PHYTOCHEMICAL INVESTIGATIONS ON SEA BUCKTHORN (*Hippophae rhamnoides*) BERRIES

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BY

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DECLARATION

I hereby declare that the thesis entitled 'PHYTOCHEMICAL INVESTIGATIONS ON SEA BUCKTHORN *(HIPPOPHAE RHAMNOIDES)* BERRIES' embodies the results of investigations carried out by me at Agroprocessing and Natural Products Division of National Institute for Interdisciplinary Science and Technology (CSIR), Thiruvananthapuram, as a full-time Research Scholar under the supervision of Dr. C. Arumughan and that no part of this thesis has been presented before for any other degree.

Ranjith A

Thiruvananthapuram
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CERTIFICATE

This is to certify that the thesis entitled 'PHYTOCHEMICAL INVESTIGATIONS ON SEA BUCKTHORN (Hippophae rhamnoides) BERRIES' is an authentic record of research work carried out by Mr. Ranjith A under my supervision in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Chemistry of Cochin University of Science and Technology, and further that no part of this thesis has been submitted elsewhere for any other degree.

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Dedicated to
My Parents, Teachers and Friends

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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.

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 C_1 to C_{10} 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, cardio active 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 (6,7)

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 1.1. Classification of phenolic compounds based on structure.

Structure	Class
C_6	Simple phenolics
C_6-C_1	Phenolic acids and aldehydes
C_6-C_2	Acetophenones and phenyl acetic acids
C_6-C_3	Cinnamic acids, cinnamyl alcohols and cinnamyl aldehydes
C_6-C_1-C_6	Benzophenones
C_6-C2-C_6	Stilbenes
C_6-C_3-C_6	Flavonoids
C_{18}	Betacyanins
C_{30}	Biflavonoids
C_6, C_{10} & C_{14}	Quinines
Dimmers or oligomers	Lignans and neolignans
Oligomers or polymers	Tannins
Polymers	Phlobaphens

(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 dihydrochalocones 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 gallocatechin 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.

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**2**), quercetin (5,7,3′,4′ hydroxyflavone), and myricetin (5,7,3′,4′,5′hydroxyflavone).

Flavonoids with pyrilium cation are refered 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 *Fabaceau*. Genestein from soybean and coumstrol from *Medicago sativa* are phytoestrogens (13).

(v) Biflavonyls: Biflavonyls 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 cardioprotecive 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.

(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 elligitannins (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 *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 $C_4 \longrightarrow C_8$ and/or $C_4 \longrightarrow C_6$ (B type) or linked through an ether bond between $C_2 \longrightarrow C_7$ (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

