plants has lead to the discovery of new medicinal drugs which have efficient protection and treatment roles against various diseases, including cancer and Alzheimer's disease (Soma *et al*., 2010).

1.1 Phytochemical screening

Medicinal plants have been a major source of treatment for human diseases since immemorial. The world's one-fourth population are dependent on traditional medicines, particularly plant drug for curing various ailments (Kumari *et al*., 2011). It is generally estimated that over 6000 plants in India are used in traditional, folklore and herbal medicine, representing about 75% of the medicinal needs of the third world countries (Rajashekharan, 2002). Majority of rural dwellers in developing countries still depend on medicinal plants to prevent or eliminate diseases (Parekha and Chanda, 2008). Medicinal herbs are moving from fringe to main stream use with a greater number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals. Recently, considerable attention has been paid to utilize ecofriendly and biofriendly plant based products for the prevention and cure of different human diseases. Considering the adverse effects of synthetic drugs, people are looking for natural remedies which are safe and effective (Johnson and William, 2002).

World plant diversity is the largest source of herbal medicine and it is now clear that, the medicinal values of these plants lies in the bioactive phytochemical constituents that produce definite physiological effects on human body (Santhi *et al*., 2011). These natural drugs formed the base of modern drugs as we use today (Edeoga *et al*., 2005). Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. Phytochemicals are basically divided into two groups that is primary and secondary metabolites; according to their functions in plant metabolism. Primary metabolites comprise common sugars, amino acids, proteins and chlorophyll while secondary metabolites consist of alkaloids, flavonoids, tannins and so on (Kumar *et al*., 2009).

In the recent past, there has been growing interest in exploiting the biological activities of different ayurvedic medicinal herbs, owing to their natural origin, cost effectiveness and lesser side effects (Chopra *et al*., 2009). Most of the medicinal plants, even today, are collected from wild. Commercial cultivation will put a check on the continued exploitation from wild sources and serve as an effective means to conserve the rare floristic wealth and genetic diversity (Naik *et al*., 2005).

Medicinal plants are nature's priceless gift to human. The development in the field of modern medicine temporarily subdued the traditional herbal medicine. But it has now staged a comeback and a "herbal renaissance" is blooming across the world. The acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has lead researchers to investigate the antimicrobial activity of plant extracts (Sumathi and Parvathi, 2010).

Herbal medicines are safer than synthetic medicines because the antimicrobial agents phytochemicals in the plant extract target the biochemical pathway present only in the microbes and not the biochemical pathway of plants and animals. Medicinal plants have been used all over the world for the treatment and prevention of various ailments, particularly in developing countries where infectious diseases are endemic and modern health facilities and services are inadequate (Zaidan *et al*., 2005).

Phytochemicals like carotenoids, tocopherols, ascorbates and phenols (Figure 3) present in the plants are strong antioxidants and have an important role in the health care system (Dhan *et al*., 2007). A main stream medicine is increasingly receptive to use of antimicrobials and other drugs derived from plants as traditional antibiotics (Amin and Kapadnis, 2005).

Medicinal principles are present in different parts of the plant like root, stem, bark, heartwood, leaf, flower, fruit or plant exudates. These medicinal principles are separated by different processes; the most common being extraction. Extraction is the separation of the required constituents from plant materials using a solvent. (i) Where it is sufficient to achieve within set limits equilibrium of concentration between drug components and the solution, *e.g*., tinctures, decoction, teas, *etc*. (ii) Where it is necessary to extract the drug to exhaustion, *i.e*. until all solvent extractables are removed by the solvent (Soma *et al*., 2010).

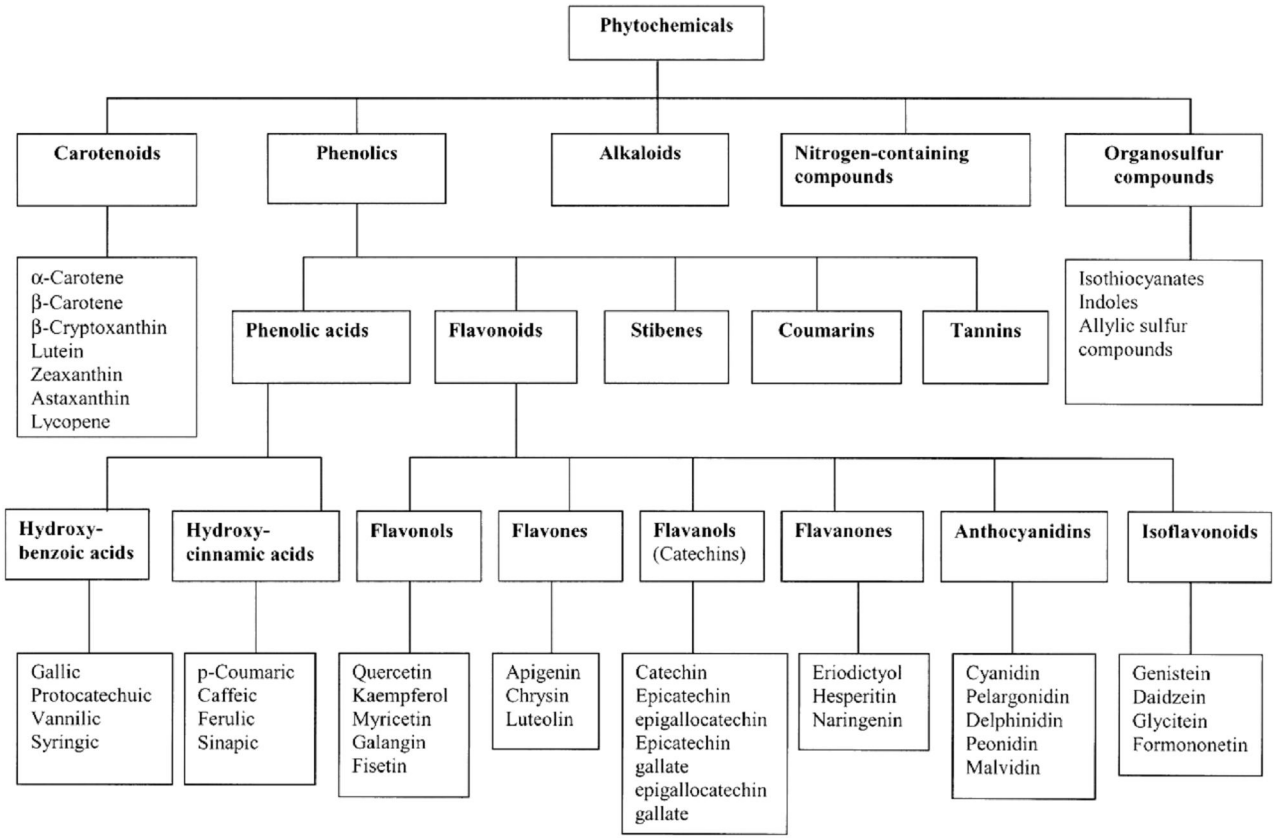

Figure 3: Classification of phytochemicals (Dhan *et al*., 2007)

Both the methods are employed depending on the requirement, although in industry the latter method is mostly used. In all industrial procedures, the raw material is pretreated with solvent outside the extractor before changing the latter. This prevents sudden bulk volume changes (which are the main cause of channelling during extraction) and facilitates the breaking up of the cell walls to release the extractables. To facilitate the extraction, the solvent should diffuse inside the cell and the substance must be sufficiently soluble in the solvent. The ideal solvent for complete extraction is one that is most selective, has the best capacity for extraction and is compatible with the properties of the material to be extracted. The increase in prevalence of multiple drug resistance has slowed down the development of new synthetic antimicrobial drugs, and has necessitated the search for new antimicrobials from alternative sources (Soma *et al*., 2010).

1.1.1 Carbohydrates

Complex carbohydrates obtained from natural sources such as plants have shown diverse biological activities such as wound healing, enhancement of the reticuloendothelial system, stimulation of the immune system, treatment of tumours and effects on the hematopoietic system (Talmadge *et al*., 2004). *Aloe vera* (L.) Burm.f. (*Aloe barbadensis* Miller) is a perennial succulent xerophyte, which develops water storage tissue in the leaves to survive in dry areas of low or erratic rainfall. Therefore, the thick fleshy leaves of aloe plants contain not only cell wall carbohydrates such as cellulose and hemicellulose but also storage carbohydrates such as acetylated mannans (Ni *et al*., 2004).

Aloe vera has been used for many centuries for its curative and therapeutic properties and although over 75 active ingredients from the inner gel have been identified, therapeutic effects have not been correlated well with each individual component (Habeeb *et al*., 2007). Many of the medicinal effects of aloe leaf extracts have been attributed to the polysaccharides found in the inner leaf parenchymatous tissue (Ni *et al*., 2004).

Macroalgae are regarded as a rich source of sulfated polysaccharides and the particular type of polysaccharide is different depending on the taxonomic group. Different carbohydrates including agar, carrageenan, or alginates are extracted from macroalgae and these carbohydrates are used widely in the food and pharmaceutical industries as functional ingredients such as stabilizers. Among their associated bioactive properties, immunomodulating, anticancer, anti-inflammatory, antiviral, or antioxidant activities have been pointed out (Li *et al*., 2008). Other minor sulfated polysaccharides, typically from particular species, are also known, such as porphyrans produced by *Porphyra*. In general, these polysaccharides can significantly vary in their composition, and therefore their related properties. For instance, the bioactivity might differ depending on the degree of sulfation, molecular weight, type of sugar found predominantly, and/or glycosidic branching (Qi *et al*., 2005).

1.1.2 Amino acids

Amino acid utilization and demand differ between the healthy state and various disease states. In the healthy state most circulating amino acids are derived from dietary proteins that are stored and broken down in the gut and released gradually into the portal circulation, and from continuous turnover of body protein. In disease states, the composition of amino acids derived from peripheral protein breakdown and released in the circulation, is different, for example because a substantial part of the branched-chain amino acids is broken down to yield glutamine and alanine, which are released in the circulation (Peter *et al*., 2004).

At the most simple level, an individual's requirement for amino acids can be divided into those necessary for growth itself and those that must be supplied to maintain both the body protein equilibrium and optimum physiological functions. Both are sensitive to the adequacy of amino acid supply (Peter and Peter, 2003).

Many chronic diseases are associated with deficiencies and imbalances of particular amino acids causing specific changes in requirements (Peter and Peter, 2004). Administration of extra nutritional support can alter the tumor-host nutritional balance so that host repletion may occur. Although the usefulness of nutritional support for correcting malnutrition in cancer patients is clear, the specificity and sensitivity and optimal choices of constituents for nutritional support continue to evolve (Landel *et al*., 1985).

