Chapter 1.0

INTRODUCTION
The highest education is that which does not merely give us information but makes our life in harmony with all existence.

.....Tagore
1.1. Preamble

Overwhelming epidemiological evidences indicate that plant based diets afford protection against the development of certain chronic diseases, particularly cancer. Plants provide rich source of phytochemicals and phytopharmaceuticals and have been used since antiquity to treat and manage different diseases. The active plant constituents developed as a part of their own defense mechanism seems to contribute to man’s health. Phytochemicals are secondary metabolites in plants and many of them are incorporated into foods or used as food supplements or nutraceuticals or as pharmaceuticals that can function in vivo to complement or boost the endogenous defense system. Thus functional foods, nutraceuticals or phytoceuticals capable of providing additional physiological benefits such as preventing or delaying the onset of chronic diseases are now as considered alternative health care.

The mounting cost of modern health care and contra indications of chemical entities based therapies coupled with recent experimental and epidemiological evidences in favor of phytochemicals have encouraged people to accept the concept of alternative health care. The world trade in herbal products are growing at a faster pace as a result of popularization of complimentary and alternative medicinal systems, but the product’s true information suffer from lack of active principles, validation etc. Since the plants and plant products are subjected to wide variation in their phytochemical profile due to variety, geo climatic conditions, maturity, post harvest processing, storage, stability etc, it is extremely important to conduct detailed investigations on the composition and physiological significance of medicinal plants and standardize the formulations based on ingredients.
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Although natural products have been a well spring of drugs and drug leads for decades, synthetic drugs have dominated in modern western medicine for the past two decades due to the emergence of promising and exciting advancements and technologies associated with combinatorial chemistry. The diminished productivity of de novo molecules towards drugs or drug leads and recent developments in the separation and analytical techniques reclaims the attention of researchers on natural product drug discovery. According to a recent survey 61% of small-molecule new chemical entities introduced as drugs world wide during 1981-2002 can be traced to or were inspired from natural products. In case of antibacterials and anticancer agents the productivity was higher than 70%. Despite the spectacular results obtained during the past years in the development of new drugs through biotechnology, genetic engineering and bioinformatics, natural products still have a very important role to play in drug discovery. In fact, only about 10% of the existing 350,000 plant species have been investigated from a phytochemical and pharmacological point of view. Nature’s architecture (not only plants but also marine organisms, insects and even animals) provides an unpredictable range of skeletal types and novel substances that it is of immense value to evaluate as many natural products as possible in order to find sources of new drugs, new lead compounds, new pesticides and new compounds for animal health.
1.2. Phytochemicals in Health Care

All living organisms are required to transform and interconvert a vast number of organic compounds to enable them to live, grow, reproduce and survive. They need to provide themselves with energy and supply precursors to construct their own tissues. An integrated network of enzyme mediated and carefully regulated reactions are involved for this purpose. Collectively they are referred as metabolism and the reaction steps involved are termed metabolic pathways. Despite the extremely varied characteristics of living organisms, the pathways for the modification and synthesize of carbohydrates, proteins, fats, and nucleic acids are in general essentially the same in all organisms collectively described as primary metabolism, the compounds involved in the pathways are termed primary metabolites. Glycolysis, oxidation of fatty acids (β-oxidation), digestion of proteins etc thus, are classified as primary metabolism. The metabolism concerned with the compounds which have a limited distribution in nature and is specific to certain organism is referred to as secondary metabolism and such compounds are called secondary metabolites. Secondary metabolites are generally produced in response to climatic stress, infection, defense against predators, for propagation etc. Most of the pharmacologically active natural products such as phenolic compounds, alkaloids, terpenoids etc are products of secondary metabolism. The phytochemicals those generally known as secondary metabolites of pharmacological significance are discussed below.

1.2.1. Lipids and their derivatives

Water insoluble biomolecules but soluble in non-polar solvents are broadly termed as lipids. There are some limitations in this classification as most organic compounds fall in this category and many classical fatty acids
have significant solubility in water. A more constricted classification of lipids is to simply classify them as fatty acids and their derivatives and to treat other hydrocarbons based natural products separately

1.2.1.1. Hydrocarbons: Hydrocarbons comprise a relatively small group of natural products with least polar nature. Aliphatic hydrocarbons in plants usually have odd number of carbon atoms and are mainly derived through the decarboxylation of fatty acids (1). Aliphatic and aromatic as well as saturated and unsaturated hydrocarbons are seen in plants. The highly branched hydrocarbons derived from isoprene are considered as a separate group (terpenoids). Saturated hydrocarbons are widely distributed as the waxy coating on leaves and surface of fruits. As the chain length and unsaturation increase hydrocarbon become more waxy and solid in room temperature. The simplest unsaturated hydrocarbon ethylene is an important plant hormone inducing abscission and ripening of fruits. Larger unsaturated hydrocarbons are also common in plants. Polyacetylenes are unique group of plant hydrocarbons with one or more acetylene linkages. Polyacetylenes are likely to be derived through the enzymatic dehydrogenation of corresponding olefins. Polyacetylenes with 14 to 18 carbon atoms are found in higher plants. Most of the polyacetylenes are toxic in nature and are mainly involved in plants defense mechanism. Cicutoxin in Oceanthe crocata (2) and falcarinol (Fig. 1.3) in domestic carrot (Daucos carota) root (3) are examples.

1.2.1.2. Alcohols: Large varieties of volatile aliphatic alcohols occur in small quantities in plants as a part of essential oils. All of the straight chain alcohols from C₁ to C₁₀ are found in plants either in free or esterified forms. Several larger alcohols such as ceryl alcohol are regular constituents of cuticular wax. Aliphatic alcohols containing cis-3-hexene-1-ol moiety have characteristic odors and are of interest to the fragrance industry (4).
1.2.1.3. **Aldehydes and ketones:** Low and medium molecular weight aldehydes and ketones occur as a part of volatile oils in plants. Citrus plants and bergamot (*Monarda didyma*) on cold pressing yield essential oils rich in aldehydes and ketones. Citral, nootketone, octanal etc are examples of industrially important carbonyl compounds (5).

1.2.1.4. **Fatty acids (FAs):** Natural FAs may contain from 4 to 30, or even more, carbon atoms, the most abundant being those with 16 or 18 carbons in plants. FAs are mainly found as esters with glycerol and are called fats or oils, depending on whether they are solid or liquid at room temperature. Most natural fats and oils are composed largely of mixed triglycerides. Animal fats contain a high proportion of glycerides of saturated FAs and tend to be solids, whilst those from plants and marine organisms contain predominantly unsaturated FA esters and tend to be liquids. Selective cis isomerisation of FAs in plants diminishes the close association of molecules and facilitates to maintain fluidity of cellular membranes.

1.2.1.5. **Terpenoids:** The terpenoids form a large and structurally diverse family of natural products derived from C$_5$ isoprene units, joined in a head to tail fashion therefore these are also referred to as ‘isoprenoids’. Typical structures contain carbon skeletons represented by (C$_5$)$_n$, and are classified as hemiterpenes (C$_5$), monoterpenes (C$_{10}$, eg; geraniol) (Fig-1.3), sesquiterpenes (C$_{15}$, eg; farnesol), diterpenes (C$_{20}$, eg; geranylgeraniol), sesterterpenes (C$_{25}$), triterpenes (C$_{30}$, eg; Squalene) and tetraterpenes (C$_{40}$, eg; phytene). Higher polymers are encountered in materials such as rubber. Relatively few of the natural terpenoids exactly encounter the simple concept of a linear head-to-tail combination of isoprene units. Most terpenoids are modified further by cyclization reactions e.g. menthol, bisabolene and taxadiene. The linear arrangement of isoprene units can be more difficult to appreciate in many other structures like sterols when rearrangement reactions have taken place.
1.2.1.6. Steroids: The steroids are modified triterpenoids containing the tetracyclic ring system of lanosterol. Cholesterol (Fig. 1.3) exemplifies the fundamental structure of steroids. Modifications on the side-chain result in a wide range of biologically important natural products. Steroids include a variety of bioactive compounds such as sterols, steroidal saponins, cardioactive glycosides, bile acids, corticosteroids and mammalian sex hormones. Many natural steroids together with a considerable number of synthetic and semi-synthetic steroidal compounds are routinely employed in medicine.

1.2.1.7. Carotenoids: Carotenoids are the most common tetraterpenoids with wide distribution in plant kingdom. Carotenoids are generally derived from lycopene through the cyclization of end groups. β-carotene (Fig-1.3) is the most common carotenoid in higher plants. Carotenoids not only impart bright colors to plants, but also involved in photosynthesis and antioxidant (AO) defense mechanism.

1.2.2. Aromatics

Compounds with aromatic rings contribute a major share of natural products. Aromatic compounds are involved in a wide spectrum of physiological functions of plants such as color, photosynthesis, microbial deterrence and structural composition. Aromatic compounds are formed through several biosynthetic routes including polyketide and shikimate pathways as well as from terpenoids. The vast majority of aromatic compounds are phenols.

1.2.2.1. Phenolic Compounds

They are compounds that have one or more hydroxyl groups attached directly to an aromatic ring. Because of the aromatic ring, the hydrogen of the phenolic hydroxyl is labile, which makes phenols weakly acidic. Polyphenols are compounds that have more than one phenolic hydroxyl group attached to
one or more benzene rings. Phenolic compounds are characteristic of plants and as a group they are usually found as esters or glycosides rather than as free compounds. The term ‘phenolics’ covers a large and diverse group of chemical compounds. These compounds can be classified in a number of ways. Harborne and Simmonds classified these compounds into groups based on the number of carbons in the molecule (Table 1.1) (8). Origins of various phenolics from simple phenyl propanoids are illustrated in Figure 1.1.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Class</th>
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<tbody>
<tr>
<td>C6</td>
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<tr>
<td>C6-C1</td>
<td>Phenolic acids and aldehydes</td>
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<tr>
<td>C6-C2</td>
<td>Acetophenones and phenyl acetic acids</td>
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<tr>
<td>C6-C3</td>
<td>Cinnamic acids, cinnamyl alcohols and cinnamyl aldehydes</td>
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<td>C6-C3-C6</td>
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<td>Betacyanins</td>
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<td>C30</td>
<td>Biflavonoids</td>
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<td>Oligomers or polymers</td>
<td>Tannins</td>
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<td>Polymers</td>
<td>Phlobaphens</td>
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(i) Simple phenolics: Simple phenolics are substituted phenols. Catechol (1,2-dihydroxybenzene), resorcinol (1,3-dihydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene) etc are typical examples for simple phenolics.
(ii) **Phenolic acids:** Phenolic acids are important phenolic compounds of non flavonoid family, which constitute a large group of phenolic compounds in plants. Phenolic acids includes two main groups namely, hydroxybenzoic acid and hydroxycinnamic acid derivatives. They differ according to the number and position of hydroxylation and methoxylation in aromatic ring (Figure-1.3). Phenolic acids are distributed as their free and bound forms in nature, more often bound forms occur as their esters and glycosides. Phenolic acids are reported to have a wide spectrum of pharmacological activities including AO, antimutagenic, antitumor and anticarcinogenic properties (9,10).

(iii) **Coumarins:** Coumarins have a C6-C3 skeleton, with an oxygen heterocycle as part of the C3-unit. There are numerous coumarins, many of which play a role in disease and pest resistance, as well as UV-tolerance. Umbelliferone (Figure 1.3) is a popular coumarin used in enzyme assays. Isocoumarins, such as bergenin have a structure similar to coumarins, but the position of the oxygen and carbonyl groups within the oxygen heterocycle are reversed. Isocoumarins also play a role in defense responses (11).

(iv) **Flavonoids:** Flavonoids are C15 compounds all of which have the structure C6-C3-C6. Flavonoids may be grouped into different classes based on their general structure (Figure 1.3).

Chalcones and dihydrochalcones have a linear C3-chain connecting the two rings. The C3-chain of chalcones contains a double bond, whereas the C3-chain of dihydrochalcones is saturated. Chalcones, such as butein, are yellow pigments in flowers. An example of a dihydrochalcone is phloridzin (phloretin-2′-O-D-glucoside), a compound found in apple leaves, and which has been reported to have anti-tumor activity (12). Aurones are formed by cyclization of chalcones, whereby the meta-hydroxyl group reacts with the α-
carbon to form a five-member heterocycle. Aurones are also yellow pigments present in flowers.