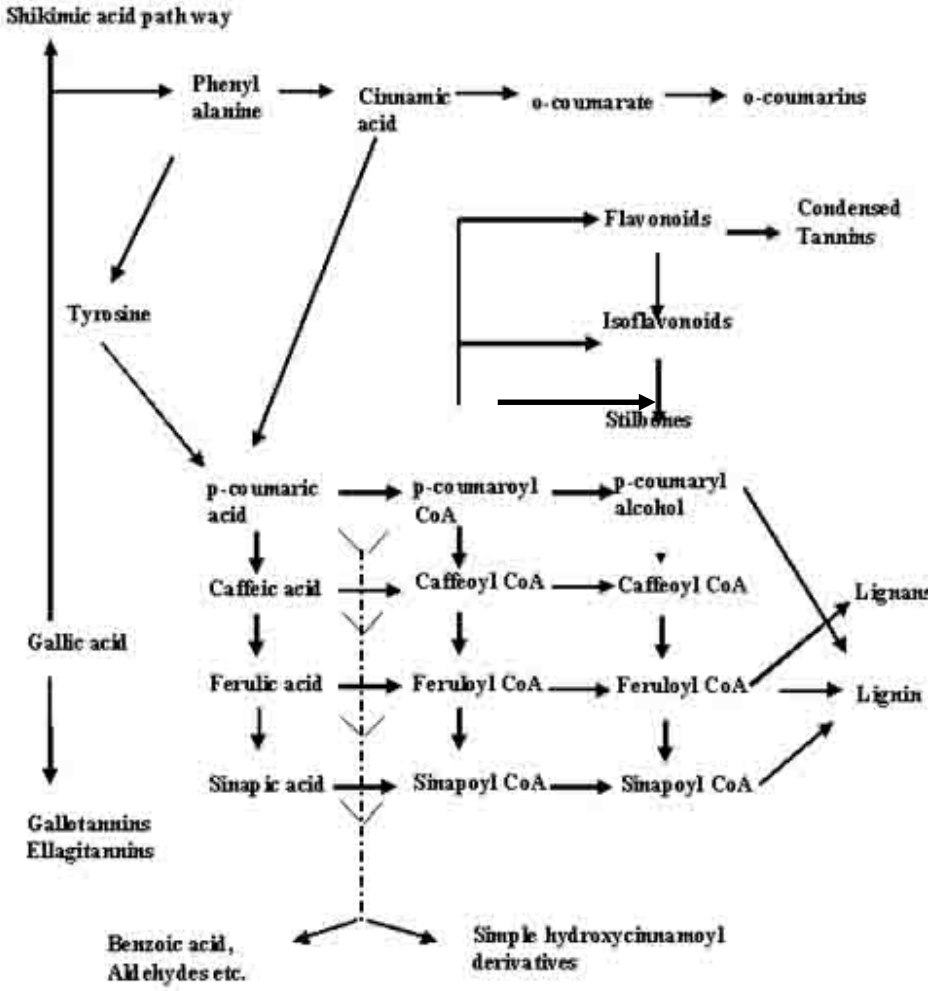
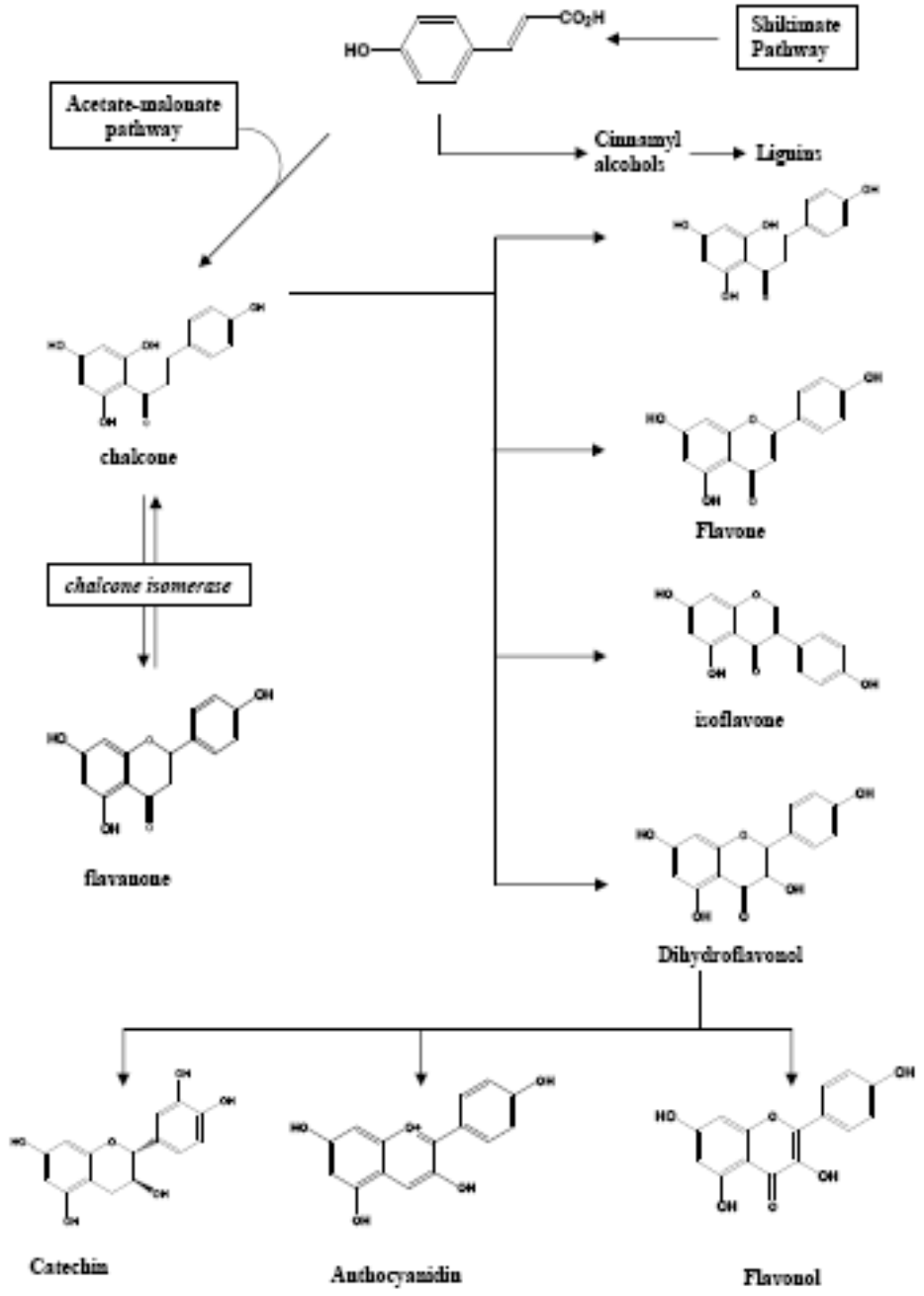