1.1.3 Glycosides

Cyanogenic glycosides are widely distributed among 100 families of flowering plants. They are also found in some species of ferns, fungi and bacteria (Harborne, 1993). There are many economical important plants highly cyanogenic, including white clover, linum, almond, sorghum, the rubber tree and cassava (Tokarnia *et al*., 1994).

Phenylethanoid glycosides represent a group of natural products, which have been reported to have various biological activities. In a study conducted by Aydin *et al*. (2004), the mutagenic and/or antimutagenic activities of lavandulifolioside, verbascoside, leucosceptoside A and martynoside isolated from *Stachys macrantha* were investigated in human lymphocytes by single cell gel electrophoresis or 'comet' assay. The lymphocytes incubated with different concentrations of phenylethanoid glycosides with or without mitomycin C and DNA strand breakage was measured by comet assay (Cheeke, 1995).

At the phenylethanoid glycoside concentrations of 10, 50, 100, and 200 µg/ml, no additional DNA damage was observed whereas the frequency of damaged cells induced by mitomycin C was significantly decreased by phenylethanoid glycoside treatment. It seemed that in human lymphocytes, phenylethanoid glycosides showed protective effects on DNA damage induced by mitomycin C in a concentration dependent manner (Aydin *et al*., 2004).

1.1.4 Saponins

Saponins are a large family of structurally-related compounds of steroid or triterpenoid aglycone (sapogenin) linked to one or more oligosaccharide moieties by glycosidic linkage. The carbohydrate moiety consists of pentoses, hexoses, or uronic acids. The presence of both polar (sugar) and nonpolar (steroid or triterpene) groups provide saponins with strong surface-active properties (Makkar *et al*., 2007). Their physiochemical and biological properties feature structural diversity, which have led to a number of traditional and industrial applications (Martin and Briones, 1999). Many saponins exhibit distinct foaming properties. They are also added to shampoos, liquid detergents, toothpastes and beverages as emulsifier and long-lasting foaming agent (Tanaka *et al*., 1996). In addition, saponins of plants possess some pharmacological effects, such as molluscicidal (Huang *et al*., 2003), anti-inflammatory (Takagi *et al*., 1980), antimicrobial (Tamura *et al*., 2001), antihelmintic, antidermatophytic, antitussives and cytotoxic activities (Sparg *et al*., 2004). One research use of the saponin class of natural products involves their complexation with cholesterol to form pores in cell membrane bilayers, *e.g.* in red cell (erythrocyte) membranes, where complexation leads to red cell lysis (hemolysis) on intravenous injection (Francis *et al*., 2002). There is evidence of the presence of saponins in traditional medicine preparations, where oral administrations might be expected to lead to hydrolysis of glycoside from terpenoid (Asl and Hossein, 2008).

Camellia oleifera is an important source of edible oil in tropical and sub-tropical regions of Asia, especially in China. It is used in cooking and as a medicine for the treatment of intestinal disorders and burn injuries (Chen *et al*., 2007). The defatted seed meal, which contains saponins, is extensively used in aquaculture to eliminate unwanted fish and harmful insects in prawn ponds (Tang, 1961). The major active ingredients of the defatted seed meal of *C. oleifera* are saponins. Previous phytochemical studies have identified various types of saponins, including camelliasaponin, theasaponin E1, theasaponin E2, and sasanquasaponin from the defatted seed meal of *C. oleifera* (Chaicharoenpon and Petsom, 2009).

1.1.5 Tannins

Tannins have a characteristic strange smell and astringent taste and could bind to proteins and consequently precipitate proteins through the effective formation of strong complexes with proteins and other macromolecules. Thus, they could have a major impact on animal nutrition, including inhibition of digestive enzymes responsible for growth (Bennick, 2002). Tannins have been implicated with various pharmaco-therapeutic effects (Ferreira *et al*., 2008). Tannins, in the form of proanthocyanidins, have a beneficial effect on vascular health. Topical applications of tannins help to drain out all irritants from the skin. They are useful as anti-inflammatory agents in the treatment of burns and other wounds based on their antihemorrhagic and antiseptic potentials. In particular, tannin-rich remedies are used as antihelmintics (Ketzis *et al*., 2006), antioxidants (Koleckar *et al*., 2008), antimicrobials and antivirals (Buzzini *et al*., 2008), in cancer chemotherapy (Chung *et al*., 1998) and to chelate dietary iron (Clauss *et al*., 2007).

Tannins, polyphenols with widely varying chemistry, are one of the major phytochemicals found in many higher plants. Although, tannins are widely distributed in almost all plant foods (Serrano *et al*., 2009), the primary source of tannins used as active pharmaceutical ingredients are the medicinal plants, implying that the pharmacologic effect of tannins is dependent on plant type (Okuda, 2005).

Samanea saman is a large canopied tree *S. saman* has local aesthetic properties. The leaf is made into an infusion in Jamaica for treating blood pressure and the seeds are chewed in tropical Africa for treating inflammation of the gums and throat. A study conducted by Ukoha *et al*. (2011), revealed that *S. saman* can be used as a significant source of natural antimicrobial and antifungal in the form of tannins.

1.1.6 Terpenoids

Terpenoids are the largest and most widespread class of secondary metabolites, mainly in plants and lower invertebrates. These compounds are found in higher plants, mosses, liverworts, algae and lichens, as well as in insects, microbes or marine organisms. Terpenoids are derived from a common biosynthetic pathway based on mevalonate as parent, and are named terpenoids, terpenes or isoprenoids with the subgroup of steroids among them as a class (Bohlmann and Keeling, 2008). In general, the term terpene is used to denote compounds containing an integral number of C_5 units and chemically all terpenoids can be considered to be derived from the basic branched C_5 unit isoprene (2-methyl-1,3-butadiene). It is useful to classify terpenoids according to the number of such C_5 units present in the molecule, from which they are biogenetically derived. Thus, terpenoids are classified into hemi-,mono-, sesqui-, di- sester-, tri- and tetra terpenoids (carotenoids) having 1, 2, 3, 4, 5, 6 and 8 isoprenoid C_5 residues, respectively (Tholl, 2006).

Monoterpenes are substances derived from isoprene hydrocarbons (2-methyl-1,3-butadiene) and originated by the attachment of two or more isoprene molecules. They are of plant origin and can exert a wide spectrum of biological actions of great importance in many different areas from food chemistry and chemical ecology to pharmacology and pharmaceutics (Perillo and Zygadlo, 1992). The action mechanism of some terpenes involves membrane receptor-mediated effects and stereo specificity was demonstrated in one case (Granger *et al*., 2005).

In pharmaceutics, it is known to maintain effective plasma concentrations of drugs as well as to reduce dose and dose-dependent toxicity and adequate zero-order delivery. Hence, the transdermal route is a better alternative to per oral administration, which additionally provides better patient compliance. However, transdermal permeability is poor for hydrophilic drugs. In order to enhance the transdermal permeation rate of several drug molecules, oxygenated terpenes, claimed generally regarded as safe (GRAS) status, have been extensively used (Narishetty and Panchagnula, 2004).

1.1.7 Phytosterols

Phytosterols, which encompass plant sterols and stanols, are steroid compounds similar to cholesterol which occur in plants and vary only in carbon side chains and/or presence or absence of a double bond. Stanols are saturated sterols, having no double bonds in the sterol ring structure. More than 200 sterols and related compounds have been identified. Free phytosterols extracted from oils are insoluble in water, relatively insoluble in oil, and soluble in alcohols (Akhisa and Kokke, 1991).

The richest naturally occurring sources of phytosterols are vegetable oils and products made from them. Nuts, which are rich in phytosterols, are often eaten in smaller amounts but can still significantly contribute to total phytosterol intake. Cereal products, vegetables, fruit and berries which are not as rich in phytosterols may also be significant sources of phytosterols due to their higher intakes (Valsta *et al*., 2007). The intake of naturally occurring phytosterols ranges between 150-450 mg/day (Ostlund and Richard, 2002) depending on eating habit. Specially designed vegetarian experimental diets having been produced yielding upwards of 700mg/day. The most commonly occurring phytosterols in the human diet are β-sitosterol, campesterol and stigma sterol which account for approximately 65%, 30% and 3% of diet contents, respectively (Weihrauch and Gardner, 1978). The most common plant stanols in the human diet are sitostanol and campestanol which combined make up about 5% of dietary phytosterols (Andersson *et al*., 2004).

1.1.7.1 *Cholesterol lowering property of phytosterols*

The ability of phytosterols to reduce cholesterol levels was first demonstrated in humans (Pollak, 1953). They were subsequently marketed as a pharmaceutical under the name Cytellin as a treatment for elevated cholesterol from 1954-1982 (Jones, 2007). Unlike the statins, where cholesterol lowering has been proven to reduce cardiovascular disease (CVD) risk and overall mortality under well defined circumstances, no such effect has ever been documented with phytosterol enriched foods or phytosterol OTC medications (Genser *et al*., 2012). While cholesterol lowering was frequently used as a surrogate endpoint for beneficial effects on CVD counter-examples exist where specific medications for cholesterol lowering were found to have unfavorable effect on clinical endpoints such as with ezetimibe. Co-administration of statins with phytosterol enriched foods increases the cholesterol lowering effect of phytosterols, again without any proof of clinical benefit and with anecdotal evidence of dangerous adverse effects (Weingartner *et al*., 2008). Statins work by reducing cholesterol synthesis by inhibiting the rate-limiting HMG-CoA reductase enzyme. Phytosterols reduce cholesterol levels by competing with cholesterol absorption in the gut, a mechanism which complements statins. Phytosterols further reduce cholesterol levels by about 9% to 17% in statin users (Scholle *et al*., 2009). The type or dose of statin does not appear to affect phytosterols' cholesterol lowering efficacy (Katan *et al*., 2003).

Elevated triglyceride levels are a risk factor for CVD (Malloy and Kane, 2001). Beyond LDL-C lowering, growing evidence suggest that phytosterols reduce triglyceride levels as well. Triglycerides were found to be reduced by 14% in individuals supplementing 1.6g/day of plant sterols in a fermented milk beverage for 6 weeks (Plana *et al*., 2008). The proposed mechanism behind the triglyceride lowering effect of phytosterols is due to a reduction in triglyceride rich VLDL particle produced by the liver (Plat and Mensink, 2009). The triglyceride lowering effects of phytosterols may be more pronounced in individuals with elevated triglycerides (Theuwissen *et al*., 2009).