Typical flavonoids, such as flavanone, have a six-membered heterocycle. The A-ring originates from the condensation of three malonyl-CoA molecules, and the B-ring originates from $p$-coumaroyl-CoA. These origins explain why the A-ring of most flavonoids is either $m$-dihydroxylated or $m$-trihydroxylated. In typical flavonoids one of the $m$-hydroxyl groups of the A-ring contributes the oxygen to the six member heterocycle. The oxygen heterocycle of typical flavonoids may be a pyran, pyrylium, or pyrone ring. The B-ring is typically monohydroxylated, $o$-dihydroxylated, or vic-trihydroxylated. The B-ring may also have methyl ethers as substituents.

The heterocycle of flavanones also contains a ketone group with saturated C$_2$-C$_3$ carbon-carbon bond (eg; naringenin). Dihydroflavonols are known as flavanonols and often occur in association with tannins in heartwood (eg: Taxifolin or dihydroquercetin).

Flavan-3,4-cis-diols are referred to as leucoanthocyanidins. They are synthesized from flavanonols via a reduction of the ketone moiety on C4. Examples are leucocyanidin and leucodelphinidin. These compounds are often present in wood and play a role in the formation of condensed tannins. Because of their completely saturated heterocycle, leucoanthocyanidins, together with flavan-3-ols are referred to as flavans. Catechin and gallicatechin are examples of flavan-3-ol. The ‘gallo’ in the latter compound refers to the vic-tri-hydroxy substitution pattern on the B-ring. Unlike most other flavonoids, the flavans are present as free aglycones or as polymers of aglycones. Catechins can also be found as gallic acid esters that are esterified at the 3’ hydroxyl group.
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The heterocycle of flavones contains a ketone group, and has an unsaturated carbon-carbon bond. Flavones are common in angiosperms. The most widely distributed flavones in nature are kaempherol (5,7,4′hydroxyflavone), quercetin (5,7,3′,4′ hydroxyflavone), and myricetin (5,7,3′,4′,5′hydroxyflavone).

Flavonoids with pyrilium cation are referred to as anthocyanidins. Widely distributed, colored anthocyanins such as pelargonidin, cyanidin, delphinidin, petunidin, and malvidin are found as their aglycones. The most common anthocyanidin is cyanidin. These compounds are present in the vacuoles of colored plant tissues such as leaves or flower petals. The color of the pigment depends on the pH, metal ions present, and the combination of substituted sugars and acylesters. Different colors can also result from the presence of combinations of several anthocyanidins.

Isoflavonoids possess a rearranged flavonoid skeleton. Variety of structural modifications of the skeleton lead to different groups including isoflavones, isoflavonones and rotenone. The isoflavones are common in legume family Fabaceae. Genestein from soybean and coumstrol from Medicago sativa are phytoestrogens (13).

(v) Biflavonoids: Biflavonoids have a C30 skeleton. They are dimers of flavones such as apigenin or methylated derivatives and are found in gymnosperms. The most familiar is ginkgetin from Ginkgo biloba.

(vi) Benzophenones and xanthones: Benzophenones and xanthones have a C6-C1-C6 structure. Xanthones are yellow pigments in flowers.
(vii) **Stilbenes:** Stilbenes have a C6-C2-C6 structure and are localized in woody stems and seeds. Resveratrol, a well known stilbene found in grape is reported to have cardioprotective effects.

(viii) **Benzoquinones, anthraquinones and naphthaquinones:**
Benzoquinones, such as 2,6-dimethoxybenzoquinone are present in root exudates of maize and stimulate parasitic plants to form haustoria (14). Ubiquinones, the benzoquinone with isoprenoid sidechains, are also known as Coenzyme Q and have a role in electron transport in the mitochondria. Naphthaquinones are rare. Among the naphthaquinones juglone is relatively common and it is found in walnuts. Anthraquinone is the most widely distributed of the quinones in higher plants and fungi. The anthtraquinone emodin occurs as a rhamnoside in rhubarb roots.

(ix) **Betacyanins:** Betacyanins are red pigments and account for the red color of beets (*Beta vulgaris*). They are unique compounds to the *Centrospermae* with absorption spectra that resembles anthocyanins, but they contain nitrogen eg; betanidin. Betacyanins are normally found as their glycosides.

(x) **Lignans:** Lignans are dimers or oligomers that result from the coupling of monolignols – *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, with coniferyl alcohol being the most common monolignol used in lignan biosynthesis. Lignans are present in ferns, gymnosperms and angiosperms. They are localized in woody stems and in seeds and play a major role in insect deterrence. The term lignan typically refers to dimers of monolignols that are linked via an 8-8′ (β-β′) bond, whereas the term neolignan refers to dimers and oligomers that contain bonds other than the 8-8′ bond. Most lignans are optically active, and typically only one enantiomer is found in a given species. Examples of lignans include (+)-pinoresinol, (+)-sesamin, and (−)-plicatic acid.
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(xi) Lignin: Lignin, the second most abundant bio-polymer on earth (after cellulose) is a phenolic polymer. It provides structural support to plants, facilitates water transport through the vascular tissue and acts as a physical barrier against insects and fungi. Lignin is synthesized primarily from three monolignol precursors: \( p \)-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Comparatively small amounts of additional compounds such as coniferaldehyde, sinapaldehyde, dihydroconiferyl alcohol, 5-hydroxyconiferyl alcohol, tyramine ferulate, \( p \)-hydroxy-3-methoxybenzaldehyde, \( p \)-hydroxybenzoate, \( p \)-coumarate and acetate are also found in lignin (15).

(xii) Tannins: Tannins comprise a group of compounds with a wide diversity in structure and commonality in their ability to bind and precipitate proteins. The process of tanning animal skin to make leather utilize tannin and hence the name tanning. In Indian, Japanese and Chinese natural medicine tannins have been used as anti-inflammatory and antiseptic compounds. In wine and beer production, tannins are used to precipitate proteins. Tannins are abundant in many different plant species particularly in oak (Quercus spp.), chestnut (Castanea spp.), staghorn sumac (Rhus typhina), and fringe cups (Tellima grandiflora). Tannins are also present in leaves, bark, and fruits, and are known to protect the plant against infection and herbivory. Tannins can be classified under three groups: condensed tannins, hydrolysable tannins, and complex tannins. Hydrolysable tannins are further divided in to gallotannins (GT) and ellagitannins (ET).

(a) Gallotannins (GTs): GTs (Figure 1.4) are hydrolysable tannins with a polyol core (a compound with multiple hydroxyl groups) substituted with 2-12 gallic acid residues. GT contains the characteristic \textit{meta}-depside bonds between gallic acid residues. This bond is more labile than an aliphatic ester bond, and can be methanolyzed with a weak acid in methanol. The most
commonly found polyol is D-glucose, although some GT contain catechin and triterpenoid units as the core polyol.

(b) Condensed tannins: Condensed tannins are also referred to as proanthocyanidins (PAs). PA are mixtures of oligomers and polymers of flavan-3-ols, mainly formed through \( \text{C}_4 \rightarrow \text{C}_8 \) and/or \( \text{C}_4 \rightarrow \text{C}_6 \) (B type) or linked through an ether bond between \( \text{C}_2 \rightarrow \text{C}_7 \) (A type). On hydrolysis under harsh conditions, such as heating in acid, PA yields anthocyanidins. An example of PA is procyanidin B2 (epicatechin-(4β→8′)-epicatechin). Polymers are formed through the action of acids or enzymes. Polymers made up of more than 50 catechin units have been identified. The protein precipitation capacity of PA has importance in wine making, where a high level of condensed tannins, especially in red wines, can result in the dry feeling in mouth.

(c) Ellagitannins (ETs): ETs are also hydrolysable tannins derived from pentagalloylglucose, but unlike GT, they contain additional C-C bonds between adjacent galloyl moieties in the pentagalloylglucose molecule. This C-C linkage is formed through oxidative coupling between the two adjacent galloyl residues, and results in the formation of a hexahydroxydiphenoyl (HHDP) unit. The name ET is derived from ellagic acid, which is formed spontaneously from hexahydroxydiphenic acid in aqueous solution via an intra-molecular esterification reaction.

(d) Complex tannins. Complex tannins are defined as tannins in which a catechin unit is bound glycosidically to either a GT or an ET unit. As the name implies, the structure of these compounds can be very complex (eg; acutissimin A (Figure 1.4)). Acutissimin A is a flavogallonyl unit bound glucosidically to C-1, with an additional three hydrolyzable ester bonds to a D-glucose-derived open-chain polyol. This complex tannin is formed during
the aging process of red wine, whereby the catechin unit originates from the grapes, and the ET originates from the oak barrels. Acutissimin A has been shown to be a powerful inhibitor of DNA topoisomerase II, an enzyme required for the division of cancer cells, and a target for chemotherapeutic drugs (16).

(e) Phlobaphenes: Phlobaphenes are phenolic polymers present in floral organs of maize (Zea mays L.), Accumulation of phlobaphenes results in red pigmentation. Certain lines of sorghum (Sorghum bicolor L. (Moench)) also produce phlobaphenes. The structure of phlobaphenes is poorly understood. These compounds are believed to be polymers of flavan-4-ols, notably apiferol and luteoferol. The polymerization is thought to be under chemical, rather than enzymatic control, and give rise to a polymer in which the monomers are linked via a 4-8′ linkage. The C-C bonds between the flavan-4-ol monomers are difficult to break, which makes the structural elucidation of phlobaphenes difficult.
Figure 1.1 Schematic illustration of the origin of various phenolics from simple phenyl propanoids
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Figure 1.2 Interrelationships among flavonoid monotypes
1.2.3. Amines and alkaloids

1.2.3.1 Amines: Compounds containing nitrogen as a part of their side chain are generally referred to as amines. These molecules impart fishy odor and believed to act as insect attractants. Aliphatic polyamines such as putriscine (NH$_2$(CH$_2$)$_4$NH$_2$), agmatin (NH$_2$(CH$_2$)$_4$NHC(=NH)NH$_2$), and spermidine (NH$_2$(CH$_2$)$_3$NH(CH$_2$)$_4$NH$_2$) are also found in plant kingdom. These polyamines are thought to have many biological functions in plant including acting as plant hormones and are invariably found complexed with nucleic acids (17). Most of the plant aromatic amines are physiologically active. Mescaline (from *Lophophora willimasii*) is a potent hallucinogen. Noradrenaline, histamine and serotonin occurring in common plants are critical to brain metabolism (18).

1.2.3.2 Alkaloids: Alkaloids are classically defined as the plant derived basic compounds with pharmacological effects and contain one or more nitrogen atoms. In practice most of the nitrogen containing secondary metabolites are considered as alkaloids unless they may be readily classified otherwise. Alkaloids impart a wide spectrum of physiological effects in plants and animals. Alkaloids in plants serve as chemo protective, anti-herbivory agents or as growth regulator such as indole-3-acetic acid. Reserpine (from *Rauwolfia serpentina*) is an antihypertensive alkaloid. Cocaine is a local anesthetic and a potent central nervous system stimulant. Caffeine is one of the world’s most popular additive drugs. Quinine derived from cinchona trees have been used as an anti-malarial drug. Morphine, the principal alkaloid of opium poppy (*Papaver somniferum*) is a potential narcotic analgesic. Strychnine from *Strychnos nux vomica* is a strong poison. Colchicine from *Colchicum autmuale* has been used to treat gout for 2000 years. Atropine as a smooth muscle relaxant is used to dilate the pupil before eye examination and is also used for the treatment of ambylopia (lazy eye). Camptothecine, a quinoline alkaloid from Chinese tree of joy (*Camptotheca accuminata*) is well known for its antiapoptotic activity. Papaverine is used as a vasodialator (19).
Figure 1.3: Structures of some biologically active phytochemicals

Falcarinol

Geraniol

Cholesterol

β-carotene

Squalene

Gallic acid

Caffeic acid

Umbelliferone

Quercetin

Ellagic acid

Reserpine

Camptothecin
Figure 1.4 Structures of tannins and hexahydroxydiphenyl unit

Gallotannin

Condensed tannin (proanthocyanidin)

Hexahydroxydiphenoyl (HHDP) unit

Complex tannin

Phlobaphenes
1.3. Phytochemicals as antioxidants

1.3.1 Free Radicals

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons. This unpaired electron(s) usually seeks other electron(s) to become paired and thus, the free radicals in general are highly reactive and less stable. Their life times are extremely short in solutions, but they can be kept frozen for relatively long periods within the crystal lattice of other molecules. The lifetime of a radical depends not only on its inherent stability, but also on the conditions under which it is generated. A stable radical is inherently stable; a persistent radical has a relatively long lifetime under the conditions at which it is generated, although it may not be very stable.