Figure 1.2 Interrelationships among flavonoid monotypes

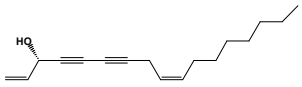


1.2.3. Amines and alkaloids

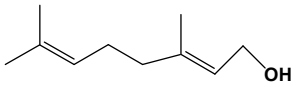
1.2.3.1 Amines: Compounds containing nitrogen as a part of their side chain are generally referred to as amines. These molecules impart fishy odor and believed to act as insect attractants. Aliphatic polyamines such as putriscine ($NH_2(CH_2)_4NH_2$), agmatin ($NH_2(CH_2)_4NHC(=NH)NH_2$), and spermidine ($NH_2(CH_2)_3NH(CH_2)_4NH_2$) 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. Mescalin (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 (*Papver somniferum*) is a potential narcotic analgesic. Strychnine from S*trychnos nux vomica* is a strong poison. Colchinine 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 antiapoptic activity. Papaverine is used as a vasodialator (19).

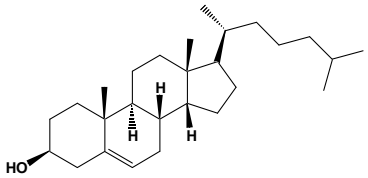
Figure 1.3: Structures of some biologically active phytochemicals