1.1.7.2 *Phytosterols as a marker for cholesterol absorption*

The use of serum plant sterol levels to predict cholesterol absorption was first proposed by Tilvis and Miettinen (1986). These researchers showed that serum levels of campesterol when normalized for total serum cholesterol, correlated positively with cholesterol absorption in healthy populations. Phytosterols should also not be used as surrogates for cholesterol absorption in situations when phytosterols are being supplemented (Vanstone and Jones, 2004). Phytosterols may inhibit lung, stomach, ovarian and breast cancers (Woyengo *et al*., 2009). 24-epibrassinolide, a brassinosteroid, modulates superoxide dismutase, catalase, and glutathione peroxidase activity (Carange, 2011).

1.1.8 Phenolic compounds

Phenolic compounds are secondary plant metabolites, which are important determinants in the sensory and nutritional quality of fruits, vegetables and other plants (Lapornik *et al*., 2005). They are

widely spread throughout the plant kingdom, have been associated with color, sensory qualities, and nutritional and antioxidant properties of food. These compounds are one of the most widely occurring groups of phytochemicals, are of considerable physiological and morphological importance in plants. Phenolics may acts as phytoalexins (Popa *et al*., 2008), antifeedants, attractants for pollinators, contributors to plant pigmentation, antioxidants and protective agents against UV light (Naczk and Shahidi, 2006).

More than 8000 polyphenolics, including over 4000 flavonoids have been identified, and the number is still growing (Harborne *et al*., 1999). Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C_6-C_3-C_6 configuration. Essentially the structure consists of two aromatic rings, A and B, joined by a 3-carbon bridge, usually in the form of a heterocyclic ring C. The aromatic ring A is derived from acetate/ malonate pathway, while ring B is derived from phenyl alanine through the shikimate pathway (Merken and Beecher, 2000).

Several relationships between chemical structure and antioxidant activity of phenolic compounds have been explained (Lopez *et al*., 2003):

- The antioxidant potential of phenolics depends on the number and arrangement of the hydroxyl groups.
- The enhanced ABTS values for the catechins-gallate esters in relation to catechins reflect the additional contribution from the trihydroxy benzoate gallic acid.
- The contribution of the 3- hydroxyl group in flavonoids is very significant. Blocking the 3-hydroxyl group in the B ring decreases the antioxidant activity.
- The presence of an ortho-dihydroxy substitution in the B ring confers higher stability to the radical structure and participates in electron delocalization and plays an important role in the antioxidant activity.

Classification of phenolic compounds has been shown in Figure 4, which is given below:

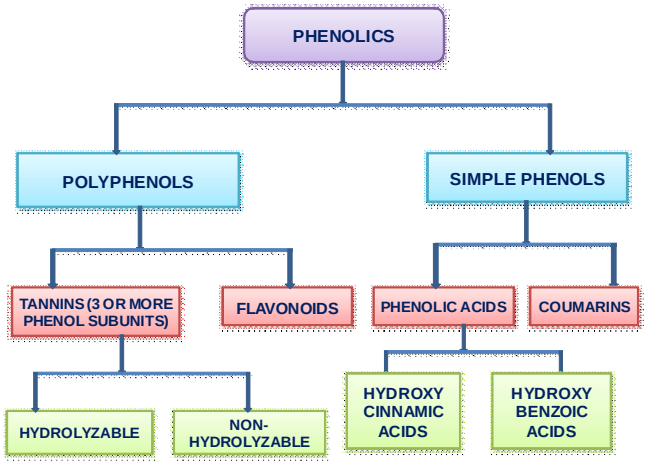

Figure 4: Classification of phenolic compounds (Harborne *et al*., 1999)

There are several mechanisms for antioxidant activity, but it is believed that radical scavenging *via* hydrogen atom donation is the predominant mode. Other established antioxidant mechanisms involve radical complexing of pro-oxidant metals, as well as quenching through electron donation and singlet oxygen quenching. Polyphenols are considered chemopreventive agents because they can quench or prevent the formation of ROS and RNS, which play important roles in carcinogenesis (D'Angelo *et al*., 2009). Polyphenols, abundant micronutrients in our diet, play a role in the prevention of various diseases associated with oxidative stress, *viz*. cancer, cardiovascular disorders and neurodegenerative diseases. Polyphenols are diverse in nature, but generally can be classified into phenolic acids, flavonoids and lignans. This group of compounds have been shown to possess varying degrees of antioxidant activity (Atawodi *et al*., 2009).

1.1.9 Alkaloids

One of the largest groups of chemical compounds produced by plants is alkaloid. Alkaloids are more or less toxic substances. They act primarily on the central nervous system (CNS). They have a basic character, containing heterocyclic nitrogen, and are synthesized in plants from amino acids or their immediate derivatives. In most cases, they are of limited distribution in the plant kingdom. Alkaloids are very important in the medicinal world and are used as powerful drugs mainly due to their sedative properties and powerful effect on the nervous system. Many current drugs are derived from alkaloids. Alkaloids are produced in actively growing tissue and rarely occur in dead tissue. Researches on production of useful alkaloids by plant tissue culture have also been carried out for more than 25 years (Okwu, 2005).

1.1.10 Flavonoids

Flavonoids are another group of plant secondary metabolites which are present almost universally in higher plants and contribute to the color of flowers and fruits. They impart mostly red, yellow, blue and violet colour to plant organs. Chemically they are phenolic compounds and most of them have flavone nucleus with two side aromatic rings (Figure 5). All flavonoids share a basic C_6-C_3-C_6 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). Division into further groups is made on the basis of the central ring oxidation and on the presence of specific hydroxyl groups. Most common flavonoids are flavones (with a C_2-C_3 double bond and a C4-oxo function), flavonols (flavones with a 3-OH group) and flavanones (flavone analogues but with a C_2-C_3 single bond), and abundant isoflavonoids include isoflavones (the analogue of flavones). 4-arylcoumarin (a neoflavonoid with a C_3-C_4 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 C_6-C_3-C_6 backbone, and are henceforth included in the general group of flavonoids (Pinheiro and Justino, 2010).

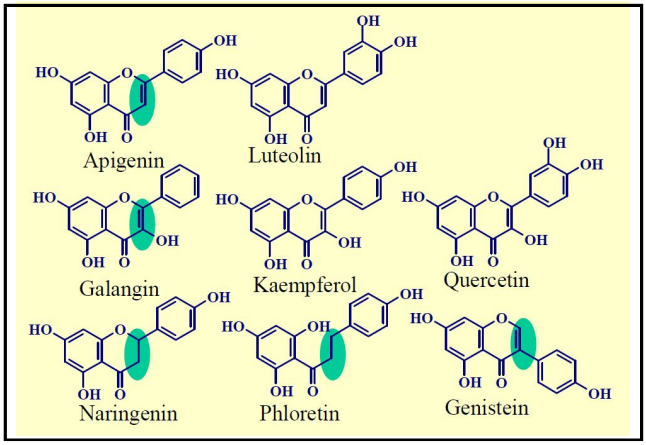

Figure 5: Structure of some common flavonoids in herbs and spices (Pinheiro and Justino, 2010)

1.1.10.1 *General health benefits of flavonoids*

Food phenolic compounds, particularly flavonoids, are thought to play important roles in human health (Huang *et al*., 1994). *In vitro* and animal studies have demonstrated that flavonoids have antioxidant and antimutagenic activities (Aherne and Obrien, 2002) and may reduce the risk of cardiovascular disease and stroke (Duthie *et al*., 2000). Isoflavonoids, such as phytoestrogens, have a wide range of hormonal and non hormonal activities in animals or *in vitro*, suggesting potential human health benefits of diets rich in these compounds (Cassidy *et al*., 2000). Flavonoids may act as antioxidants to inhibit free-radical mediated cytotoxicity and lipid peroxidation, as antiproliferative agents to inhibit tumor, growth or as weak estrogen agonists or antagonists to modulate endogenous hormone activity. In this way, flavonoids may confer protection against chronic diseases such as atherosclerosis and cancer and assist in the management of menopausal symptoms. Thus, flavonoids have been referred to as semi-essential food components (Kuhnau, 1976).

Early studies have uncovered some properties of tea polyphenols related to human health (Chow and Kramer, 1990), including a capillary-strengthening property, an antioxidative property responsible for the radioprotective effect, and antimicrobial property. Hara (1992) showed that the habit of tea drinking could prevent cardiovascular diseases by increasing plasma antioxidant capacity in humans. Flavonoids have shown many biological properties that account for cancer chemoprevention. In recent years, considerable attention has been paid to their abilities to inhibit the cell cycle, cell proliferation, and oxidative stress, and to induce detoxification enzymes, apoptosis, and activate the immune system (Birt *et al*., 1999).

Cardiotonic activity

Flavonoids have been reported to have action on the heart. The unsubstituted parent flavone exerts coronary dilatory activity and was commercially available under the name ‘Chromocor’ and its combination with rutine and isoquercetin was also available with brand name ‘flavoce’, useful in the treatment of atherosclerosis. 3-methyl quercetin has positive chronotropic effect on guinea pig right atrium and antiarrhythmic effect on left atrium (Lackeman *et al*., 1986). A study on mouse reported that the cardiotoxicity (negative inotropic effect) of doxorubicin on the mouse left atrium has been inhibited by flavonoids, 7-monohydroxy ethyl rutoside and 7-1,3',4'- trihydroxyethyl rutoside (Huesken *et al*., 1995).

Lipid lowering activity

Oxidative modification of low-density lipoproteins (LDL) by free radicals is an early event in the pathogenesis of atherosclerosis. The rapid uptake of oxidatively modified LDL *via* a scavenger receptor leads to the formation of foam cells. Oxidized LDL also has a number of other atherogenic properties. A number of mechanisms are likely to contribute to inhibition of LDL oxidation by flavonoids. Flavonoids may directly scavenge some radical species by acting as chain breaking antioxidants (De-Whalley *et al*., 1990). In addition, they may recycle other chain-breaking antioxidants such as α-tocopherol by donating a hydrogen atom to the tocopheryl radical (Francel *et al*., 1993). Transition metals such as iron and copper are important pro-oxidants, and some flavonoids can chelate divalent metal ions, hence preventing free radical formation. The ability of quercetin, and the quercetin glycosides, to protect LDL against oxidative modification has shown a significant protective effect (Mc Anlis *et al*., 1997).