Radicals can be characterized by several techniques, such as spin resonance spectroscopy, mass spectrometry (20), and Step-Scan Time-Resolved Infrared Spectroscopy (21). Electron Spin Resonance (ESR) also called Electron Paramagnetic Resonance (EPR) spectroscopy is an important general technique used for the characterization of free radicals. ESR spectroscopy utilizes the resultant magnetic moment of the unpaired electrons and their behavior in a strong magnetic field. Since only the free radicals respond to ESR, the method can be used to detect and quantify the free radicals. Another important magnetic spectroscopy NMR can also be used to detect the presence of free radicals during the course of reactions, through examining the variations in NMR signals due to chemically induced dynamic molecular polarization (CIDNP) (22).

The stability of free radicals depends on the field strengths, hyperconjugation, resonance possibilities and steric factors. Generally the
order of the stability of simple free radicals are primary <secondary <tertiary <allyl <benzyl. Triphenyl radical is stable enough to exist in solution. Steric hindrance to dimerisation is thought to be the major reason for its stability. Diphenyl picryl hydrazyl (DPPH) (Figure 1.6) is a stable free radical that can be kept for years and used as a probe for radical scavenging assays. TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl free radical) (Figure 1.6) is a stable nitrosyl radical used in chemical reactions and for spin trapping.

A number of biradical (or diradicals) are also known. Biradicals are short lived species with life time less than 1s. Biradical of 3,5-di-tert-butyl-3’(N-tert-N-aminoxy)-4-oxybiphenyl is reported to be stable for weeks. Radicals with both electrons on same carbon are known as carbenes. Carbenes are highly reactive species with very short life time (< 1s). Methylene and dichloromethylene are examples for carbenes which are extensively used in organic chemistry.

Many metabolic reactions in biological systems involve oxygen, and most of the oxygen is reduced to water. However, if the reduction is incomplete a series of reactive free radicals are formed. Free radicals thus formed may trigger free radical chain reactions. Environmental factors such as ionizing radiations, food, cigarette smoke, trace metals etc also contribute to the free radical generations. The common free radicals in our body include reactive oxygen species (ROS), reactive nitrogen species (RNS) and some sulphur-centered radicals.

1.3.1.1 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) ie; radicals derived from oxygen, represent the most important class of radical species generated in living system. Oxygen itself is a diradical with 2 unpaired electrons residing in antibonding \( \pi \) orbitals. This distribution of electrons makes it impossible for
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Oxygen to accept a spin-matched pair of electrons. However one of its unpaired electrons may undergo a spontaneous spin-reversal to make the pairing possible. At ordinary collision frequency, the period of contact is too short to spin reversal to occur, imposing a kinetic barrier to oxidative reaction. This kinetic barrier saves us from reacting explosively with an atmosphere of huge thermodynamic oxidizing potential.

Occasionally, under normal biological conditions, oxygen does manage to capture electrons from other molecules resulting in the formation of free radicals. The one electron reduction product of oxygen is the superoxide anion radical (O$_2^•$) (Figure 1.5). It is formed in many autoxidation reactions and by the electron transport chain and is considered as a primary ROS. It is less reactive and less harmful in physiological systems but can react with other molecules to generate secondary ROS either directly or through enzymatic or non-enzymatic process. It undergoes dismutation to form hydrogen peroxide (Figure 1.5) spontaneously or by enzymatic catalysis. It can release Fe$^{2+}$ from iron-sulphur proteins and ferritin (23). If two electrons are transferred, the product is hydrogen peroxide (H$_2$O$_2$). It is mainly formed by dismutation of O$_2^-$ or by direct reduction of O$_2$. It is lipid soluble and thus able to diffuse across membranes. Even though H$_2$O$_2$ is a stable non-radical, it act as precursor for the most reactive ROS namely hydroxyl radical (OH$^•$). Hydroxyl radical is the three electron reduction state of oxygen. It is one of the most potent oxidant known and has a very short half-life of approximately 10$^{-9}$ S. Fenton’s reactions and decomposition of peroxynitrite result hydroxyl radicals. It is extremely reactive and attacks most of the cellular components. Alkoxy (RO$^•$) and peroxy (ROO$^•$) radicals and organic hydro peroxide (ROOH) are formed in association with lipid oxidation reactions. Hypochlorous acid (HOCl) is formed from hydrogen peroxide by myeloperoxidase. It is lipid soluble and highly reactive. It readily oxidize protein constituents, including thiol groups, amino groups and methionine.
Peroxynitrate (OONO\(^-\)) formed in a rapid reaction between O\(_2\)- and NO- is also considered as active oxygen species. Protonation of peroxynitrate forms peroxynitrous acid, which can undergo hemolytic cleavage to form OH\(^\bullet\) and NO\(_2\). Chemical formation and exogenous sources of various ROS are summarized in Table 1.2

**Figure 1.5. Molecular orbital diagram of different oxygen derived species.**

<table>
<thead>
<tr>
<th>Molecular Orbital</th>
<th>State</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Sigma^*2P )</td>
<td></td>
<td>Ground State O(_2)</td>
</tr>
<tr>
<td>( \Pi^*2P )</td>
<td></td>
<td>Singlet O(_2)</td>
</tr>
<tr>
<td>( \Pi^2P )</td>
<td></td>
<td>( ^1\Delta_g ) O(_2)</td>
</tr>
<tr>
<td>( \Sigma2p )</td>
<td></td>
<td>Superoxide ion</td>
</tr>
<tr>
<td>( \Sigma^*2S )</td>
<td></td>
<td>Peroxide ion</td>
</tr>
<tr>
<td>( \Sigma2S )</td>
<td></td>
<td>( ^1\Sigma_g^+ ) O(_2)</td>
</tr>
<tr>
<td>( \Sigma^*1S )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Sigma1S )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 1.3.1.2. Reactive Nitrogen Species (RNS)

Nitric oxide (NO\(^\bullet\)), is a small molecule that contains one unpaired electron and is therefore a radical. Nitric oxide is generated in biological tissues by specific nitric oxide synthases (NOSs) which metabolize arginine to citrulline with the formation of NO\(^\bullet\) via five electron oxidative reaction. It is a highly reactive molecule with a half-life of only a few seconds in an aqueous environment. It has a greater stability in an environment with a lower oxygen
Introduction

concentration. As it is soluble in aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membrane.

Table 1.2. Chemical sources of ROS and RNS (24-26)

<table>
<thead>
<tr>
<th>ROS/RNS</th>
<th>Chemical formation/exogenous sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Singlet oxygen</td>
<td>Photosensitized oxidation. The reactions like</td>
</tr>
<tr>
<td></td>
<td>$\text{OCl}^{-} + \text{H}_2\text{O}_2 \rightarrow \text{Cl}^{-} + \text{H}_2\text{O} + ^1\text{O}_2$</td>
</tr>
<tr>
<td>Superoxide anion</td>
<td>Univalent reduction of $\text{O}_2$ by hydrated electrons</td>
</tr>
<tr>
<td>Peroxide ion</td>
<td>Two electron reduction product of $\text{O}_2$</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>Radiolysis or photolysis of $\text{H}_2\text{O}$. Dismutation reactions. $\text{OH}^* + \text{OH}^* \rightarrow \text{H}_2\text{O}_2$. $2\text{O}_2^* + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2$</td>
</tr>
<tr>
<td>Hydroxyl radical</td>
<td>Radiolysis or photolysis of $\text{H}_2\text{O}_2$, Fenton reaction $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{OH}^* + \text{OH}^- + \text{Fe}^{3+}$</td>
</tr>
<tr>
<td>Ozone</td>
<td>Polluted air and photochemical reactions like Solar energy $\text{O}_2 \rightarrow 2\text{O}^<em>$ $\text{O}_2 + \text{O}^</em> \rightarrow \text{O}_3$</td>
</tr>
<tr>
<td>Hypochlorous acid</td>
<td>Enzymatic reactions</td>
</tr>
<tr>
<td>Peroxyl and alkoxy radicals</td>
<td>Lipid peroxidation</td>
</tr>
<tr>
<td>Organic hydroperoxide</td>
<td></td>
</tr>
<tr>
<td>Nitric oxide.</td>
<td>Smoke</td>
</tr>
<tr>
<td>Nitrogen dioxide</td>
<td></td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>Smoke. By the reaction $\text{O}_2^* + \text{NO} \rightarrow \text{ONOO}^-$</td>
</tr>
</tbody>
</table>

1.3.2. Sources of free radicals in physiological systems

Both cellular metabolism and environmental factors serve as the source of free radicals and other ROS in our body. Major biological sources of ROS and RNS are given in Table 1.3
### Introduction

Table 1.3 Biological sources of ROS and RNS (24-26).

<table>
<thead>
<tr>
<th>ROS/RNS</th>
<th>Endogenous sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Singlet oxygen ($^1\text{O}_2$)</td>
<td>Photosensitized oxidation &amp; action of myeloperoxidase (MPO) during phagocytosis</td>
</tr>
<tr>
<td></td>
<td>$\text{H}_2\text{O} + \text{Cl}^- + \text{H}^+ \rightarrow \text{H}_2\text{O} + \text{HOCl}$</td>
</tr>
<tr>
<td></td>
<td>$\text{HOCl} \rightarrow \text{H}^+ + \text{OCl}^-$</td>
</tr>
<tr>
<td></td>
<td>$\text{OCl}^- + \text{H}_2\text{O}_2 \rightarrow \text{Cl}^- + \text{H}_2\text{O} + ^1\text{O}_2$</td>
</tr>
<tr>
<td>Superoxide anion ($\text{O}_2^{\bullet-}$)</td>
<td>Enzymatic reactions (eg; xanthine/XO), autooxidation reaction of oxyhaemoglobin, electron transport chains of mitochondria, endoplasmic reticulum, phagocytosis etc.</td>
</tr>
<tr>
<td>Hydrogen peroxide ($\text{H}_2\text{O}_2$)</td>
<td>Enzyme systems (eg; glycollate oxidase), phagocytosis, dismutation of $\text{O}_2^{\bullet-}$ etc.</td>
</tr>
<tr>
<td>Hydroxyl radical ($\text{OH}^\bullet$)</td>
<td>Radiolysis of $\text{H}_2\text{O}_2$, Fenton reaction etc.</td>
</tr>
<tr>
<td>Hypochlorous acid (HOCl)</td>
<td>Action of MPO during phagocytosis.</td>
</tr>
<tr>
<td>Peroxyl radicals (RO$^\bullet$)</td>
<td>Lipid peroxidation</td>
</tr>
<tr>
<td>Nitric oxide (NO)</td>
<td>From vascular endothelial cells catalysed by nitric oxide synthase.</td>
</tr>
<tr>
<td>Nitrogen dioxide (NO$_2$)</td>
<td>Combination of NO with O$_2$ at body temperature</td>
</tr>
<tr>
<td>Peroxynitrite (ONOO$^-$)</td>
<td>From vascular endothelial cells</td>
</tr>
</tbody>
</table>

### 1.3.3. Biological effects of free radicals

Aerobic organisms are exposed to a wide range of oxidants as part of their metabolism. Free radicals and other reactive oxygen species are also generated in the body as a result of stress, exercise, food habits, exposure to pollution, radiation, pesticides etc. The free radicals formed in our body serve important biological functions such as phagocytosis, cell signaling, apoptosis etc. Free radicals and ROS are useful only when they are produced in right
amounts and at right place and time. They in excess have the potential to
damage biomolecules such as proteins, lipids, and DNA.