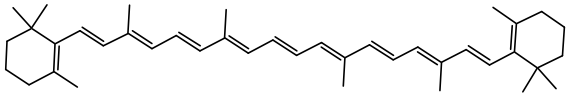
Falcarinol



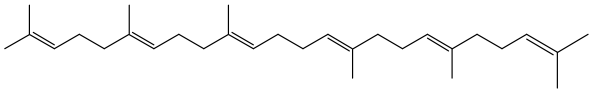
Geraniol



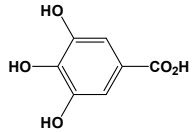
Cholesterol



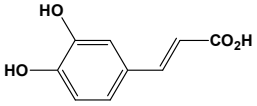
β-carotene



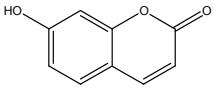
Squalene



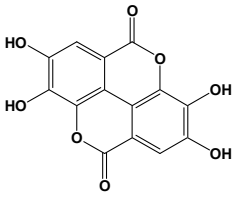
Gallic acid



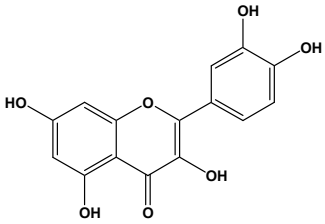
Caffiec acid



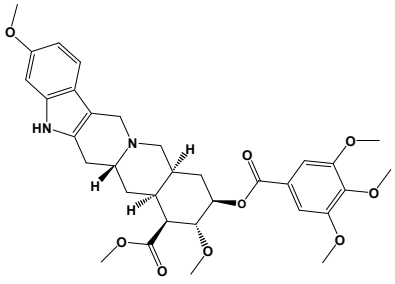
Umbelliferone



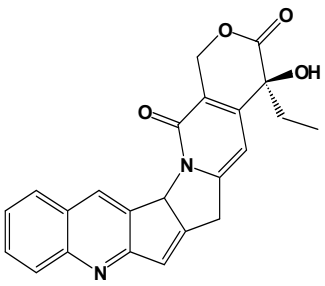
Ellagic acid



Quercetin

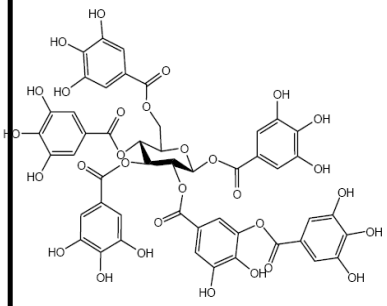


reserpine

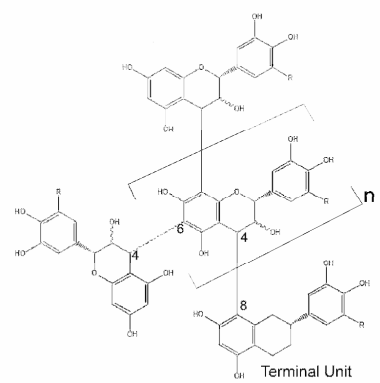


camptothecin

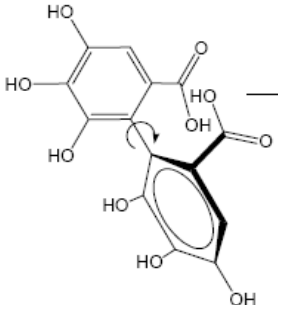
Figure 1.4 Structures of tannins and hexahydroxydiphenyl unit



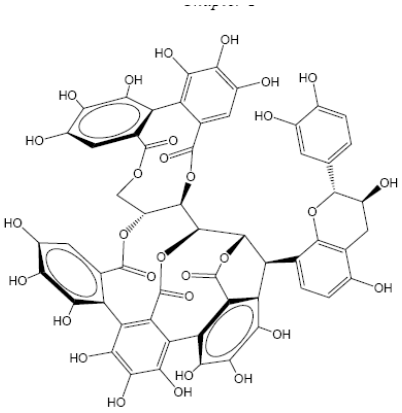
Gallotannin



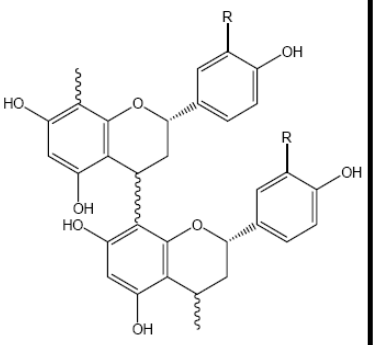
Condensed tannin (proanthocyanidin)



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, resonation 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 recative 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)