Liquiritigenin showed a significant fall in serum cholesterol, LDL-cholesterol and atherogenic index. Influence of flavonoids on blood coagulation has been studied. The anticoagulant action of heparin was antagonized by flavonoids extracted from *T. hircanicum*. Ability of different flavonoids to inhibit the procoagulant activity of adherent human monocytes has been studied and hinokiflavone, a bioflavonoid has been found to be most active in inhibiting the interleukin-1b induced expression of tissue factor on human monocytes (Raj and Shalini, 1999). Silymarin counteracts ethanol-induced lipid peroxidation injury by reducing liver MDA and raising GSH levels (Parmar and Shikha, 1998).

Antioxidant activity

Free radical production in animal cells can either be accidental or deliberate. With the increasing acceptance of free radicals as common place and important biochemical intermediates, they have been implicated in a large number of human diseases (Ares and Outt, 1998). Quercetin, kaempferol, morin, myricetin and rutin by acting as antioxidants exhibited several beneficial effects, such as anti-inflammatory, antiallergic, antiviral as well as an anticancer activity. They have also been suggested to play a protective role in liver diseases, cataracts, and cardiovascular diseases. Quercetin and silybin acting as free radical scavengers were shown to exert a protective effect in reperfusion ischemic tissue damage (Hillwell, 1994). The scavenging activity of flavonoids has been reported to be in the order: myricetin > quercetin > rhamnetin > morin > diosmetin > naringenin > apigenin > catechin > 5,7- dihydroxy-3',4',5'-trimethoxyflavone > robinin > kaempferol > flavon3 (Ratty, 1988). Stabilization of meat lipids with flavonoids has been studied and morin, myricetin, kaempferol and quercetin at a level of 200 ppm were found to be most effective (Robak and Glyglewski, 1988). Induction period of lipid oxidation in canola oil was delayed with the flavonoid myricetin up to fifteen days. Formation of oxidation products was also inhibited by 69%, during this period. Morin, myricetin, kaempferol and quercetin have also been suggested as stabilizers for fish oil as an alternative to synthetic antioxidants (Husain *et al*., 1987).

Anti-inflammatory activity

A number of flavonoids are reported to possess anti-inflammatory activity. Hesperidin, a citrus flavonoid possesses significant anti-inflammatory and analgesic effects. Recently, apigenin, luteolin and quercitin have been reported to exhibit anti-inflammatory activity. Quercetin, gallic acid ethyl ester and some as yet unidentified flavonoids might account for the antinociceptive action reported for the hydroalcoholic extract of *Phyllanthus caroliniensis*. Treatment with silymarin demonstrated reversal of the carrageenan-induced biochemical changes (Shahidi *et al*., 1998).

Antimicrobial activity

Flavonoids and esters of phenolic acids were investigated for their antibacterial, antifungal and antiviral activities. All samples were active against the fungal and gram-positive bacteria test strains and most showed antiviral activity (Wild and Fasal, 1980).

(i) Antibacterial activity: Antibacterial activity has been displayed by a number of flavonoids. Twenty five out of one hundred and eighty two flavonoids were found to be active against many bacteria. Most of the flavonones having no sugar moiety showed antimicrobial activities whereas none of the flavonols and flavonolignans tested showed inhibitory activity on the microorganisms (Wild and Fasal, 1980).

(ii) Antifungal activity: Number of flavonoids isolated from peel of tangerine orange, when tested for fungistatic activity towards *Deuterophoma tracheiphila* showed promising activity. Chlorflavonin was the first chlorine-containing flavonoid type antifungal antibiotic produced by strains of *Aspergillus candidus* (Tencate *et al*., 1973).

(iii) Antiviral activity: Flavonoids also displayed antiviral, including anti-HIV activity. It has been found that flavonols are more active than flavones against herpes simplex virus type 1 and the order of importance was galangin > kaempferol > quercitin (Thomas *et al*., 1988). Recently, a natural plant flavonoid polymer of molecular weight 2100 Daltons was found to have antiviral activity against two strains of type-1 herpes type simplex virus, including a thymidine-kinase deficient strain and type -2 herpes simplex virus (Loewenstein, 1979). Out of twenty eight fiavonoids tested, flavan-3-ol was more effective than flavones and flavonones in selective inhibition of HIV-1, HIV-2 and similar immunodeficiency virus infections (Gerdin and Srensso, 1983).

1.2 Extraction of bioactive compounds

Considering the great variations among bioactive compounds and huge number of plant species, it is necessary to build up a standard and integrated approach to screen out these compounds carrying human health benefits. Extraction, a pharmaceutically used term can be defined as the technique used for the separation of therapeutically desired active constituent(s) and elimination of unwanted insoluble material by treatment with selective solvents. Extraction mainly involves the release of complex plant constituents and solubilization of secondary metabolites from the matrix, followed by separation of soluble target compounds from the crude extract through selective use of solvents. Different extraction techniques should be used in diverse conditions for understanding the extraction selectivity from various natural sources. Different techniques, many of them remain almost same through hundreds of years; can also be used to extract bioactive compounds. All these techniques have some common objectives, (i) to extract targeted bioactive compounds from complex plant sample, (ii) to increase selectivity of analytical methods, (iii) to increase sensitivity of bioassay by increasing the concentration of targeted compounds, (iv) to convert the bioactive compounds into a more suitable form for detection and separation, and (v) to provide a strong and reproducible method that is independent of variations in the sample matrix (Naczk and Shahidi, 2004).

The basic parameters influencing the quality of an extract are - part of the plant used as starting material, the solvent used for extraction, the extraction technology used with the type of equipment employed, and crude drug: extract ratio. Other important parameters affecting the yield of the extraction procedure are the moisture content of the plant material and temperature. Traditional extraction processes involve extraction with water or organic solvents. Water is almost the solvent used universally to extract activity. Initial screening of plants for possible antimicrobial activities typically begins by using crude aqueous or alcohol extractions. Starches, polypeptides and lectins are better extracted in water. Coumarins and fatty acids are better extracted in ether, whereas methanol is good for extracting lactones and phenones (Cowan, 1999).

1.3 Conventional extraction techniques

Bioactive compounds from plant materials can be extracted by various classical extraction techniques. Most of these techniques are based on the extracting power of different solvents in use and the application of heat and/or mixing. In order to obtain bioactive compounds from plants, the existing classical techniques are: (i) Soxhlet extraction, (ii) Maceration and (iii) Hydrodistillation (Cowan, 1999).

Soxhlet extractor was first proposed by German chemist Franz Ritter Von Soxhlet in 1879. It was designed mainly for extraction of lipids but now it is not limited for this only. The Soxhlet extraction has widely been used for extracting valuable bioactive compounds from various natural sources. It is used as a model for the comparison of new extraction alternatives. Generally, a small amount of dry sample is placed in a thimble. The thimble is then placed in distillation flask which contains the solvent of particular interest. After reaching to an overflow level, the solution of the thimble-holder is aspirated by a siphon. Siphon unloads the solution back into the distillation flask. This solution carries extracted solutes into the bulk liquid. Solute is remained in the distillation flask and solvent passes back to the solid bed of plant. The process runs repeatedly until the extraction is completed.

Maceration was used in homemade preparation of tonic from a long time. It became a popular and inexpensive way to get essential oils and bioactive compounds. For small scale extraction, maceration generally consists of several steps. Firstly, grinding of plant materials into small particle is used to increase the surface area for proper mixing with solvent. Secondly, in maceration process, appropriate solvent named as menstruum (a solvent used in extracting compounds from plant and animal tissues) is added in a closed vessel. Thirdly, the liquid is strained off but the marc which is the solid residue of this extraction process is pressed to recover large amount of occluded solutions. The obtained strained and the press out liquid are mixed and separated from impurities by

filtration. Occasional shaking in maceration facilitates extraction by two ways; (i) increase diffusion, (ii) remove concentrated solution from the sample surface for bringing new solvent to the menstruum for more extraction yield (Cowan, 1999).

Hydrodistillation is a traditional method for extraction of bioactive compounds and essential oils from plants. Organic solvents are not involved and it can be performed before dehydration of plant materials. There are three types of hydrodistillation: water distillation, water and steam distillation and direct steam distillation. In hydrodistillation, first, the plant materials are packed in a still compartment; second, water is added in sufficient amount and then brought to boil. Alternatively, direct steam is injected into the plant sample. Hot water and steam act as the main influential factors to free bioactive compounds of plant tissue. Indirect cooling by water condenses the vapor mixture of water and oil. Condensed mixture flows from condenser to a separator, where oil and bioactive compounds separate automatically from the water. Hydrodistillation involves three main physicochemical processes; hydrodiffusion, hydrolysis and decomposition by heat. At a high extraction temperature, some volatile components may be lost. This drawback limits its use for thermo labile compound extraction. Extraction efficiency of any conventional method mainly depends on the choice of solvents (Cowan, 1999).

Expansion of the chemistry of natural products is indicative of organic chemist's interest in the plant kingdom for finding new phytoconstituents of therapeutic value, precursors for the synthesis of complex chemical substances, or new sources of compounds of economic value. Wide phytochemical surveys of alkaloids, saponins, tannins, *etc*., have been carried out in different countries. There are more than 4,20,000 distinct plant species, yet less than 10% of them have been fully analyzed. The isolation and purification of these distinct species is a major goal of the biotechnology and pharmaceutical industries with screening procedures for phytochemical analysis. However, many of the plant secondary metabolites have been successfully isolated /fractionated and purified using various methods (Farnsworth, 1996).

1.4 Fractionation of active extracts

Once a particular activity has been confirmed to be possessed by an extract (crude preparation), investigators try to analyze it further to know its composition. Efforts are made to isolate the active component(s) responsible for the activity. A variety of separation techniques are available for fractionation of the crude extracts. After separation is achieved, fractions (simpler mixtures) obtained are subjected to a number of further analytical investigations in order to obtain more information on the properties of their constituent substances (Houghton and Raman, 1998). Broadly, further investigations involve:

(i) Qualitative chemical analyses-determination of the nature of the constituents of a mixture or the type of an isolated compound.

(ii) Quantitative chemical analyses-determination of the purity of an isolated substance or the concentration of a single substance or a group of substances in a mixture.

(iii) Bioassay- determination of the biological activity of substances and the dose range over which they exert their effects.

The amount of fraction available is possibly the most important factor in making decisions about its future treatment and analysis. Investigative methods can be either nondestructive or destructive. Usually chemical tests for different chemical classes, *i.e*. phytochemical screening, is followed by suitable chromatographic and spectroscopic methods. In identifying a plant constituent, it is necessary first to determine the class of compound and then to find out which particular substance it is within that class. The class of compound is usually clear from its response to color tests, its solubility and R_f properties and its UV spectral characteristics. Complete identification within the class depends on measuring other properties and then comparing these data with those in the literature. These properties include melting point/boiling point, optical rotation and retardation factor (R_f) or retention factor (R_t) under standard conditions (Harborne, 1998).