1.3.3.1. Oxidative stress

Free radicals and other ROS are constantly formed in the human body. Some is chemical accident, such as generation of hydroxyl radical by our constant exposure to low levels of radiation from the environment and of superoxide radical by leakage of electrons from electron transport chain. Other production of these species is deliberate and beneficial. They can be harmful when produced in excess and free radicals have been implicated in the pathology of several human diseases including cancer, atherosclerosis, rheumatoid arthritis and neurodegenerative diseases.

Human body is equipped with AO defense mechanisms to minimize the effects of oxidants. Most cells can tolerate mild oxidative stress as they have repair systems which recognize and replace oxidatively damaged molecules. In addition cells may increase the AO defenses in response to the stress. The disturbance in the balance between oxidants and AOs towards oxidants is called ‘oxidation stress’. Oxidative stress causes oxidation of vital biomolecules. Severe oxidative stress results in cell damage and death. It has been implicated in numerous human diseases. Oxidative stress is thought to be playing a major role in degenerative disorders such as CVD, rheumatoid arthritis, neurodegenerative diseases, cancer and aging. The role of free radical mediated reactions in degenerative diseases is tabulated in Table 1.4. The role of free radical reaction in disease/ageing, toxicology, biology (Table 1.5) and in the deterioration of food (lipid peroxidation) has become an area of intensive investigation.
**Table 1.4 Role of free radical reactions in biology** (24-26).

<table>
<thead>
<tr>
<th>ROS/RNS</th>
<th>Deleterious Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Singlet oxygen</td>
<td>Oxidation of lipids and proteins especially in the retina of eye leading to cataract and age related molecular degeneration (AMD).</td>
</tr>
<tr>
<td>Superoxide anion</td>
<td>Oxidation of membrane lipids, reduction of cytochrome C</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>Inactivation of enzymes</td>
</tr>
<tr>
<td>Hydroxyl radical</td>
<td>Membrane damage, DNA damage &amp; strand breakage</td>
</tr>
<tr>
<td>Ozone</td>
<td>Irritating eyes, nose and lungs, cross linking of proteins, peroxidation of lipids, DNA damage etc.</td>
</tr>
<tr>
<td>Hypochlorous acid</td>
<td>Damage to proteins, tissues &amp; DNA, cell death caused by cholesterol chlorohydrins.</td>
</tr>
<tr>
<td>Peroxyl radicals</td>
<td>Lipid peroxidation, LDL oxidation.</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>Excess NO causes hypotension &amp; insufficient amounts cause hypertension.</td>
</tr>
<tr>
<td>Nitrogen dioxide</td>
<td>Peroxidation of membrane lipids.</td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>Damage to proteins, lipids and DNA.</td>
</tr>
</tbody>
</table>

**Table 1.5 Role of free radical reactions in diseases and ageing** (24-26).

<table>
<thead>
<tr>
<th>Disease/Disorder</th>
<th>Free radical related event</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular diseases</td>
<td>Oxidative modification of LDL after initial damage to vascular endothelium by other means.</td>
</tr>
<tr>
<td>Cancer</td>
<td>DNA damage</td>
</tr>
<tr>
<td>Adult respiratory distress syndrome (ARDS)</td>
<td>Damage to lungs</td>
</tr>
<tr>
<td>Radiation damage</td>
<td>Damage to proteins, lipids and DNA.</td>
</tr>
<tr>
<td>Brain &amp; spinal cord injuries</td>
<td>Tissue damage after the injury</td>
</tr>
<tr>
<td>Keshan disease</td>
<td>Mediated by oxidative stress</td>
</tr>
<tr>
<td>Cataract</td>
<td>Oxidation of lens proteins</td>
</tr>
<tr>
<td>Ageing</td>
<td>Damage to proteins, lipids, DNA and tissues.</td>
</tr>
</tbody>
</table>
1.3.3.2. Lipid peroxidation: Oxidation of lipids have significant role in deterioration of oils and fatty foods. Lipid peroxidation is an important pathophysiological process occurring in numerous diseases and stress conditions and results in a series of degradative processes affecting the organization and function of cellular components. PUFA are more prone to lipid peroxidation. The process begins when a free radical such as hydroxyl radical captures a hydrogen atom from the methylene carbon in the polyalkyl chain of the FA. Under aerobic conditions, a FA with an unpaired electron undergoes a molecular rearrangement on reaction with oxygen, generate peroxy radical. The peroxy radicals are highly reactive and can combine with other peroxy radicals to alter the membranes. They can also capture hydrogen molecules from adjacent FAs to form a lipid hydroperoxide thereby inducing the propagation of lipid peroxidation. Thus, the peroxidation of unsaturated FAs can induce the conversion of several FA side chains into lipid hydroperoxides, thereby forming a reaction chain with the final product of lipid peroxidation being malondialdehyde (MDA) (27). The major aldehyde product of lipid peroxidation other than malondialdehyde is 4-hydroxy-2-nonenal (HNE). Latter is weakly mutagenic but appears to be the major toxic product of lipid peroxidation. The MDA thus formed, reacts with the free amino group of proteins, nucleic acids and phospholipids to produce inter and intra molecular 1-amino-3-iminopropene (AIP) bridges. They can also produce a structural modification of these biomolecules. The immune system recognizes these MDA induced structures as non-self leading to an auto immune response (24).

Initiation

\[ LH + OH^* \rightarrow L^* + H_2O \]

Propagation

\[ L^* + O_2 \rightarrow LOO^* \]
\[ LOO^* + LH \rightarrow LOOH + L^* \]
Hydroperoxide decomposition

LOOH → LO* → MDA.

Moreover, the FA moieties of the plasma LDL can also be oxidized during oxidative stress.

1.3.3.3. Beneficial Role of Free Radicals

Oxidation and production of radicals and ROS are an integral part of metabolic processes. The free radicals may exert toxic effects but at the same time, they are also essential for several biological functions. For example, the process of phagocytosis is facilitated by free radicals (27). One of its many steps involves the production of superoxide and hydrogen peroxide in the extracellular space. Hydrogen peroxide is toxic to microorganisms either directly by promoting several oxidations or indirectly by forming hydroxyl radical or hypochlorite which in turn are more reactive.

ROS and RNS are known to play important roles in signal transduction. The capacitation of spermatozoa by superoxide is an example for the involvement of ROS in signal transduction. ROS may have beneficial or detrimental effects on sperm fractions depending on the nature and concentration of the ROS involved, as well as the moment and the location of exposure. In particular, low concentrations of superoxide trigger this phenomenon; whereas excessive generation of hydrogen peroxide in semen could be a cause for infertility. The RNS, nitric oxide, as an intracellular messenger plays an important role in the nervous system, where it acts as a neuromodulator and plays an important role in synaptic plasticity and long term memory. In vascular system, it controls vasodilation and hence the blood pressure. It is also associated with the immune system (28).
Programmed cell death (apoptosis) is needed for proper development and to destroy cells that represent a threat to the integrity of the organism. Apoptosis of a cell is initiated by the disturbance in balance between the positive signals which help the survival of the cells (e.g., growth factors for neurons) and the negative signals (e.g., increased level of oxidants within the cell) (29). ROS are also used as therapeutic agents too. In photodynamic therapy (PDT) the photosensitizer localized in the target tissue, is activated by light to produce oxygen intermediates such as singlet oxygen that can destroy target tissue cells. The easy access of skin to visible light and molecular oxygen has led PDT successful in the treatment of basal cell carcinoma and Bower’s disease. The most popular photosensitiser used in PDT is 8-aminolevulinic acid.

1.3.4. Anti oxidants (AOs)

Free radicals and ROS are useful only when they are produced in right amount, at right place and time. Otherwise, as mentioned, they will cause in oxidative damage to the physiological systems. To protect the body from harmful effects of free radicals and other oxidants all aerobic organisms are endowed with powerful AO systems. These include physical defenses, preventative and repair mechanisms and AO defenses (23). Human beings and other living organism have developed powerful and complex AO systems. AOs as defined by Halliwell and Gutteridge are group of substances which, when present at low concentrations in relation to oxidizable substrates, significantly inhibit or delay oxidative processes (30). Thus it is very important to maintain equilibrium between pro oxidants and AOs. It can not be solely maintained by endogenous AO system and therefore requires external supply of AOs.

AOs intercept free radical mediated reactions and prevent oxidative damage of cells. AOs function by a number of modes:
**Chain Breaking mechanism** – A number of AOs are able to quench the free radicals by donating hydrogen radicals or electrons and thus break the free radical chain reactions. Thus AOs form new radicals with more stability which leads to stable molecules (24,30,31). Such AOs are called primary antioxidants. Examples include ascorbic acid and α-tocopherol. α-tocopherol can stop lipid peroxidation by donating an electron to the peroxyl radical of fatty acid there by stopping the propagation steps.

**Scavenging initiating radicals** - Certain AOs can react with the initiating radicals, (or inhibiting the initiating enzymes) and such AOs are called secondary AOs. Enzymes (eg: superoxide dismutase (SOD)) and the compounds which inhibit enzymes such as xanthine oxidase (XO), cyclooxygenase etc are typical examples. β-carotene is an efficient singlet oxygen scavenger (24,30,31).

**Metal chelation**: Free metals can catalyse the formation of free radicals. Certain AOs are able to chelate the transition metal and prevent them from catalyzing free radical reactions. For example, albumin and ferritin are good Fe$^{2+}$ chelators. Some low molecular weight compounds such as polyphenols, in addition to their ability to donate hydrogen, can also chelate transition metal ions (24,30,31)

### 1.3.4.1. Classification of AOs

AOs are classified based on several criteria such as their origin, solubility in lipid or water; physical and chemical characteristics etc. Based on their origin, AOs can be classified into synthetic and natural AOs.

#### 1.3.4.1a. Synthetic AOs.

In general synthetic AOs are compounds with phenolic structures of various degrees of alkyl substitution. Synthetic AOs currently permitted for
use in food include BHT, BHA, PG, TBHQ etc. BHA and BHT (Figure 1.6) are fat soluble, volatile monohydric phenolics. These are extensively used in packaged foods. TBHQ is regarded as the best AO for protecting frying oils and fats. PG is sparingly water soluble and functions well in stabilizing animal fats and vegetable oils. The use of synthetic antioxidants is becoming increasingly restricted as many of them are reported to be carcinogenic and this has resulted in an increased interest in the investigation for newer sources of natural AOs.

Figure 1.6 Structures of some stable free radicals and synthetic AO compounds

Triphenyl radical  TEMPO  DPPH radical

BHT  BHA  TBHQ  PG
1.3.4.1b. Natural AOs.

Natural AOs are in turn divided into two main classes: enzymatic and non enzymatic AOs.

(i) Enzymatic AOs:

Cells have developed enzymatic systems which convert oxidants into harmless molecules, thus protecting the organism from the deleterious effects of oxidative stress. AO enzymes therefore have the capacity to lower the free radical burden. They can inhibit the generation of free radicals during all the stages of free radical reaction viz initiation, propagation and termination. The various AO enzymes include;

**Superoxide dismutase (SOD):** SOD is one of the most effective intracellular enzymatic AOs that catalyzes the dismutation of $\text{O}_2\cdot-\rightarrow\text{O}_2 + \text{H}_2\text{O}_2$.

$$2 \text{O}_2\cdot- + 2\text{H} + \text{SOD} \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$$

SOD is the first line in cell defense against oxidative stress. SOD exists in several iso forms, differing in their nature of active metal centre and amino acid composition, as well as their number of subunits, co-factors and other features (23). In humans, there are three forms of SOD are reported: cytosolic Cu-Zn SOD, mitochondrial Mn SOD, and extra cellular SOD. (32). SOD destroys $\text{O}_2\cdot-$ with remarkably high reaction rates by successive oxidation and reduction of the transition metal ions at the active site (33).