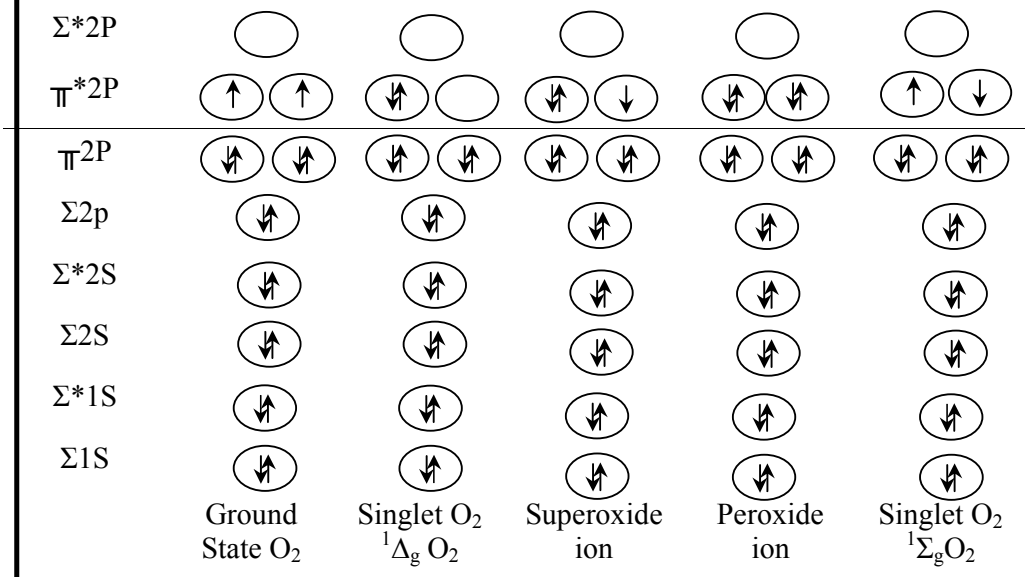
Reactive oxygen species (ROS) ie; radicals derived from oxygen, represent the most important class of radical species generated in living system. Oxygen itself is a diradical with 2 unpaired electrons residing in antibonding π orbitals. This distribution of electrons makes it impossible for

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 ($O_2^{\bullet -}$) (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_2O_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_2O_2 is a stable non-radical, it act as precursor for the most reactive ROS namely hydroxyl radical ($OH^{\bullet}$). 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 ($RO^{\bullet}$) and peroxy ($ROO^{\bullet}$) 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.



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

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)

ROS/RNS	Chemical formation/exogenous sources
Singlet oxygen	Photosensitized oxidation. The reactions like
	$OCl^- + H_2O_2 \longrightarrow Cl^- + H_2O + {}^1O_2$
Superoxide anion	Univalent reduction of O_2 by hydrated electrons
Peroxide ion	Two electron reduction product of O_2
Hydrogen peroxide	Radiolysis or photolysis of H_2O.
	Dismutation reactions.
	$OH^\bullet + OH^\bullet \longrightarrow H_2O_2$.
	$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2$
Hydroxyl radical	Radiolysis or photolysis of H_2O_2, Fenton reaction
	$Fe^{2+} + H_2O_2 \longrightarrow OH^\bullet + OH^- + Fe^{3+}$
Ozone	Polluted air and photochemical reactions like
	Solar energy
	$O_2 \longrightarrow 2O^*$
	$O_2 + O^* \longrightarrow O_3$
Hypochlorous acid	Enzymatic reactions
Peroxyl and alkoxy radicals	Lipid peroxidation
Organic hydroperoxide	
Nitric oxide.	Smoke
Nitrogen dioxide	
Peroxynitrite	Smoke.
	By the reaction $O_2^{\bullet-} + NO \longrightarrow ONOO^-$