1.4.1 Column chromatography

Column chromatography is one of the most useful methods for the separation and purification of plant constituents individually. It is another solid-liquid technique in which the two phases are: a solid (stationary phase) and a liquid (mobile phase). The theory of column chromatography is analogous to that of thin-layer chromatography. The most common adsorbents - silica gel and alumina - are the same ones used in TLC. The sample is dissolved in a small quantity of solvent (the eluent) and applied to the top of the column. The eluent, instead of rising by capillary action up a thin layer, flows down through the column filled with the adsorbent. Just as in TLC, there is an equilibrium established between the solute adsorbed on the silica gel or alumina and the eluting solvent flowing down through the column. Column chromatography is generally used as a purification technique and isolates desired compounds from a mixture (Houghton and Raman, 1998).

1.4.2 Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC) is widely used for analysis of plant products as it is a rapid method, and provides qualitative as well as semi-quantitative information on the sample constituents. It provides a chromatographic fingerprint, hence suitable for monitoring the identity and purity of phytochemical preparations when comparing Rf values of a component with those quoted in the literature, or those recorded in one's own previous experiments, it is important to remember that variations may occur due to slight changes in temperature, stationary phase or mobile phase composition and saturation status of the TLC tank. Reproducible TLC preparations can be guaranteed only if standardized adsorption layers are used. Silica gel is an efficient adsorbent for the separation of most plant extracts (Wagner and Bladt, 1996). The system selection entails choosing the mobile phase, the stationary phase and the detection method. Ethanol, methanol and water can dissolve appreciable amounts of silica gel which appears as a residue when the eluent is concentrated (Houghton and Raman, 1998).

Analytical TLC plates can deal only with a small amount of sample, for applying larger sample amounts, preparative plates (thickness 0.5-1 mm) are available. However, resolution in some cases may not be satisfactory on preparative plates. Even on preparative plates, only up to 50 mg of a sample mixture can be separated (Houghton and Raman, 1998). For detection of separated substances, use of UV lamps offer a non-destructive approach. Chromatograms are viewed in short (254 nm) and long (365 nm)

wave UV light. If a fluorescent layer such as silica gel GF254 is exposed to short wave UV light, all substances which absorb in this region stand out distinctly as dark zones on the green fluorescing layer as background. Details of TLC have been dealt within the most comprehensive fashion by Stahl 1969. Use of TLC for investigation of bioactive plant products, including those with antioxidant and/or antimicrobial properties, has been reported by numerous workers (Horwath *et al*., 2002).

1.5 High pressure (performance) liquid chromatography (HPLC)

High pressure (performance) liquid chromatography (HPLC) has the ability to provide both quantitative and qualitative data in a single operation. HPLC is mainly used for those classes of compounds which are non-volatile, *e.g*. higher terpenoids, phenolics of all types, alkaloids, lipids and sugars. It works best for compounds which can be detected in the ultraviolet or visible regions of the spectrum. It is possible to carry out most separations using either a silica microporous particle column (for non-polar compounds) or a reverse-phase C18 bonded phase column (for polar compounds). The mobile phases used in HPLC range from organic solvents to mixtures of water or buffer with water-miscible organic modifiers such as methanol, acetonitrile and tetrahydrofuran. Due to the small volumes needed (5-100 μL), it is important that the sample solution is sufficiently concentrated for the amounts applied to be detectable. The mobile phase is to be used as the sample solvent wherever possible, and in all cases, the sample solvent and the mobile phase must be miscible. HPLC is advantageously used for the quantitative estimation of various flavonoids in plant extracts. Identification of vibrioicidal compounds in plant extracts through HPLC has been reported by Sharma *et al*. (2009). This technique has been used for the study of bioactive tannins, flavonoids and their derivatives in different plant preparations (Sharma *et al*., 2008).

1.6 High performance thin layer chromatography (HPTLC)

High performance thin layer chromatography (HPTLC), advanced version of thin layer chromatography, now also called planar chromatography is, like all chromatographic techniques, a multistage distribution process. This process involves: a suitable adsorbent (the stationary phase), solvents or solvent mixtures (the mobile phase or eluent), and the sample molecules. For thin layer chromatography, the adsorbent is coated as a thin layer onto a suitable support (*e.g*., glass plate, polyester or aluminium sheet). On this layer, the substance mixture is separated by elution with a suitable solvent. The principle of TLC is known for more than 100 years now (Beyerinck, 1889).

High pressure (performance) liquid chromatography (HPLC) uses a stationary phase which is a thin layer (0.25-2.0mm) of silica on a metal foil or a glass plate. The sample is applied with the help of an automated applicator as a thin streak or sample can be applied by spraying with the help of nitrogen gas. Since, the mass distribution is uniform over the full range of the bands, densitometric estimation can be done by scanning. The plate is then developed in a saturated chamber containing the developing solvents. The separation of compounds may be based on adsorption, partition, ion exchange or molecular exclusion principles (Harbone, 1998). The separation rates of compounds are based on their distribution coefficients between the stationary phase and mobile phase. Once the mobile phase reaches the front end of the plate, retardation factor (R_f) or retention factor (R_t) is calculated as:

$$R_f = \frac{\text{Distance moved by the analyte from the origin}}{\text{Distance moved by the mobile phase from the origin}}$$

1.7 Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance spectroscopy is an extremely powerful analytical technique for the determination of flavonoid structures (Andersen and Fossen, 2003). Recent developments have, however, made NMR arguably the most important tool for complete structure elucidation of flavonoids. Today, it is possible to make complete assignments of all proton and carbon signals in NMR spectra of most flavonoids isolated in the low milligram range. These assignments are based on chemical shifts (d) and coupling constants (J) observed in 1D 1H and 13C NMR spectra combined with correlations observed as crosspeaks in homo and heteronuclear 2D NMR experiments. Recent MS and two dimensional (2D) NMR techniques have, in particular, become important for the determination of many flavonoids. Natural abundance 17O NMR spectra have been recorded for 11 methoxyflavones (Tsai et al., 2004) and 17O NMR data for some 3-arylidenechromanones and flavanones have recently been discussed in terms of mesomeric and steric substituent interactions. 17O NMR spectroscopy has also been used to study the effect of sugar on anthocyanin degradation and water mobility in a roselle anthocyanin model system. The most frequently used NMR solvents for flavonoid analyses are hexadeuterodimethylsulfoxide (DMSO-d6) and tetradeuteromethanol (CD3OD). Advances in computing power have been an important factor for the success of more advanced NMR techniques. Running many scans and accumulating data may enhance weak signals since baseline noise, which is random, tends to cancel out. One of the main advantages to be gained from signal averaging combined with the use of Fourier transform methods and high-field superconducting magnets (up to 21 T) is the ability to routinely obtain 13C NMR spectra (Exarchou et al., 2003).

1.8 *In silico* approach

Computer-based docking screens which are now widely used to discover new ligands for targets of known structure are common in molecular discovery. They offer a valuable alternative to the costly and time consuming process of random screening (Ekins *et al*., 2007). In biomedical arena, computer-aided or *in silico* design which uses computational techniques in drug discovery and development process is being utilized to expedite and facilitate hit identification, hit-to-lead selection, optimize the absorption, distribution, metabolism, excretion and toxicity profile and avoid safety issues. Computational approaches when applied to potentially large number of families of related proteins expand the structural knowledge and, thus, help to fill the gap between the number of known protein sequences and the known structures. On the basis of sequence and structural similarities, bioinformatics offers numerous approaches for the prediction of structure and function of proteins. The protein sequence to structure and function relationship is well established and reveals that the structural details at atomic level help to understand the molecular function of proteins (Siew *et al*., 2000).

One of the main challenges of computational science is to predict the overall fold of a protein from its sequence. Homology modeling or comparative modeling methods are able to predict the 3D structure of a protein sequence by using information derived from

a homologous protein of known structure. In recent years, molecular docking techniques have become one of the most popular and successful approaches in drug discovery. However, almost all docking programs are protein based (Li *et al*., 2010). The receptor proteins already complexed with ligands are used as targets and during protein preparation, using the module of the Schrodinger software, these ligands along with the hydrophilic water molecules are removed displaying the active site to which the drugs will bind when compatible. Protein databank provides access to retrieve the three dimensional (3D) crystal structure of the target protein (Rajkannan and Malar, 2007).

1.9 Phytochemical screening assays

1.9.1 Detection of carbohydrates

The qualitative analysis of various extracts of plants for carbohydrates was carried out by the methods of Ramakrishnan et al. (1994). Extract (10mg) was dissolved in 10 ml of water, filtered and the filtrate was subjected to tests for carbohydrates.

- **Molish test**: To 2 ml of the test solution, two drops of Molish reagent (5% á-naphtol in alcohol) were added, shaken well, 1ml of conc. sulphuric acid was added slowly along the sides of the test tube, violet ring indicated the presence of carbohydrates.
- **Barfoed's test**: To 1ml of filtrate, 1ml of Barfoed's reagent (6.5% cupric acetate in distilled water, filtered and added 0.9 ml of glacial acetic acid) was added and heated in a boiling water bath for 2min. Red precipitate indicated the presence of reducing monosaccharides.
- **Benedict's test**: To 0.5 ml of filtrate, 0.5 ml of Benedict's reagent (173 g sodium citrate and 100 g sodium carbonate were dissolved in 800 ml of distilled water and boiled to make it clear. 17.3 g cupric sulphate dissolved in 100 ml distilled water was added to it) was added and heated in a boiling water bath for 2 min. Red precipitate indicated the presence of reducing sugars.
- **Fehling's test**: One ml filtrate was boiled in a water bath with 1ml each of Fehling's solution A (7% cupric sulphate in distilled water) and B (35% potassium sodium tartarate and 10% sodium hydroxide in water). A red precipitate indicated the presence of reducing sugars.

1.9.2 Detection of amino acids

The qualitative analysis for amino acids in various extracts of plants was carried out by the method of Ruthmann (1970). The extract (10 mg) was dissolved in 10 ml of distilled water, filtered through Whatman no.1 filter paper and the filtrate was subjected to qualitative tests for amino acids.