**Catalase:** Catalase is an enzyme present in the cells of plants, animals and aerobic bacteria. It is located in the cytoplasm of RBC but compartmentalized in the peroxisomes of the other cells. These enzymes catalyze the conversion of $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ and molecular oxygen. Catalase also inhibits initiation phase of free radical reaction (33).
Glutathione peroxidase: There are 2 forms of the glutathione peroxidase enzymes namely selenium independent (glutathione-S-transferase, GST) and selenium dependent (GPx) glutathione peroxidase (33). GPx act in conjunction with the tripeptide glutathione (GSH) present in the cells. GPx converts lipid hydroperoxides (LOOH) to their corresponding alcohols (LOH) with the simultaneous oxidation of GSH.

\[
\text{LOOH} + 2\text{GSH} \xrightarrow{\text{GPx}} \text{LOH} + \text{GSSG} + \text{H}_2\text{O}
\]

GPx is also involved in the removal of \(\text{H}_2\text{O}_2\)

\[
2\text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{GPx}} \text{GSSG} + 2\text{H}_2\text{O}
\]

Thus GPx competes with catalase for \(\text{H}_2\text{O}_2\) and is the major source of protection against low levels of oxidative stress.

(ii) Non-enzymatic AOs:

Vitamin C: Vitamin C or ascorbic acid (AA) is a very important and powerful AO that works in aqueous environments (23). It is found principally in fresh vegetables and fruits and its deficiency is responsible for scurvy. AA cooperates with Vitamin E to regenerate \(\alpha\)-tocopherol from \(\alpha\)-tocopheryl radical in membrane and lipoproteins (34). AA is a di-acid as it has two ionisable hydroxyl groups. At physiological pH, 99.9% of AA is present as \(\text{ASC}^-\) and only very small proportion as \(\text{ASC}_2\) (0.05%) and \(\text{ASC}^{2-}\) (0.004%). \(\text{ASC}^-\) is an electron donor AO and reacts with radicals to produce resonance stabilized tricarbonyl ascorbate free radical (ASCH\(^\bullet\)). ASCH\(^\bullet\) has a pK -0.86, and hence it is not protonated but it is present in the form of semi dehydroascorbate radical (Asc\(^\bullet\)) a poorly reactive radical.

AA is known to protect membrane against oxidation. Recent in-vivo and ex-vivo studies revealed that AA in plasma increases resistance to lipid-peroxidation in a dose dependent manner, even in the presence of redox active iron or copper and \(\text{H}_2\text{O}_2\) (35). AA also prevents endothelial dysfunction in chronic heart disease by inhibiting NO degradation (36). It neutralizes
oxidants which are produced during macrophage activation by tobacco smoke. Supplementation with AA showed a reduction in markers of oxidative DNA, protein and lipid damage in a number of in vivo studies (37). However, ascorbate is also shown to have pro oxidant properties and this effect of ascorbate was attributed to the release of metal ions from damaged cells (38).

**Figure 1.7. Structures of L-ascorbic acid and dehydroascorbic acid**

![ Structures of L-ascorbic acid and dehydroascorbic acid ]

**Vitamin E:** Vitamin E is a fat soluable vitamin that exists in eight different forms such as- dα, dβ, dγ and dδ tocopherol and dα, dβ, dγ and dδ tocotrienols. α-tocopherol is the most active form of vitamin E in humans and is a powerful antioxidant (39). Tocopherol (Figure 1.3) inhibits lipid peroxidation because they scavenge lipid peroxyl radicals much faster than these radicals can react with adjacent fatty acid side chains or with membrane proteins

\[
\alpha\text{-tocopherol} + \text{lipid-O}_2 \rightarrow \alpha\text{-tocopherol} + \text{lipid-O}_2\text{H}
\]

The –OH group of the tocopherol gives up its hydrogen atom to the peroxyl radical, converting it to lipid peroxide. This leaves an unpaired electron on the O to produce a tocopheryl radical. Thus α-tocopherol breaks the chain reaction of lipid peroxidation. The α-tocopheryl radical can be reduced to the original α-tocopherol form by ascorbic acid (34).
**Introduction**

**Carotenoids:** Carotenoids impart bright color to fruits, flowers and other plant parts and are widely distributed in nature. β-carotene (Figure 1.3), the most important carotenoid with wide distribution in plant kingdom is a provitamin A. Carotenoids are considered as potent AOs. The presence of lengthy conjugate double bond system enables carotenoids to act as effective singlet oxygen quenchers.

\[ ^1\text{O}_2 + \beta\text{-carotene} \rightarrow ^3\text{O}_2 + \beta\text{-carotene} \]

Thus β-carotene and other carotenoids in plants, serve as AOs and help to scavenge singlet oxygen that can be generated by the interaction of light with the plant pigment chlorophyll. β-carotene may also exert other AO effects such as scavenging of free radicals in lipid systems (40).

**Thiol AOs**

**Glutathione:** The tripeptide glutathione (γ -L-glutamyl -L-cysteinyl- L-glycine) is the major thiol AO. It is present in large quantities in organs exposed to toxins such as the kidney, the liver, the lungs and the intestine, but very little is found in body fluids. The reduced form of glutathione is GSH (glutathione) and oxidized form is GSSG (Glutathione disulphide) tripeptide. The AO capacity of glutathione and other thiol compound is due to sulphur atom which can easily accommodate the loss of electrons. Also the life time of sulfur radical species formed (GS●) is significantly longer than many other radicals generated during the stress.

\[ \text{GSH} + \text{R}^\bullet \rightarrow \text{GS}^\bullet + \text{RH} \]
\[ \text{GS}^\bullet + \text{GS}^\bullet \rightarrow \text{GSSG} \]

GSH also serves as a cofactor of several detoxifying enzymes such as glutathione peroxidase and glutathione transferase against oxidative stress. GSH participate in amino acid transport through the plasma membrane. GSH is an effective scavenger of hydroxyl radical and singlet oxygen. GSH is also able to regenerate vitamin C and E back to their active forms. GSH in the
nucleus helps to maintain the redox state of protein sulfahdryls that are necessary for DNA repair and expression (41,42).

**Thioredoxin and glutaredoxin systems:** The thioredoxin system comprises of thioredoxin reductase (TS) and thioredoxin (TrX) a small multi functional disulfide containing redox protein NADPH. It reduces the disulfide bond of several proteins and oxidized GSH to thiol groups. The thioredoxin system thus regulates the activity of proteins such as the transcription factors NFkB and AP-1 (41). Thioredoxin can also reduce other compounds especially lipid hydroperoxides. Trx and TR contribute to maintain the redox status in the plasma by acting as electron donors for the blood plasma peroxidase (43). The mechanism of action of the glutaredoxin system is similar to the thioredoxin system and the former is composed of glutaredoxin (Grx), glutaredoxin reductase (GR) and NADPH. It also needs the presence of glutathione (44). It was suggested that it plays an important role in the intra cellular balance between GSH/GSSG and in the glutathionylation of proteins such as carbonic anhydrase (41, 44).

**Taurine and hypotaurine:** Taurine and its precursor hypotaurine are β amino acids derived from cysteine metabolism (45). Hypotaurine has shown to have the capacity to scavenge hydroxyl radical and to inhibit lipid peroxidation. Taurine supplementation has been shown to decrease lipid peroxidation and it also protects the heart during reperfusion post ischemic (46).

![Figure 1.8 Structures of taurine and hypotaurine](image)
α-Lipoic acid: α-Lipoic acid or thiothic acid is a disulphide derivative of octanoic acid. α-Lipoic acid is both water and fat soluble and hence is widely distributed in both cellular membrane and the cytosol. It exists in cells as lipoamide which is covalently bound to different cytoplasmic protein. Lipoate is reduced to dihydrolipoate by glutathione reductase, thioredoxin reductase or dihydrolipoamia dehydrogenase. Both α-Lipoic acid and dihydrolipoic acid are powerful AOs (47). Their AO functions involve quenching of ROS, regeneration of endogenous and exogenous AOs involving vitamin C and E and glutathione, chelation of redox metals, and repair of oxidized proteins (48).

1.3.5. AO related medicinal properties of phytochemicals
The AO nature of phytochemicals is largely responsible for their anticancer, cardioprotective, immunomodulator, neuroprotective, and antimicrobial properties (49-52). Phenolic compounds and fruit extracts have been reported to have positive effects on cancer, CVD, immune disorders, microbial infections, neurodegenerative disease and viral infections (53-59). Vitamin C, tocopherols and phenolics are the major classes of phytochemicals with AO properties.
1.3.5.1. AO activity of phytochemicals

The AO activity of phytochemicals is related to a) scavenging free radicals, b) chelating transition-metals involved in free-radical production and c) inhibiting the enzymes participating in free-radical generation (24,30,31,60-62). The free radical scavenging activity of phenolic compounds is generally attributed to their ability to donate a hydrogen atom to reduce ROS radicals (63). In doing so, the phenolic compounds (ArOH) are converted to oxidized phenoxy radicals (ArO•) that are stable due to resonance-stabilized delocalization of the unpaired electron over the aromatic ring (61,64). For example, the reduction of peroxyl and hydroxyl radicals by phenolic compounds can be represented as follows:

\[
\begin{align*}
ROO^• + ArOH & \rightarrow ROOH + ArO^• \\
HO^• + ArOH & \rightarrow HOH + ArO^•
\end{align*}
\]

The phenoxy radical intermediates are relatively stable and therefore further oxidation reactions are not easily initiated. Non-radical products may also be formed by the coupling of ROS radicals with phenoxy radicals (63).

\[
ROO^• + ArO^• \rightarrow ROOArO
\]

Phenolic compounds may also enhance the AO activity of non-phenolic AOs by regenerating the oxidized forms of these compounds. For example, phenolic compounds have been reported to regenerate ascorbic acid from its oxidized form (65). The synergistic AO interactions between flavonoids and α-tocopherol in vitro have been demonstrated by Pedrielli and Skibsted (66). (+)-catechin, (-)-epicatechin and quercetin are found to enhance the induction period for oxidation of α-tocopherol and results in longer and increased inhibition of oxidation. The regeneration of α-tocopherol from its oxidized form through the hydrogen atom transfer between flavonoids and tocopherol was suggested as the mechanism for this action.

Metal chelating effects of phenolics due to their catechol, galloyl, or 1,3 positioned hydroxyl and carbonyl moieties also imparts secondary AO
effects by inactivating metals (31, 61). Phenolic acids and flavonoids have been shown to complex with iron (67) and copper ions (68) to provide secondary AO effects.

1.3.5.2. Medicinal Effects of Phytochemicals

There are numerous in vitro and in vivo studies that have indicated the potential medicinal benefits of phenolic compounds. Excellent reviews on this topic have been published (50, 69-73)

Oxidation of LDL is a key event leading to the formation of atherosclerotic plaques (74). It is believed that ROS as well as enzymes, such as lipoxygenase and myeloperoxidase, are involved in the oxidation of LDL (71,75). Some studies have shown the role of dietary phenolics in preventing CVD through their AO effects (69,76,77). Flavonoids have also been reported to exert positive effects on the cardiovascular system through modulation of the nitric oxide synthase system (78,79). The anthocyanins extracted from bilberry (Vaccinium myrtillus) juice have been reported to protect the vascular system in vivo by increasing the permeability of capillary blood vessels (80). Phenolic compounds in red wine are hypothesized to be the beneficial components responsible for inhibiting the oxidation of LDL and thus, providing protection against atherosclerosis (81). Resveratrol, a stilbene has been reported to exhibit AO (82), cardioprotective (83) and anti-inflammatory properties (84). However, not all epidemiological studies favor a protective effect of dietary phenolic compounds against CVD (84,85).

Consumption of green vegetables and fruits found to reduce greatly the risk of cancer (86). Yen and Chen showed that aqueous extracts of black, green, Oolong and Pouchong tea inhibit > 90% of the mutagenicity of a selected number of chemicals toward S. typhimurium TA98 and TA100 (87). Epigallocatechin gallate (EGCG), a phenolic component of green tea, has
been found to reduce the incidence of chemically induced tumors in the esophagus, liver, lungs, skin and stomach of experimental animals (88). EGCG was also reported to possess effective AO properties and can provide protection in vitro against both peroxyl radical and hydroxyl radical induced oxidation of DNA (89). Anthocyanins have been shown to have stimulatory effect on the secretion of tumour necrosis factor-α in macrophages in vitro (90) which are responsible for cytostatic and cytotoxic activities on malignant cells (91). Citrus flavonoids, in particular, flavanones have been shown to display in vitro anticarcinogenic and antiallergenic (92) activities. Hibasami et al; reported that catechin-rich persimmon induced apoptosis in human lymphoid leukemia cells (93). Wang et al; reported that lingonberries had potent free radical scavenging and apoptosis inducing activities (94).