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

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

ROS/RNS	Endogenous sources
Singlet oxygen ($^1O_2$)	Photosensitized oxidation & action of myeloperoxidase (MPO) during phagocytosis MPO $H_2O + Cl^- + H^+ \longrightarrow H_2O + HOCl$ $HOCl \longrightarrow H^+ + OCl^-$ $OCl^- + H_2O_2 \longrightarrow Cl^- + H_2O + {}^1O_2$
Superoxide anion ($O_2^{\bullet-}$)	Enzymatic reactions (eg; xanthine/XO), autooxidation reaction of oxyhaemoglobin, electron transport chains of mitochondria, endoplasmic reticulam, phagocytosis etc.
Hydrogen peroxide (H_2O_2)	Enzyme systems (eg; glycollate oxidase), phagocytosis, dismutation of $O_2^{\bullet-}$ etc.
Hydroxyl radical ($OH^{\bullet}$)	Radiolysis of H_2O_2, Fenton reaction etc.
Hypochlorous acid (HOCl)	Action of MPO during phagocytosis.
Peroxyl radicals ($ROO^{\bullet}$)	Lipid peroxidation
Nitric oxide (NO)	From vascular endothelial cells catalysed by nitric oxide synthase.
Nitrogen dioxide (NO_2)	Combination of NO with O_2 at body temperature
Peroxynitrite ($ONOO^-$)	From vascular endothelial cells

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).

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

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

Disease/Disorder	Free radical related event
Cardiovascular diseases	Oxidative modification of LDL after initial damage to vascular endothelium by other means.
Cancer	DNA damage
Adult respiratory distress syndrome (ARDS)	Damage to lungs
Radiation damage	Damage to proteins, lipids and DNA.
Brain & spinal cord injuries	Tissue damage after the injury
Keshan disease	Mediated by oxidative stress
Cataract	Oxidation of lens proteins
Ageing	Damage to proteins, lipids, DNA and tissues.

1.3.3.2. Lipid peroxidation: Oxidation of lipids have significant role in deterioration of oils and fatty foods. Lipid peroxidation is an important patho-physiological 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

$$LH + OH^{\bullet} \longrightarrow L^{\bullet} + H_2O$$

Propagation

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + LH \longrightarrow LOOH + L^{\bullet}$$

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 extra cellular space. Hydrogen peroxide is toxic to micro organisms 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; where as 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 vasodialation and hence the blood pressure. It is also associated with 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 (eg; growth factors for neurons) and the negative signals (eg; 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

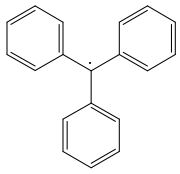
AOs are classified based on several criteria such as their origin, solubility in lipid or water; physical and chemical characteristics etc. Based on their origin, AOs can be classified into synthetic and natural AOs.

1.3.4.1a. Synthetic AOs.

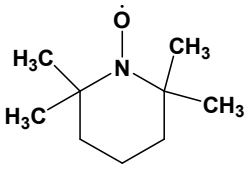
In general synthetic AOs are compounds with phenolic structures of various degrees of alkyl substitution. Synthetic AOs currently permitted for

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.

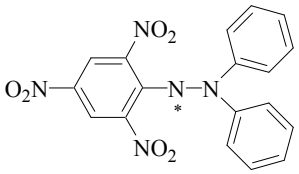
Figure 1.6 Structures of some stable free radicals and synthetic AO compounds



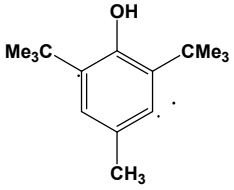
Triphenyl radical



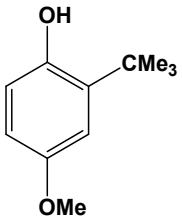
TEMPO



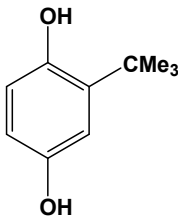
DPPH radical



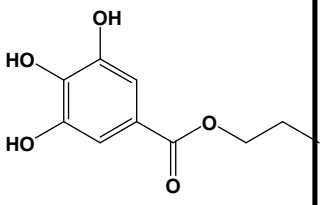
BHT



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 $O_2^{\bullet -}$ to O_2 and to the less toxic H_2O_2.

$$2\ O_2^{\bullet -} + 2H + \xrightarrow{SOD} O_2 + H_2\ O_2$$

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 $O_2^{\bullet -}$ 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 H_2O_2 to H_2O 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.

$$LOOH + 2GSH \xrightarrow{GPx} LOH + GSSG + H_2O$$

GPx is also involved in the removal of H_2O_2

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

Thus GPx competes with catalase for H_2O_2 and is the major source of protection against low levels of oxidative stress.