- **Million's test**: To 1ml of the test solution, few drops of Million's reagent (1g mercury in 9 ml of fuming nitric acid) were added; red color indicated the presence of aromatic amino acid-tyrosine.
- **Hopkin cole test**: To 2 ml of the test solution, 2 ml of glacial acetic acid and 2 ml of conc. H_2SO_4 were added through the sides of the test tube, appearance of purple ring indicated the presence of aromatic amino acid-tryptophan.
- **Ehrlich test**: To 1ml of the test solution, 2 ml of Ehrlich reagent (10% p-dimethyl amino benzaldehyde in conc. HCl) were added. The appearance of pink color indicated the presence of aromatic amino acid- tryptophan
- **Pauly's test**: To 2 ml of test solution, 1ml of 1% 2sulphanilic acid was added followed by 1ml of 5% sodium nitrate solution. After 3 min, solution was made alkaline by adding 2 ml of 1% Na_2Co_3 solution. The appearance of yellow, orange and red color indicated the presence of tyrosine, tryptophan and histidine, respectively.
- **Nitroprusside test**: To 1ml of the test solution, 1ml of aqueous solution of freshly prepared 2% sodium nitroprusside and 40% NaOH solution were added. Appearance of blood red / dark pink color indicated the presence of sulphur containing amino acids.

1.9.3 Detection of flavonoids

The qualitative analysis for flavonoids in various extracts of plants was carried out by the method of Edeoga *et al*. (2005).

- **Sodium hydroxide test:** The extract was treated with 20% sodium hydroxide; formation of yellow colour indicated the presence of flavonoids.
- **Sulphuric acid test:** The extract was treated with conc. sulphuric acid; formation of yellow or orange colour indicated the presence of flavones.
- **Sodium acetate test:** Little quantity of extract was treated with amyl alcohol, sodium acetate and ferric chloride. Yellow colour solution formed disappears on the addition of an acid indicated the presence of flavonoids.

1.9.4 Detection of sterols and terpenoids

The qualitative analysis of various samples for sterols and triterpenoids was carried out (Finar, 1986).

- **Libermann-Buchard's test**: To 1mg of test substance dissolved in few drops of chloroform, 3 ml of acetic anhydride, 3 ml of glacial acetic acid were added, warmed and cooled under the tap and few drops of conc. H_2SO_4 were added along the sides of the test tube. Appearance of bluish-green color showed the presence of sterols.
- **Salkowski test**: To 1mg of test substance dissolved in few drops of chloroform, equal volume of conc. H_2SO_4 was added. Formation of bluish-red to cherry color in chloroform layer and green fluorescence in the acid layer indicated the sterol components in the extract.

1.9.5 Detection of tannins and phenol compounds

The qualitative analysis of various samples for tannins and phenolic compounds was carried out (Herborne, 1973).

- **Ferric chloride test:** To the test solution, few drops of neutral 5% ferric chloride solution were added, dark green color indicated presence of phenolic compounds.
- **Potassium ferricyanide test:** A small quantity of the test solution was treated with 2% potassium ferricyanide and ammonia solution. A deep red color indicated the presence of tannins.

- **Potassium dichromate test:** To the test solution, 2% potassium dichromate solution was added. A yellow precipitate indicated the presence of tannins.

1.9.6 Detection of glycosides

The qualitative analysis of various samples for glycosides was carried out by the methods given by Sofowora (1993). The test solution was prepared by dissolving the extract in alcohol or boiling with hydro-alcoholic solution.

- **Baljet's test**: The test solution was treated with 2% sodium picrate. Appearance of yellow to orange color indicated the presence of glycosides.
- **Raymond's test**: The test solution was treated with dinitrobenzene in hot methanolic alkali. Appearance of violet color indicated the presence of glycosides.
- **Bromine water test**: Test solution when mixed with bromine water gave yellow precipitate indicating the presence of glycosides.
- **Keller-Killani test for digitoxose**: The test solution was treated with few drops of 5% $FeCl_3$ solution, mixed, and then H_2SO_4 containing $FeCl_3$ solution was added, it formed two layers where the lower layer was reddish brown and upper layer turned bluish green.
- **Legal's test**: Test solution was treated with pyridine and made alkaline; addition of 2% sodium nitroprusside gave pink to red color indicating the presence of glycosides.

1.9.7 Detection of anthroquinone glycosides

- **Borntrager's test**: Test solution was treated with 5 ml of 10% sulphuric acid for 5 min., filtered while hot, cooled, and the filtrate was shaken gently with equal volume of benzene. Benzene layer was separated and then treated with half of its volume with a solution of 10% ammonia and allowed to separate it. The rose pink color in ammonical layer indicated the presence of anthroquinones.

1.9.8 Detection of organic acids

Aqueous extract was neutralized with dilute NH_4OH solution and then the following tests were performed (Jaiswal *et al*., 2012).

- **Calcium chloride test:** To 2 ml of the test solution, few drops of 5% $CaCl_2$ solution were added. Formation of white precipitate immediately, indicated the presence of oxalic acid. Precipitate observed on shaking showed the presence of tartaric acid, precipitate observed on boiling and cooling showed the presence of citric acid.
- **Test for oxalic acid:** To 2 ml of test solution, few drops of 5% lead acetate solution were added, white precipitate confirmed the presence of oxalic acid.
- **Test for citric acid:** To 2 ml test solution, one drop of dilute NH_4OH and excess $AgNO_3$ solution were added, boiled for 15 min. Blackish silver mirror formed, indicated the presence of citric acid.

1.9.9 Detection of saponins

- **Foam test:** Test solution when shaken, showed the formation of foam, which was stable for at least 5 min, confirmed the presence of saponins.

1.10 Types of bioactivity

Screening the plant extracts for the desired bioactivity is among the most crucial operations in medicinal plant research (Vlietinck and Vanden, 1991). It is important to note in context of traditional medicines derived from the flowering plants that they still form the basis for primary healthcare for 80% of the world's population (WHO). Some cultures, notably in India and China, have evolved a philosophy of disease and medicine which is very sophisticated and can explain the use of particular plants. Plants have been investigated for different types of bioactivities, *viz.* antimicrobial, antioxidant, insecticidal, antitumor, antineoplastic, immunosuppressive activity *etc*. Specialized bioassays are needed for the detection of each of the bioactivity. In recent years, secondary metabolites are being used, either directly as precursors or as lead compounds in the pharmaceutical industry and it is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens. However, very little information is available on such activity of medicinal plants and, of the 400,000 plant species on Earth, only a small percentage has been systematically investigated for the biological activities (Houghton and Raman, 1998).

1.10.1 *In vitro* methods to determine antioxidant activity

A variety of tests suggested for expressing antioxidant potency of food components can be categorized into two groups: assays for radical scavenging ability and assays that test the ability to inhibit lipid oxidation under accelerated conditions (Frankel and Meyer, 2000). The essential features of any test are a suitable substrate, an oxidation initiator and an appropriate measure of the end-point. Measurement of any one of these can be used to assess antioxidant activity (Antolovich *et al*., 2002). The main methods comprise superoxide radicals scavenging (O^-_2); hydrogen peroxide scavenging (H_2O_2); hypochlorous acid scavenging (HOCl); hydroxyl radical scavenging (HO^-); peroxyl radical scavenging (ROO^-). Among them are the methods that use azo-compounds to generate peroxyl radicals, such as the ''TRAP'' method (Total Radical-Trapping Antioxidant Parameter) and the ''ORAC'' method (Oxygen- Radical Absorbance Capacity); the scavenging of radical cation 2,2-azinobis-(3-ethylbenzothiazoline-6- sulphonate) or the ABTS or the ''TEAC'' method (Trolox Equivalent Antioxidant Capacity); the scavenging of stable radical 2,2-diphenyl-1-picrylhydrazyl or DPPH method and the scavenging of radical cation N,N-dimethyl-p-phenylenediamine or DMPD method as mentioned below:

1.10.1.1 *ABTS (2, 2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) scavenging assay*

The ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)) assay is based on the inhibition by antioxidants of the absorbance of the radical cation 2,2- azinobis-(3-ethylbenzothiazoline-6-sulphonate) $ABTS^+$, which has a characteristic long-wavelength absorption spectrum showing main absorption maxima at 415 nm, and secondary absorption maxima at 660, 734, and 820 nm. The original method was based on the activation of metmyoglobin, acting as peroxidase, with H_2O_2 *via* the formation

of the ferrylmyoglobin radical, which then oxidizes the phenothiazine compound ABTS, forming the ($ABTS^{+}$) radical cation. In terms of assay design, several different analytical strategies are used: decolorization and inhibition strategies, in which the absorbance of the reaction mixture is read when the color of the incubation mixture is stable or at a fixed time point, respectively, and lag phase strategy, in which the length of lag phase before the antioxidant reaction starts is measured (Rice-Evans and Miller, 1997).

Recently, a modification of this method has been introduced by a decolorization technique in which the radical is generated directly in a stable form using potassium persulphate (Re *et al*., 1999). Afterwards, the formed radical is mixed with the antioxidant in the reaction medium and the percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of concentration of antioxidants. Arnao *et al*. (2001) introduced some modifications in this assay: the $ABTS^{+}$ radical cation is generated enzymatically using the system formed by H_2O_2 and horseradish peroxidase (ABTS/ H_2O_2/HRP). The reaction is monitored at a wavelength selected between 400 and 750 nm, to avoid exogenous interference. Two strategies may be used: i) the antioxidant compounds are added previously to the formation of the $ABTS^{+}$ radical cation and then the lag phase, caused by different antioxidants in the production of the $ABTS^{+}$ radical cation, is measured. ii) the $ABTS^{+}$ radical cation is pregenerated and when stable absorbance is obtained the antioxidant sample is added to the reaction medium, and the antioxidant activity is measured in terms of decolorization. Several authors (Campos and Lissi, 1996) proposed a modification based on pre-generation of the $ABTS^{+}$ radical anions by heating the radical cation 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate ($ABTS^{+}$) with a thermolabile azo compound ABAP (2,2'-azo-bis (2-amidino-propane)hydrochloride). On the basis of pregeneration of the $ABTS^{+}$ radical cation with a thermolabile azo compound ABAP, lipophilic and hydrophilic compounds with antioxidant capacity, several wines, and crystal malt extracts were tested. Also, the effect of moderate consumption of beer, red wine and spirits on plasma antioxidants in middle-aged men was evaluated (Woffenden *et al*., 2001).