Other studies have shown that resveratrol from grape can inhibit cellular events associated with carcinogenesis including tumour initiation, promotion and progression (95). In vivo studies have indicated that resveratrol has anticancer effects against intestinal (96), colon (97) and skin cancer (73). In contrast, some other studies reported no anticancer effect and even carcinogenic properties at high dosage levels of resveratrol (98,99). Furthermore, it is noted that the very low concentration of resveratrol in red wine (0.3 to 2.0 mg/L) makes the attribution of protective effects to this compound unlikely (100,101). Combination of resveratrol and quercetin synergistically induce apoptosis in human leukemia cells (86). Other studies have suggested that caffeic acid and some of its esters possess antitumour activity against colon carcinogenesis (102,103).

Lignans are recognized as phytoestrogens due to their estrogen moderating activity (104). There is evidence that lignans from flaxseed can reduce mammary tumor size and number in rats (105,106). Genistein, a soy derived ‘phytoestrogen’ has the potential as chemotherapeutic agents capable
of inducing apoptosis by inhibiting DNA topoisomerase and angiogenesis (107) or suppressing tumor promoting proteins such as COX-2 (108). Correlation between the consumption of phenolic compounds and improved health has been reported in epidemiological studies (73, 109).  

Drug phytochemicals interactions also result in varying effects on cancer treatment. For example quercetin has been found to enhance the activity of anticancer drugs such as triazofurin and carboxytriazole on human cancer cells (110,111). The apoptotic inducing activity of EGCG on lung cancer PC-9 cells have been synergistically enhanced by other chemopreventive agents such as sulindac and tamoxifen (112,113). Many cancer drugs are synthesized on the basis of novel structures provided by natural products. Many natural products such as taxanes, vinca alkaloids, podophyllotaxins and camptothecins are served as chemotypes for anticancer drugs (114) 

It has been proposed that the phenolic AOs that are consumed through foods, medical foods and/or dietary supplements seldom reach levels in vivo that are sufficient to function as effective AOs. According to Hensley et al; the level of external phenolic AOs can range from concentrations of high nM to low µM concentrations in cells and blood plasma (62). These authors suggested that the minimum concentration for effective free radical scavenging activity of these AOs, however, is in the medium to high µM concentration range (i.e.50 to greater than 100 µM). In spite of the limited AO levels in foods, they noted physiological benefits of phenolic free radical scavengers in inhibiting diseases associated with oxidative stress. It was proposed that the protective effect of many phenolic AOs, however, may be largely due to inhibition of enzyme systems or signal transducers that are responsible for the generation of ROS. In support of this hypothesis for in vivo AO activity, others have reported that phenolic compounds show marked
inhibition of enzymes responsible for the generation of ROS such as nitric oxide synthase (78,79,90), tyrosine kinase (115) and xanthine oxidase (116). It is important to note that under certain conditions phenolic AOs may act as promoters of free radicals and thus, act as pro-oxidants. Such conditions have been reported to include high concentrations of the phenoxy radicals resulting from low concentrations of synergistic AOs or a lack of reducing enzymes to regenerate the AO from its phenoxy radical state (117).

Tea phenolic compounds have also been also reported to exhibit some other physiological activities. Green tea phenolic components inhibit intestinal uptake of glucose through rabbit intestinal epithelial cells and thus may contribute to the reduction of blood glucose levels (118). Various in vitro studies have been reported that flavonols inhibit many pathogenic microorganisms including *Vibrio cholerae* (70), *Streptococcus mutans* (119) and *Shigella* (120). PA rich extracts from grape seeds also display anticataract activity in rats and the inhibition of cataract progression was suggested to be due to the AO activity of the grape seed PA (121). In other studies, grape seed extracts were found to possess antiulcer properties in rats (99). Caffeic acid is known to selectively block the biosynthesis of leukotrienes, components involved in immunoregulation diseases, asthma and allergic reactions (122). Caffeic and ferulic acid derivatives have been suggested to be potential protective agents against photooxidative skin damage (123). Quercetin has been reported to be a potent inhibitor of human immunodeficiency virus (HIV)-1 protease (58). This compound has also been shown to decrease the infectivity of herpes simplex virus type I, poliovirus type I and parainfluenza virus in vitro (124).
1.4. Sea Buckthorn (*Hippophae rhamnoides*)
Sea buckthorn (SB) (*Hippophae rhamnoides*), is an economically important medicinal plant belonging to *Elaeagnaceae* family. It grows in cold regions of Asia, Europe, and North America. The distribution ranges from Himalayan regions including India, Nepal, Bhutan, Pakistan and Afghanistan, to China, Mongolia, Russia, Kazakstan, Hungary, Romania, Switzerland, Germany, France and Britain, and northwards to Finland, Sweden and Norway (125-128). The wide distribution of SB is reflected in its habit-related variation not only in morphology, yield, growth rhythms and cold hardiness, but also in berry related characters such as fresh weight, chemical and sensory attributes (126, 129). SB is known in different languages as Chharma (Hindi), Shaji (Chinese), and Sanddrome (German). In Greece, SB leaves and twigs are added in fodder to gain weight and shiny coat for the horse. This gives the name to the bush ‘*Hippophae*’. ‘Hippo’ means horse and ‘phoas’ means shine. SB includes 5 or more species namely *H. rhamnoides*, *H. salicifolia*, *H. tibetana*, *H. neurocarpa* and *H. goniocarpa*. In these *H. rhamnoides* is the most common and important species and is further classified into nine subspecies (ssp.) namely *rhamnoides*, *fluviatilis*, *carpatica*, *caucasica*, *mongolica*, *yunnanensis*, *gyatsensis*, *Sinensis*, and *turkestanica* (130).

The SB is a hardy deciduous shrub with narrow leaves, reaching 2 to 4 meters in height and develops a tree-like appearance since usually only the upper buds sprout and branch (131). SB is adapted to cold, drought as well as saline and alkaline soils. Consistent with other members of the *Elaeagnaceae* family, SB is also a nitrogen fixer (132). Its vigorous production of vegetation and its strong and complex root system with nitrogen-fixing nodules make SB an optimal pioneer plant for water and soil conservation in eroded land. Over one million SB seedlings reported to have been planted in the Canadian Prairies since 1982 as a part of land reclamation program (133). The shrub is also planted in British Columbia, Newfoundland and Quebec (127).
In summer, plentiful round yellow-orange berries cover the female plants. SB berries consist of a fairly tough skin and juicy pulp enveloping a small, hard, oval seed. Ripe berries are orange-red in color and have a diameter of 10–15 mm and a soft outer fleshy tissue and hard seed. A natural SB can yield 750 to 1500 kg fruit/ha (134). SB fruit has attracted considerable attention, mainly for its medicinal value and great economic potential.

The fruit and leaves have been used for more than one thousand years in traditional medicines in India, China, Mongolia and Tibet (135). In Asia and Russia fruit and seed extracts have been used for the treatment of burns, cancer, CVD, gastric ulcers and oral inflammation (136,137). SB oil is approved for clinical use in hospitals in China and Russia, where, in 1977, it was formally listed in the Pharmacopoeia (134, 135). More than ten different drugs have been developed from SB in these countries (134).

1.4.1 Sea Buckthorn in India

In India, SB is found in Himalayan regions of Himachal Pradesh, Ladakh (J&K), Uttarakhand, Sikkim and Arunachal Pradesh. SB cultivation in India is mainly concentrated in the cold deserts of Trans-Himalayas (Ladakh, Lahaul and Spiti) at an altitude from 2500 m to 4500 m. Indian Himalayas host world’s second or third largest area under SB (30,000 ha). In Ladakh it grows approximately 12,000 hector area and covers major part of the forest area in the region. The SB-growing areas in the Trans-Himalaya host unique geoclimatic conditions of high altitude coupled with extreme temperature variations (–30 to 30°C), low precipitation, low oxygen in air and arid soil. Mainly three species of SB viz; *H. rhamnoides var. turkestanica*, *H. salcifolia* and *H. tibetana* are found in India. Among these *H. rhamnoides var. Turkestanica* is the most common in India. The SB-growing areas in the Trans-Himalaya host unique geo-climatic conditions of high altitude coupled
with extreme temperature variations (–30 to 30°C), low precipitation, and low oxygen in air (138).

**1.4.2. Chemical Composition of SB berries**

SB berry is recognized for its nutritional benefits, being rich in amino acids, carbohydrates, organic acids, protein, vitamins and phenolic compounds (131). A number of studies have shown that the chemical composition of the fruit varies greatly according to the species, and climatic and geological conditions of the areas where the plant is grown (126,139,140).

The moisture content of SB fruit has been reported to range from 74.0 to 86.7% (140,141). Carbohydrate content in the fresh berries of Chinese origin varies between 8.0 – 14.0% with glucose and fructose as the major carbohydrates and are present in approximately equal amounts (142). The protein content of the fruit varies with the variety and geographical location and is reported to vary from 0.79 to 3.11% on fresh weight basis (143).

SB is reputed to be an excellent source of vitamin C, although a wide concentration range (2 to 2500 mg/100 g juice) has been reported (131,126,144, 146). SB berries are highly acidic in nature. Beveridge et al; reported an average pH of 3.13, and a titratable acidity of 1.97% as malic acid in juice extracted from berries grown in Canada (147). Malic acid was the major organic acid reported in Finnish SB (ssp. *rhamnoides*) with minor quantities of citric and tartaric acid (139).

Yang and Kallio determined the oil content of Chinese and Finnish SB varieties. The whole berry oil content of Chinese SB was 2.1% and 1.7% of the seed and pulp/peel respectively (129). The whole berry oil content of Finnish SB was reported to be 3.5% with an oil content of 11.3% of the seed
and 2.8% of the pulp/peel. Yang and Kallio also published an excellent review on the composition, nutritional effects, and industrial application of SB lipids (148).

The oil content and fatty acid (FA) composition of berries from two subspecies of SB (*H. rhamnoides* L.) were investigated by Yang et al (129). The berries of ssp. *rhamnoides* contained a higher proportion of oil in seeds (11.3% vs 7.3%), berries (3.5% vs 2.1%), and seedless parts (2.8% vs 1.7%) than the berries of ssp. *sinensis*. Linoleic (18:2 n-6) and linolenic acids (18:3 n-3) comprise about 70% of seed oil FA. Palmitoleic acid (16:1 n-7) comprised 12.1-39.0% of oil in pulp/peel. More linoleic acid (40.9% vs 39.1%) and less linolenic acid (26.6% vs 30.6%) are found in the seed oil of ssp. *sinensis* than those in the seed oil of ssp. *rhamnoides*. Palmitoleic acid is practically absent in seed oils.

Ozerinina and coworkers investigated the composition and structure of the triacylglycerols (TAGs) of SB seeds belonging to Russian varieties with the aid of lipase hydrolysis (149). Various SB (*H. rhamnoides* L.) climatypes are classified into two classes based on the composition, structure, and biosynthetic pattern of TAGs of their fruit mesocarp oil; (1) Siberian, Central Asian, and Baltic, and (2) Caucasian. Group 1 oils contain predominantly monosaturated-diunsaturated (SU2) TAG, which include C16:0 and C16:1 fatty acid (FA) residues. Berry mesocarp oil of SB of the Caucasian climatype includes mainly disaturated-monounsaturated (S2U) and SU2 TAGs rich in the residues of C16:0 and C18:1 isomers. As for the seeds, the climatypes studied here practically do not differ from each other in the FA composition of their TAGs (149, 150).