1.10.1.2 *DMPD (N, N-dimethyl-p-phenylenediamine) radical scavenging assay*

This assay is similar to that from Re *et al*. (1999) in which the $ABTS^{+}$ radical cation is chemically pregenerated. The main difference is the use of the hydrophilic compound N,N-dimethyl p-phenylenediamine (DMPD). In the presence of a suitable oxidant solution (ferric chloride) at an acidic pH, DMPD is converted to a stable and coloured DMPD radical cation (DMPD). The UV-visible spectrum of this compound shows maximum absorbance at 505 nm. Antioxidant compounds which are able to transfer a hydrogen atom to $DMPD^{+}$ cause a decolorization of the solution which is proportional to their concentration. This reaction is rapid (less than 10 min) and the end point, which is stable, is taken as a measure of the antioxidant efficiency. Antioxidant ability is expressed as Trolox equivalents using a calibration curve plotted with different amounts of Trolox. This method is used to measure hydrophilic compounds. It is worth noting that the high stability of the point fixed for measurement makes the effect of time variation negligible, allowing a high inter assay reproducibility (Fogliano *et al*.,1999). The presence of organic acids, especially citric acid, in some extracts may interfere with the DMPD assay, and so this assay should be used with caution in those extracts rich in organic acids (Gil *et al*., 2000).

This method is used to evaluate the antioxidant capacity of wines (Fogliano *et al*., 1999), water soluble fraction of tomatoes (Scalfi *et al*., 2000), red wines, green tea infusion and pomegranate juice (Gil *et al*., 2000).

1.10.1.3 **$DPPH^{\cdot}$** *(2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay*

This assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$). The free radical $DPPH^{\cdot}$ is reduced to the corresponding hydrazine when it reacts with hydrogen donors. This ability is evaluated using electron spin resonance spectroscopy on the basis that the $DPPH^{\cdot}$ signal intensity is inversely related to the test antioxidant concentration and to the reaction time (Chen et al., 2000; Yu, 2001), but the more frequently used technique is the decoloration assay, which evaluates the absorbance decrease at 515–528 nm produced by the addition of the antioxidant to a $DPPH^{\cdot}$ solution in ethanol or methanol. Different authors use different initial radical concentrations and different reaction times. $DPPH^{\cdot}$ assay is considered a valid and easy assay to evaluate scavenging activity of antioxidants, since the radical compound is stable and does not have to be generated as in other radical scavenging assays. Sa´nchez-Moreno and Larrauri (1998) have proposed a new methodology for the evaluation of the antiradical efficiency towards $DPPH^{\cdot}$. Their procedure takes into account not only the antioxidant concentration but also the reaction time to reach the plateau of the scavenging reaction, a modification that could be an advantage over other methods, which only consider antioxidant concentration. De Ancos et al. (2000) had applied this modified procedure to the determination of the scavenging activity of raspberries, cranberries, and carotenoids, respectively.

1.10.1.4 *Scavenging of hydrogen peroxide* **(H_2O_2)**

The generation of hydrogen peroxide H_2O_2 by activated phagocytes is known to play an important part in the killing of several bacterial and fungal strains. Additionally, H_2O_2 is generated *in vivo* by several oxidase enzymes. There is increasing evidence that H_2O_2, either directly or indirectly *via* its reduction product $OH^{\cdot}$ OH^{-}, acts as a messenger molecule in the synthesis and activation of inflammatory mediators (Auroma, 1994). Hydrogen peroxide-scavenging activity is easily and sensitively measured by using peroxidase-based assay systems. The most common assay employs horseradish peroxidase, which uses H_2O_2 to oxidize scopoletin into a nonfluorescent product. In the presence of a putative scavenger, the oxidation of scopoletin is inhibited and the H_2O_2 scavenging can be monitored (Halliwell, 1997). Following this assay, Martýnez-Tome´ *et al*. (2001a) evaluated the antioxidant activity of broccoli amino acids, and of Mediterranean spices in an aqueous medium.

1.10.1.5 *Scavenging of Hydroxyl Radical* **(HO^{-})**

Hydroxyl radical (OH^{-}) scavenging can often be calculated using the ''deoxyribose assay'': a mixture of ferric chloride ($FeCl_3$) and ethylenediamine tetra acetic acid (EDTA) in the presence of ascorbate reacts to form iron(II)-EDTA plus oxidized ascorbate, H_2O_2 then method by omitting ascorbic acid to evaluate the potential of certain tannins (methyl-gallate) to behave as pro-oxidants.

Compounds which act as pro-oxidants are thought to be detrimental since they may enhance oxidative damage. In this modification of the method, pro-oxidants substitute for ascorbic acid in the Fenton reaction and increase color formation over the ascorbic acid-free controls. The generation of HO^- in the Fenton reaction is due to the presence of the iron ions. Some compounds inhibit color formation in the deoxyribose assay, not by reacting with HO^- but by chelating iron ions and preventing HO^- formation. To identify compounds which chelate metal, the deoxyribose assay is performed without EDTA (Aruoma, 1994; Hagerman *et al*., 1998). In the absence of metal chelating test compounds, iron ions are complexed to deoxyribose and causes ''site specific'' hydroxyl radical damage. When an iron chelating test substance is present, hydroxyl radical damage and the accompanying color production is diminished. In order to elucidate if the reaction mechanism is the direct radical scavenger and not inhibition of the Fenton reaction by chelation, Yamaguchi *et al*. (2000) evaluated in the H_2O_2/NaOH/DMSO system the scavenging activity towards HO^- of some extracts from *Garcinia indica* and grape seed.

1.10.1.6 *Scavenging of hypochlorous acid* (HOCl)

Another source of strong oxidants *in vivo* is neutrophils myeloperoxidase (MPO), which catalyzes oxidation of chloride ions by H_2O_2, resulting in hypochlorous acid (HOCl) production. The cytotoxicity of this reaction contributes to the killing of bacteria in the host defence system. However, HOCl generated by MPO might also inactivate α_1-antiproteinase and contribute to proteolytic damage of healthy human tissues in inflammatory disease (Hippeli and Elstner, 1997). Antioxidants can be tested for their potential to interfere with tissue damage caused by HOCl. Many compounds can act as HOCl scavengers and are tested using this assay, such as ascorbic acid, albumin (Halliwell, 1990), gallic acid and derivatives (Aruoma *et al*., 1993) and carotenoids (Lavelli *et al*., 2000).

Scavenging of HOCl can be examined in a system in which HOCl is generated immediately by adjusting sodium hypochlorous to pH 6.2, with dilute sulphuric acid. At the end of the reaction with test antioxidant, α_1-antiproteinase is added to the reaction mixture. The inactivation of this enzyme by HOCl is followed by adding elastase, which is inactivated by any remaining α_1-antiproteinase. The remaining elastase activity is measured using elastase substrate (Nsuccinyltriala-p-nitroanilide) and monitoring increase in absorbance at 410 nm. This assay was used by Martý´nez-Tome´ *et al*. (2001a) to evaluate the antioxidant activity of broccoli amino acids and of Mediterranean spices.

1.10.1.7 *Scavenging of peroxynitrite* ($ONOO^-$)

Peroxynitrite ($ONOO^-$) is formed by the reaction of nitric oxide and superoxide. $ONOO^-$ is a cytotoxic reactive species that can be generated by endothelial cells, neutrophils, and macrophages (Balvoine and Geletti, 1999). $ONOO^-$ scavenging by the oxidation of dihydrorhodamine 123 to fluorescent rhodamine 123 is measured in the presence of potential antioxidant with a microplate fluorescent spectrophotometer with excitation at emission wavelength of 485 and 530 nm, respectively. The assay measures the potency of marine algae and green tea tannin extracts in the inhibition of DHR 123 oxidation by $ONOO^-$ (Chung *et al*., 2001).

1.10.1.8 *Scavenging of peroxyl radical* (ROO^-)

The peroxyl radical is a common free radical found in biological substrates and used in antioxidant assays. This is slightly less reactive than HO^- and, thus, possesses an extended half-life of seconds instead of nanoseconds (Halliwell, 1997). Assays of examining peroxyl radical scavenging using azo-compounds are extensively used. Naguib (2000) has used a method using 4,4-difluoro-3,5-bis-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacine as an indicator, and 2,2'-azobis-2,4 dimethylvaleronitril as generator of peroxyl radicals, to evaluate the antioxidant activity of astaxanthin and related carotenoids. Terao *et al*. (1994) have evaluated the protective effect of several polyphenols such as catechin, epigallocatechin, and quercetin in the induced lipid peroxidation of liposomes by 2,2'-diazobis-(2-amidinopropane) dihydrochloride (ABAP). Natella *et al*. (1999) have determined the scavenging ability of benzoic and cinnamic acids following absorbance decay at 443 nm of a mixture of crocin and ABAP.

Yokozawa *et al*. (2000) have studied the antioxidant protection of green tea extracts and a mixture of tannins from green tea in a culture of epithelial kidney cells (cell line LLC-PK1), using ABAP azo-compound as generator of peroxyl radical at constant rate. Rigo *et al*. (2000) studied the scavenging ability of Italian red wines using 2, 2'-azobis-(2-(2- imidazolin-2-yl) propane as generator of peroxyl radicals and linoleic acid as oxidizable substrate. The assay was validated using gallic acid as standard. Chun *et al*. (2000) evaluated the antioxidant activity of bamboo by measuring conjugated diene in a peroxidation liposome model initiated by peroxyl radical induced by 2, 2'- azobis-(2-amidinopropane) dihydrochloride (AAPH).

1.10.1.9 *Scavenging of superoxide radical* (O^-)

Xanthine oxidase is one of the main enzymatic sources of reactive oxygen species (ROS) *in vivo*. Although xanthine oxidase present in normal tissue as a dehydrogenase that transfers electrons to nicotinamide adenine dinucleotide (NAD^+) as it oxidizes xanthine or hypoxanthine to uric acid. Under certain stress conditions, the dehydrogenase is converted to an oxidase by oxidation of essential thiol groups or by limited proteolysis. Upon this conversion, the enzyme reacts with the same electron donors, but reducing oxygen instead of NAD, thus, producing superoxide and hydrogen peroxide, and contributing to the initiation and progression of a number of pathological processes (Hippeli and Elstner, 1997).The scavenging activity towards O^- of a wide range of antioxidants is measured in terms of inhibition of generation of O^- with the hypoxanthine–xanthine oxidase-superoxide generating system (HX–XO) (Yamaguchi et al., 2000). To a minor extent, O^-_2 is generated using a non-enzymatic reaction of phenazine methosulphate in the presence of NADH and molecular oxygen (Barthomeuf et al., 2001). In both strategies, O^-_2 reduces nitro-blue tetrazolium (NBT) into formazan at pH 7.4 at room temperature, and formazan generation is followed by spectrophotometry at 560 nm. Any added molecule capable of reacting with O^-_2 inhibits the production of formazan (Barthomeuf et al., 2001).

The hydrogen peroxide/sodium hydroxide/dimethyl sulfoxide (DMSO) system is designed to evaluate both water-soluble and oil-soluble free radical scavengers as a non-enzymatic and non-Fenton type ROS generating system in which not only O^-_2 and hydroxyl radicals, but also methyl radicals are generated (Yamaguchi

et al., 2000). The primary function of the HX-XO system is to oxidize xanthine or hypoxanthine to uric acid. Therefore, the inhibition of the activity of XO is measured by the evaluation of uric acid production, which is formed along with O^{-}_{2} (Kweon *et al*., 2001). In order to allow comparison among assays, it is useful to compare the obtained inhibition of the antioxidant with that obtained by the superoxide dismutase enzyme, or by standard antioxidants, such as α-tocopherol or ascorbic acid. Measurements of O^{-}_{2} scavenging should be interpreted with caution, because no equilibrium can be achieved when superoxide radicals are generated continuously during the test (Frankel and Meyer, 2000).

1.10.2 Antioxidant assays using *in vitro* models

1.10.2.1 *Ferric thiocyanate* (FTC) *assay*

In this assay, ferrous ion is oxidized by an oxidant, such as hydroperoxides to form ferric ion. This ferric ion formed is monitored as a thiocyanate complex by a spectrophotometer at 500nm. As in most studies, linoleic acid acts as a hydroperoxide source. The inhibitory effect towards oxidation of ferrous ion to ferric ion by antioxidants is evaluated by monitoring the formation of ferric thiocyanate complex. This assay is simple and highly reproducible. One drawback of this assay is that if any chemical with UV absorption around 500 nm is present, the results are overestimated or not reliable. This is true for any other assays using a spectrophotometer (Kikuzaki and Nakatani, 1993). This assay has been used to investigate natural antioxidant in combination with other assays, such as the TBA assay for fruit of mengkudu (Zin *et al*., 2007), with the DPPH assays for teas (Anesini *et al*., 2008), with DPPH and ABTS assays for essential oils (Erkan *et al*., 2008).

1.10.2.2 β-*carotene bleaching assay*

It has been long known that β-carotene reacts with the peroxyl radical to produce β-carotene epoxides (Kennedy and Liebler, 1991). Therefore, β-carotene has received attention as a radical scavenger or antioxidant (Tsuchihashi *et al*., 1995). Later, an antioxidant assay using β-carotene combined with lipids, such as linoleic acid, was established. Linoleic acid forms a peroxyl radical ($LOO^{\cdot}$) in the presence of ROS and O_2. This peroxyl radical reacts with β- carotene to form stable β-carotene radical. Subsequently, the amount of β-carotene reduces in a testing solution. If an antioxidant is present in a testing solution, it reacts competitively with the peroxyl radical. Therefore, antioxidant effects are easily monitored by bleaching the color of a test solution using a spectrophotometer at 470 nm, which is the typical absorbance by β-carotene (Takada *et al*., 2006).

1.10.2.3 *In vitro methods to assess antidiabetic activity*

Pancreatic α-amylase inhibition

Pancreatic α-amylase is a key enzyme in the digestive system and catalyses the initial step in hydrolysis of starch to a mixture of smaller oligosaccharides containing maltose, maltotriose, and a number of α-(1-6) and α-(1-4) oligoglucans. These are then acted upon by α-glucosidase and further degraded to glucose which on absorption enters the blood stream. Degradation of this dietary starch proceeds rapidly and leads to elevated post-prandial hyperglycemia. It has been shown that activity of human pancreatic α-amylase in the small intestines correlates to an increase in post prandial glucose levels, the control of which is therefore an important aspect in the treatment of type 2 diabetes. Hence, retardation of starch digestion by inhibition of enzymes such as α-amylase plays a key role in the control of diabetes. Inhibitors of pancreatic α-amylase delay carbohydrate digestion causing a reduction in the rate of glucose absorbtion and lowering the post prandial serum glucose levels (Tarling *et al*., 2008).

α-glucosidase inhibition

Mammalian α-glucosidase (α-D-glucoside glucohydrolase), located in the brush-border surface membrane of intestinal cells, is the key enzyme which catalyzes the final step in the digestive process of carbohydrates. Hence, α-glucosidase inhibitors can retard the liberation of D-glucose of oligosaccharides and disaccharides from dietary complex carbohydrates and delay glucose absorption, reducing postprandial plasma glucose levels and suppressing postprandial hyperglycemia (Lebovitz, 1997). Consequently, α-glucosidase inhibitors, *e.g.*, acarbose and miglitol, effectively compensate for defective early phase insulin release by inhibiting postprandial absorption of monosaccharides, and have been examined for clinical management of type 2 diabetes (Lembcke *et al*., 1990).

1.10.3 *In vitro* methods to assess anti-inflammatory activity

1.10.3.1 *Cyclooxygenases*

Cyclooxygenase (COX), also known as Prostaglandin H synthase (PGHS), is the rate limiting enzyme in the biosynthesis of prostanoids (Dewitt and Smith, 1990). It converts arachidonic acid to prostaglandin H_2 that gets further metabolized to tissue specifically to various prostaglandins, prostacyclin and thromboxanes together called as prostanoids. These prostanoids are extremely potent biologically active compounds with bewildering variety of actions. The enzyme has two distinct activities: a cyclooxygenase activity, which catalyzes the formation of PGG_2 to form PGH_2 (Pagels *et al*., 1983). COX exists in at least two isoforms. COX-1 is typically constitutive enzyme whereas COX-2 is expressed in most tissues and cells at very low levels unless induced by mitogenic or hormonal stimuli (Kujubu *et al*., 1991). COX-1 is expressed constitutively in nearly all mammalian tissues and is the source of prostaglandins central to "housekeeping" functions such as renal water reabsorption, vascular homeostasis, and gastric protection (Smith *et al*., 1996). COX-2 is absent from most cells but rapidly and dramatically induced in many cell types upon treatment with inflammatory cytokines, growth factors, v-*src*, and tumor promoters (Hulkower *et al*., 1994).

1.10.3.2 *Lipooxygenases* (LOX)

Lipooxygenases comprise a family of non-heme, iron containing dioxygenases which incorporate molecular oxygen into poly unsaturated fatty acids with 1-cis, 4-cis-pentadiene structures such as arachidonic acid and linoleic acid, to give rise to 1-hydroperoxy-2, 4-trans, cis-pentadiene products. Lipooxygenases catalyse conversion of arachidonic acid to hydroperoxy-eicosatetraenoic acids (HPETEs), leukotrienes (LTs) and lipoxins. Lipoxygenation involves the formation of radical intermediates, which usually remain enzyme bound. Thus, the LOX reaction itself may not be considered an efficient source of free radicals. There are five active LOXs found in humans: 5-LOX, 12*S*-LOX, 12*R*-LOX, 15-LOX-1 AND 15-LOX-2. Lipoxygenase products have been implicated in the

pathogenesis of hypersensitivity, asthma, psoriasis, atherosclerosis and cancer. Leukotrienes (LTs) play a major part in the inflammatory process. They are synthesized via the 5-LOX pathway (Morham *et al*., 1995).

1.11 *In vivo* animal studies

To overcome differences between *in vitro* test systems and the whole organism, as well as the obvious constraints of human clinical studies, animal studies are widely applied in antioxidant research (Mortensen *et al*., 2008). The capacity and efficacy of antioxidants *in vivo* may be assessed most accurately due to their effect on the level of oxidation in biological fluids and tissues, thus animal studies on polyphenols are mainly focused on serum total antioxidant capacity, lipid peroxidation and antioxidant enzymes activity measurements. Hepatoprotective studies with animal models use exogenously administered hepatotoxin such as carbon tetrachloride (CCl_4), a free radical generating compound that induces oxidative stress, and related damage of biomolecules and cell death. Polyphenols were found to be able to protect the liver against cellular oxidative damage and maintenance of intracellular level of antioxidant enzymes (Amat *et al*., 2010). Studies performed in apolipoprotein E-deficient mice proved that polyphenols from wine and tea can prevent the development and/or reduce progression of atherosclerosis, probably due to their potent antioxidative activity and ability to protect LDL against oxidation. Nevertheless, epidemiological studies are useful tool to study the health effects of polyphenols (Miura *et al*., 2001).

1.12 Human studies

At present, there is a big gap between the knowledge of *in vitro* and *in vivo* effectiveness of polyphenols as antioxidants, and human studies are still scarcer than those on animals. Oxidative damage and antioxidant protection by polyphenols have been studied in healthy people (preventive effect) or those suffering from certain disease (therapeutic effect) (Hansen *et al*., 2005). Additionally, recent clinical trials on resveratrol, as the main antioxidant in wine, have been largely focused on characterizing its pharmacokinetics and metabolism as well as the potential role in the management of diabetes, obesity, Alzheimer's disease and cancer (Patel *et al*., 2011). Considering the lack of relevant data, the future clinical trials should be aimed at identifying the health benefits of resveratrol/ polyphenols in various disease conditions. In order to verify the polyphenols as antioxidants in the *in vivo* conditions, there is still a lot of scientific research to be done. The emerging findings suggest that continued research through well-designed and adequately powered human studies that undoubtedly verify health-promoting antioxidant activity of polyphenols in the *in vivo* conditions are more than welcome (Manach *et al*., 2005).

2. Summary

Research identifying bioactive phenolic compounds and then establishing their beneficial health effect is gaining momentum. This will require advance search that integrates various scientific disciplines, culminating in well-designed large intervention trials with the compounds of interest. There are model systems that can be used for discovery of bioactive phenolic compounds of potential interest. These include assessing bioactive phenolic compounds, foods and food compounds, and whole diets in tissue/cell culture, animal feeding studies, or human feeding studies, both acute and chronic. Considering the lack of relevant data, the future clinical trials should be aimed at identifying the health benefits of polyphenols in various disease conditions. In conjunction with these model systems, important information can be generated from a combination of top-down approaches and bottom-up approaches that provide clarity about the biological effects of the multitude of potential bioactive phenolic compounds that likely exist in food. Identification of important bioactive phenolic compounds will result in the use of contemporary biotechnology to modify food stuffs to be fortified with the compound of interest. The resulting outcome will be the development of foods and food components that will have marked beneficial effects on human health.

Conflict of interest

We declare that we have no conflict of interest.

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