HPTLC and GC profiling of FA in total and individual polar lipids separated from carotenolipoprotein complexes in SB berries were reported.
The polar lipids included 61% phospholipids and 39% galactolipids, which contained mainly 16:0, 16:1 (n-7), 18:1 (n-9), 18:1 n-7 and 18:2 (n-9, 6) FA. Almost all polar lipids showed high ratios of 16:0/16:1 and 18:1 (n-9)/18:1 (n-7), and higher quantities of 18 carbon unsaturated FA than of the saturated analogue. Galactolipids were shown to be rich in 18:1 (n-9) and 18:3 (n-9, n-6, n-15) FA, while phospholipids contained higher concentrations of 16:0 and 18:1 (n-9) FAs (151).

Application of capillary supercritical fluid chromatograph (SPC), combined with a triple-quadrupole mass spectrometer (MS) via a liquid chromatography-atmospheric pressure chemical ionization (LC-APCI) interface for the analysis of SB berry oil TG has been demonstrated by Manninen et al (152). Zadernowski et al; showed that lipase activity was reduced by 40-70% and 13-50% by lipophilic and hydrophilic-EtOH extracts of SB berries of Poland origin (153).

The total sterol content was found to range from 1200 to 1800, 240 to 400 and 340 to 520 mg/kg in the oils from seeds, fresh pulp/peel and whole berries, respectively (154). Sitosterol constituted 57 to 76% and 61 to 83% of the seed and pulp/peel sterols, respectively. Salenko et al reported the presence of β-Sitosterol, 24-methylene-cycloartanol, citrostadienol, and uvaol in the unsaponifiable part of a pentane extract of the fruit pulp of common SB (155). Phytosterols in SB (*H. rhamnoides L.*) seed oil extracted by cold pressing, hexane, and supercritical carbon dioxide (SC-CO$_2$) were identified by GC-MS and FID and reported that sitosterol and δ-5-avenasterol were, quantitatively, the most important phytosterols (156).

The carotenoid content of SB fruit varies with the geoclimatic conditions but typically ranges from 30 to 40 mg/100 g fruit (131) with β-carotene accounting for approximately 45% of the total carotenoids. HPLC
quantification of free tocopherol content in whole berries of six SB cultivars grown in northeastern Poland and Belorussia was reported (157). The total free tocopherol content in oil from whole berries was 101–128 mg/100 g of oil with α-tocopherol as the predominant. α- and δ-tocopherols constituted 62.5–67.9% and 32.1–37.5% of total tocopherol, respectively, and only traces of γ-tocopherol were detected in the oil. Green berries contained a marked amount of γ-tocopherol, but its content rapidly declined to traces when the color of berries turned from green to olive-yellow.

The total phenolic content of SB fruit was reported to range from 114 to 244 mg/100 g fruit (158). These authors reported a strong positive correlation between the AO capacity of the fruit and its total phenolic and ascorbic acid contents. Phenolics, including flavonols, flavones, phenolic acids, PAs and hydrolysable tannins are reported as the major contributors to the biological properties like AO activities of SB berries and leaves (144,157).

The flavonoid content in the leaves and fruit of SB has been reported to range from 310 to 2100 mg/100 g dried leaf and 120 to 1000 mg/100 g fresh fruit, respectively (141). Analytical and preparative countercurrent chromatographic separation of flavonoid constituents from crude ethanol extract of SB dried fruits has been demonstrated (159,160). Preparative isolation and purification of flavonoids and protocatechuic acid from SB juice concentrate by high-speed counter-current chromatography was also reported (161). Zhang et al; optimized the conditions for the simultaneous determination of quercetin, kaempherol, and isorhamnetin in phytopharmaceuticals containing SB by HPLC with chemiluminescence detection (162). HPLC-DAD analysis of flavonoids in SB leaves has been described by Zu et al. and reported the presence of catechin, quercetin, isorhamnetin and rutin (163).
Jeppsson et al; investigated the variations in contents of kaempherol, quercetin and L-ascorbic acid in the berries during maturation using HPLC. The content of ascorbic acid and quercetin decreased over the time for the five cultivars studied, whereas kaempherol increased during maturation (125). Rosch et al; investigated the phenolic composition of SB fruit juice by HPLC-DAD and ECD (164). Flavonols are found to be the predominating polyphenols while phenolic acids and catechins present in minor amounts. Quantitatively isorhamnetin-3-O-glycoside is the predominant and is a poor radical scavenger as shown by ESR. Phenolic compounds such as quercetin 3-O-glycosides, catechins, and hydroxybenzoic acids with a catechol structure exhibited good AO capacities. These phenolic compounds account for less than 5% of the total AO activity of the filtered juice and ascorbic acid is shown to be the major AO in SB juice.

In another study isolation of 4 flavonol glycosides from SB pomace (H. rhamnoides) by Sephadex LH-20 gel chromatography and semi-preparative HPLC was reported. The occurrence of the major flavonol glycoside kaempferol-3-O-sophoroside-7-O-rhamnoside in SB is also reported. Most of the compounds identified were 7-O-rhamnosides of isorhamnetin, kaempferol, and quercetin, which exhibit different substitution patterns at the C-3 position, mainly glucosides, rutinosides, and sophorosides. In addition, numerous flavonol glycosides were detected lacking a sugar moiety at C-7. Finally, eight flavonol derivatives were identified that are acylated by hydroxybenzoic or hydroxycinnamic acids (165). Recently, Chen et al; reported the development of a HPLC fingerprint method for investigating and demonstrating the variance of flavonoids among different origins of SB berries (166). Thirty-four samples were analyzed including 15 H. rhamnoides ssp. sinensis samples, 7 H. rhamnoides ssp. yunnanensis samples, 5 RW H. rhamnoides ssp. wolongensis samples, 4 NS H. neurocarpa
ssp. stellatopilosa samples and 3 TI *H. tibetana* samples and 12 flavonoids are identified from HPLC chromatograms.

Rosch et al; reported the identification of monomeric flavonols and PA from SB (*H. rhamnoides*) pomace (167). Five dimeric PA are identified by HPLC-ESI-MS/MS and by acid catalyzed cleavage in the presence of phloroglucinol. Nine trimeric PA are tentatively identified by HPLC-ESI-MS/MS in the Sephadex fractions. The isolated flavan-3-ols and proanthocyanidins are potent in scavenging Fermy's salt, a synthetic free radical. They possess antioxidant capacities that are higher or comparable to that of ascorbic acid or trolox. On comparing the antioxidant capacities of monomeric flavan-3-ols and dimeric PA, no significant influence from the degree of polymerization (DP) was observed.

In another report monomeric flavan-3-ols, and dimeric and trimeric PAs are fractionated from an extract of SB (*H. rhamnoides*) pomace by Sephadex LH-20 gel chromatography and subjected to AO assays. The oligomeric fraction accounted for 84% of the total PA and 75% of the total AO activity of the SB pomace extract. Quantitative HPLC, NMR and MS investigations demonstrate (+)-gallocatechin as the predominating subunit in the oligomeric fraction and the majority of the flavan-3-ol subunits possessed a 2,3-trans configuration. The oligomers consisted mainly of prodelphinidin subunits whereas procyanidins were present in smaller amounts, indicating a very uncommon composition of the SB PA. The mean DP of the oligomeric PA is between 6 and 9 (165). In a recent report 4 monomeric flavan-3-ols (catechin, epicatechin, gallocatechin and epigallocatechin), along with 2 dimeric procyanidins, (catechin-(4α-8)-catechin and catechin-(4α-8)-epicatechin), are isolated from seeds. Polymeric PAs are also fractionated and their chemical constitutions studied by acid-catalysed degradation in the presence of toluene- α-thiol. The results showed highly heterogeneous
polymers, with catechin, epicatechin, gallocatechin and epigallocatechin as the constituent components of both the extension as well as the terminating units. The mean DP is 12.2 and the proportion of prodelphinidins was 81.2% (168).

In a recent report analysis of AO compounds such as trans-resveratrol, catechin, myricetin, quercetin, p-coumaric acid, caffeic acid, L-ascorbic acid, and gallic acid in six different varieties of SB berries (SB varieties: "Trofimovskaja (TR)," "Podarok Sadu (PS)," and "Avgustinka (AV),") is published. Trans-Resveratrol, catechin, ascorbic acid, myricetin, and quercetin were found in all SB extracts. The biggest average AA content was found in TR (740 mg/100 g of dried berries). The same varieties gave the highest quercetin content 116 mg/100 g of dried berries) (169).

Rosch et al; reported the amount of total GA and ProCA in SB berries from Finland by HPLC analysis (164). Zadernowski et al; found that the phenolic acid composition in SB berries ranged from 3570 to 4439 mg/kg on dry weight basis. They tentatively identified 17 phenolic acids in the fruit with salicylic acid accounting for 55 to 74% of the total. The phenolic acids in the fruit were mainly in their esterified and glycosylated forms, whereas the maximum free phenolic acids content was 2.3% (170).

The reported chemical composition of SB berries varies considerably. This may be because of different origins/subspecies, the climate and geographical conditions of growing areas, and agronomic practices (129). A systematic mapping of the chemical composition of SB berries of different varieties and origins is still lacking.

Nutritional qualities of different varieties of SB berries belonging to different geoclimatic conditions are compared in terms of their chemical
composition by several authors. Kallio et al; evaluated the vitamin C, tocopherols, and tocotrienols contents in berries of wild and cultivated SB (*H. rhamnoides L.*) of different origins and harvesting dates. Wild berries of ssp. *sinensis*, native to China, contained 5-10 times more vitamin C in the juice fraction than the berries of ssp. *rhamnoides* from Europe and ssp. *mongolica* from Russia (4.0 – 13.0 v/s 0.02-2.0 g/L juice). For bushes cultivated in southwest Finland, the best berry harvesting date for high vitamin C content was the end of August. The seeds of ssp. *sinensis* contained less tocopherols and tocotrienols (average 130 mg/kg) compared with seeds of ssp. *rhamnoides* (average 290 mg/kg) and *mongolica* (average 250 mg/kg). The fruit flesh of *sinensis* berries had contents of tocopherols and tocotrienols 2-3 times higher than those found in the other two subspecies (120 mg/kg vs 40 mg/kg in *rhamnoides* and 50 mg/kg in *mongolica*). The total content of tocopherols and tocotrienols in the soft parts of the berries reached the maximum level around early- to mid-September, whereas the content in seeds continued to increase until the end of November (145).

Berries and seeds of two subspecies (*ssp. sinensis and mongolica*) of SB (*H. rhamnoides L.*) have been compared in terms of TAG, glycerophospholipids (GPL), tocopherols, and tocotrienols. The study shows that the berries of ssp. *mongolica* contained less oleic acid (4.6 vs 20.2%) and more palmitic (33.9 vs 27.4%) and palmitoleic (32.8 vs 21.9%) acids in TAG than those of ssp. *sinensis*. The proportions of linoleic acid (32.1 vs 22.2%, in berries; 47.7 vs 42.7%, in seeds) and palmitic acid (21.1 vs 16.4%, in berries; 17.0 vs 14.1%, in seeds) in GPL are higher in ssp. *mongolica* than in ssp. *sinensis*, and vice versa with oleic acid (4.3 vs 18.5% in berries, 10.0 vs 22.2% in seeds). A higher proportion of linolenic acid is also found in the GPL of ssp. *sinensis* berries (16.2 vs 10.1%). Tocopherols constitute 93-98% of total tocols in seeds, and α-tocopherol alone constitutes 76-89% in berries. The total contents of tocols vary within the ranges of 84-318 and 56-140
mg/kg in seeds and whole berries, respectively. The seeds of *ssp. mongolica* are a better source of tocols than those of *ssp. sinensis* (287 vs 122 mg/kg, p < 0.001) (171).

Ts syndambev et al; evaluated the changes in the quantitative composition of TAGs in maturing SB (*H. rhamnoides* L.) seeds by lipase hydrolysis and reported that as a whole, the rate of synthesis of separate TAG classes increased in proportion to both their unsaturation and relative content (weight percent) in total TAGs (170). Essential oil and FA composition of the SB fruits (*H. rhamnoides*) L. Turkey was reported by Cakir (173).

TAG of seeds, berries, and fruit pulp/peel of different subspecies of SB (*H. rhamnoides*) has been analyzed by MS and tandem mass spectrometry (MS/MS). The study shown that the seeds contained mainly TAG with acyl carbon number (ACN) of 52 with 2-6 double bonds (DB) (20-30%), and TAG of ACN 54 with 3-9 DB (70-80%). In the pulp/peel fraction, the major TAG were species with ACN:DB of 48:1 to 48:3 (19-49%), 50:1 to 50:4 (31-41%), and 52:1 to 52:6 (9-19%). *Ssp. sinensis* differed from *ssp. mongolica* and *rhamnoides* by having a higher proportion of TAG of ACN 52 (27% vs. 21% and 22%) and a lower proportion of ACN 54 (71% vs. 79% and 78%) in seed TAG. Seed TAG of *ssp. mongolica* contained a higher proportion of more unsaturated species compared with those of the two other subspecies. Berry TAG of *ssp. mongolica* had the highest proportion of molecular species of ACN 48 due to the higher proportion of palmitic and palmitoleic acids and the lower seed content of the berries. Overall, palmitic acid favored the sn-1 and sn-3 positions. The order of preference of unsaturated FA for the sn-2 position depended at least partially on the FA combination of TAG. Seed TAG of *ssp. mongolica* contained a higher proportion of linolenic acid in the sn-2 position than those of *ssp. sinensis*. In berry TAG, *ssp. mongolica* had the highest proportions of palmitoleic and linoleic acids in the sn-2 position, and the
highest proportion of oleic/cis-vaccenic acid in the sn-2 position, among the three subspecies (174).

Abid et al; recently reported the physico-chemical characteristics and FA profiles of seed and pulp oils of SB (*H. rhamnodes L*) wildly grown in Northern Areas of Pakistan (Skardu). Pulp oil with 10.0% yield has palmitic acid (34.5 %) and almitoleic acid (33.4 %) as major FA. Oleic acid (22.1 %), linoleic acid (29.6 %) and linolenic acid (23.4 %) are the major FA in seeds oil (yield 4.5%) (175).

**1.4.2. Processing of SB berries**

Being a good source of bioactive phytochemicals, SB berries have been processed by hundreds of industries in China and Russia for nutraceutical and cosmaceutical products. Reports describing the processing of SB berries are rather limited. Beveridge et al and Zeb et al 2004 extensively reviewed the various processing techniques of SB berries and applications of products (127, 176). Both authors tabulated the available compositional data for the main products to form comprehensive sources of information on the manufacture and composition of SB products. Juice, pulp oil, seed oil, cream and pigments are the main commercial products from SB berries. Normally the processing begins with the harvesting of berries. The diseased and damaged berries and stems, leaves and other debris are removed as a part of cleaning. Washing the berries with luke warm water or with mild detergents or wetting agents are suggested to increase the juice yield. Pressing techniques such as screw pressing, cloth pressing or serpentine pressing etc are being utilized for the separation of juice from berries. Juice obtained by the conventional processing techniques reported to be turbid, with a high content of suspended solids and pulp oil (177). Juice with pulp oil leads to the formation of an undesirable oily layer on the top during storage. Centrifugation of unheated juice causes rapid separation into a floating cream
phase, an opalescent clear juice in the middle and sediment. Zhang et al.; suggested the use of a stalk centrifuge or a cream separator to separate the cream from the juice (178). Beaveredge et al.; suggested the removal of fat from the centrifuged juice with minimum contamination to adjacent layers by keeping at 4°C or lower temperature (147). Liu and Liu reported the use of pectin methyl esterase to break down pectins in the pulp to obtain clear juice (179). Heilscher and Lorber reported the use of crystalline sugar for sedimentation and subsequent centrifugation for clear juice (180). Solvent extraction has been tried for oil recovery, but it is not recommended for nutraceutical applications owing to the residual solvents and the destruction of bioactive phytochemicals during desolventisation. Fresh pressed juice separates into three phases when allowed to stand overnight in the refrigerator: an upper cream phase, juice in the middle portion, and sediment at the bottom. Enzymatic hydrolysis with commercial, broad spectrum carbohydrate hydrolyzing enzyme preparations reduces the juice viscosity, assists juice separation, and provides an opalescent juice (147).

SC-CO$_2$ extraction has been suggested for superior quality, solvent-free oils. A theoretical model of SC-CO$_2$ extraction of organic oil from SB seeds is constructed by Derevich et al.; (181). However, the berries must be dried before supercritical extraction, resulting in a loss of juice and phytonutrients during drying.

1.4.4 Physiological effects of SB berries

A wide spectrum of physiological effects of SB berries and berry products has been reported, including AO (128,144,182,183), radio-protective (184), anti tumor (185,186) inhibition of LDL cholesterol oxidation and platelet aggregation (187), anti-hypertensive (188), immunomodulation and cytoprotective effects (189), protection from gastric ulcer (190), reduction of atopic dermatitis (191,192), and wound healing (193).
Gao et al; investigated the AO activity of SB fruits and its relationship with maturity (158). The study demonstrates that capacity of phenolic and ascorbate extracts to scavenge radicals decreased significantly with increased maturation and the changes were strongly correlated with the content of total phenolics and ascorbic acid. AO capacity of the lipophilic extract increases significantly with maturation and corresponds to the increase in total carotenoids. Eccleston et al; reported that SB juice was rich in AO and moderately decreased the susceptibility of LDL to oxidation (144). Alcohol and water extracts of various SB seeds are found to possess high levels of AO and antibacterial activities and these activities are attributed to the high phenolic content in SB seeds (128,194). SB seed oil is reported to exert protection from oxidative damage caused by SO$_2$ exposure in mice (195).

Johansson and coworkers showed that SB oil inhibited platelet aggregation in humans (188). A similar inhibitory effect to aspirin on platelet aggregation induced by collagen in mouse femoral artery was reported for a total flavone extract from SB (196). This ability to prevent in vivo thrombogenesis suggested that SB fruit consumption may help prevent cardiac and cerebral thrombosis in humans.

In contrast to the in vitro studies Suomela and coworkers reported that SB flavonols, ingested with oatmeal porridge, do not have a significant effect on the levels of oxidized LDL, C-reactive protein, and homocysteine, on the plasma AO potential, or on the paraoxonase activity in human (197). They also showed that flavonols in oatmeal porridge were rapidly absorbed, and a relatively small amount of SB oil added to the porridge seemed to increase the bioavailability of flavonols considerably.

In another study Nersesyan and Muradyan shown that SB juice protects mice against genotoxic action of the anticancer drug cisplatin (198).
Geetha et al; found that concentrated (500 µg/mL) alcoholic extracts of fruit and leaves of SB could inhibit chromium-induced free radical apoptosis and DNA fragmentation and restored AO status to that of control cells in a lymphocyte in vitro model system. The leaf extracts have a cytoprotective effect against chromium induced cytotoxicity as well as immunomodulating activity (188).

The preventive effect of SB extracts on liver fibrosis was also demonstrated through a clinical study (199). SB proanthocyanidins reported to play an important role in healing of acetic acid-induced gastric lesions in mice possibly by the acceleration of the mucosal repair (200).
1.5. Relevance and Objectives of the Present Study

Epidemiological surveys have provided positive correlation between diets rich in fruits and vegetables and the delayed onset of degenerative diseases and ageing. Vast diversity and better productivity of natural products towards drugs and drug leads over de novo molecules coupled with epidemiological results favoring the therapeutic efficiency of herbals develop an increased interest in natural products among health scientists recently. This demands detailed phytochemical and pharmacological investigations on plants along with standardization and evidence based validation of herbal products. This also requisites the development of economically viable and industrially adaptable processing techniques for plant products suitable for nutraceutical and pharmaceutical applications.

SB is grown in cold regions of Asia, Europe and North America. World annual production of SB berries is approximately 200,000 tons and is being used as food and for nutraceutical and cosmaceutical applications. Complex nitrogen fixing root system enables this plant as a optimum pioneer plant for eroded areas. In India, SB is grown in the Trans-Himalayan cold deserts such as Ladakh, Lahaul, and Spiti at altitudes 2500–4500 m. *H. rhamnoides, H. salicifolia, and H. tibetana* are the predominant SB species in India. Of these, *H. rhamnoides* is widely distributed in the Trans-Himalayan region. *H. salicifolia,* and *H. tibetana* are respectively endogenous to Trans-Himalayan region and Indian Himalayas. In India 30,000 hectares of land is under SB and act as a source of food, medicine and cattle feed as well as income for villagers in under developed SB growing areas.
Detailed reports on the phytochemical compositions of SB berries are rather limited. In India, SB growing areas are under extreme climatic stress with wide temperature variations (-40 to +40), low precipitation, high sunlight and arid soil. Since the emergence of secondary metabolites in plants is associated with their defense and survival mechanisms, geo-climatic variations might reflect in their phytochemical composition. SB in Indian Trans-Himalayas has not been investigated in this perspective. In this study the commonly cultivated *H. rhamnoides* berries were investigated in detail for their chemical composition. Analytical methods suitable for the quality evaluation of berries and berry products were also standardized. Nutritional quality of the berries belonging to major species of SB grown in India namely; *H. rhamnoides*, *H. salicifolia*, and *H. tibetana* was compared using modern analytical techniques as a part of this investigation.

Even though more than hundreds of industries, mostly from China and Russia are engaged in the processing of SB berries for value added products, detailed reports with processing parameters and chemical evaluation of process streams are not available. Indian Himalayas hosts world’s second largest area under SB, however, so far, no attempt has been made to utilize SB berries grown in this region. SB berries are highly perishable and have to be processed within hours of harvesting to obtain quality products. Most of the available process involves organic solvents for oil extraction which is not suitable for ecologically fragile SB growing areas in Himalayas. This demands a green process (solvent free) with minimum technicalities applicable for fresh berries and suitable for ecologically sensitive areas. Therefore, development of a green process for the production of pulp oil and oil free clear juice with maximum retention of bioactive phytochemicals was attempted here. SC-CO$_2$ extraction conditions for oil from the seeds obtained as a byproduct of the developed process was also optimized.
A wide spectrum of biological activities has been attributed to SB berries. Apart from the reports on applications and physiological properties of SB, reports describing the bioactivities of SB berries in relation with their phytochemical compositions are limited. This study was aimed at the evaluation of AO properties of SB berries and chemical profiling of active fractions. Extracts of anatomical parts of berries were prepared and subjected to in vitro AO capacity evaluation. Active extracts were further fractionated and AO capacity was evaluated. The AO active fractions were subjected to detailed chemical composition analysis.

SB growing under unique geo-climatic conditions of Trans-Himalayan region of India has not been investigated in terms of the detailed phytochemical profile and chemical characterization, AO properties and process development for products for nutraceutical and cosmeceutical applications. Objectives of the present study was therefore framed, from the above prospective. Thus this study was undertaken with the following objectives;

1. Detailed investigations on the chemical composition of *H. rhamnoides* berries in Indian Himalayan region.
2. Development of analytical protocols for major bioactive phytochemicals in SB berries.
3. Quality evaluation of SB berries belonging to major species of SB in India
4. Development of a green integrated process for fresh SB berries for nutraceutical and cosmeceutical applications.
5. Evaluation of process streams and products in terms of their yield, efficiency and chemical compositions.
6. Evaluation of in vitro AO capacity of SB berries
7. Chemical profiling of active berry extracts.
8. Evaluation of structure-activity relationship of major AO active compounds in SB berries.

Results of the present investigation are summarized and discussed as Chapter-3-Results and Discussion. Chapter-3 is divided into 4 sections. Section 3.1 deals with the detailed chemical profiling of common SB (H. rhamnoides) berries in Indian Trans-Himalayas. Berries were separated into pulp, seed coat and kernel and analyzed in detail for their lipid profile, vitamin C content, and organic acid and phenolic composition. Analytical protocols for the profiling of major bioactive phytochemicals in SB berries were also standardized. Section 3.2 discusses the quality evaluation of berries belonging to major SB species found in India. The berries were compared in terms of their lipid profile, vitamin C and phenolic contents. Development of a green process for the integrated processing of fresh SB berries for high quality pulp oil, juice and seed oil is discussed in Section 3.3. The process parameters and chemical composition of process streams and products were evaluated. Section 3.4 deals with the AO capacity evaluation of H. rhamnoides berries and chemical profiling of active fractions. Summary and conclusion of the present study is included as Chapter 4