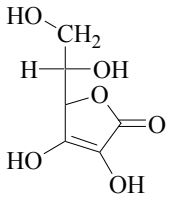
(ii) Non-enzymatic AOs:

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 α- tocopherol from α- 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 $AScH^{-}$, and only very small proportion as $AScH_2$ (0.05%) and ASc^{2-} (0.004%). $AScH^{-}$ 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.

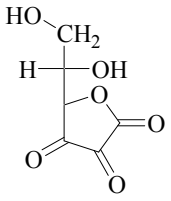
AA is known to protect membrane against oxidation. Recent in-vivo and ex-vivo studies revealed that AA in plasma increases resistance to lipid-peroxidation in a dose dependent manner, even in the presence of redox active iron or copper and H_2O_2 (35). AA also prevents endothelial dysfunction in chronic 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



L-ascorbic acid



Dehydro ascorbic 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

$$\alpha\text{–tocopherol} + \text{lipid-}O_2 \rightarrow \alpha\text{-tocopherol}^{\cdot} + \text{lipid–}O_2H$$

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 $O^{\cdot}$ 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)

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.

$$^1O_2 + \beta\text{-carotene} \longrightarrow {}^3O_2 + \beta\text{-carotene}$$

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 ($GS^\bullet$) is significantly longer than many other radicals generated during the stress.

$$GSH + R^\bullet \longrightarrow GS^\bullet + RH$$

$$GS^\bullet + GS^\bullet \longrightarrow GSSG$$

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 sulfahydryls 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).

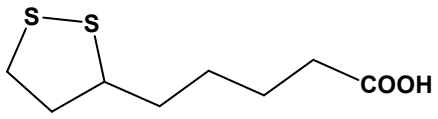
Taurine and hypotaurine: Taurine and its precursor hypotaurine are β amino acids derived from cysteine metabolism (45). Hypotaurine has shown to have the capacity to scavenge hydroxyl radical and to inhibit lipid peroxidation. Taurine supplementation has been shown to decrease lipid peroxidation and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

Figure 1.8 Structures of taurine and hypotaurine

Taurine **Hypotaurine**

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 dihydrolipoamiae 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).

Figure 1.9. Lipoic acid



1.3.5. AO related medicinal properties of phytochemicals

The AO nature of phytochemicals is largely responsible for their anticancer, cardioprotective, immunomodulatorty, 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:

$$ROO\bullet + ArOH \rightarrow ROOH + ArO\bullet$$

$$HO\bullet + ArOH \rightarrow HOH + ArO\bullet$$

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).

$$ROO\bullet + ArO\bullet \rightarrow ROOArO$$

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), Uttaranchal, Sikkim and Arunachal Pradesh. SB cultivation in India is mainly concentrated in the cold deserts of Trans-Himalyas (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*. r*hamnoides.* 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-quadruple 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/100g 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 hydoxycinnamic 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. rhamnoindes 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).

Tsydendambaev 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

lowest 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^{o} 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 approxiamtely 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 hectors 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 sun light 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 cosmaceutical 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 cosmaceutical 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

Chapter 2

MATERIALS AND METHODS

I have not failed. I've just found 10,000 ways that won't work.

.....Edison

2.1. Sea Buckthorn Berries

Berries of three SB species from major SB growing areas in the Trans-Himalayan region of India were used in this study. Fresh berries of *H. rhamnoides var, turkestanica* were collected from the Indus valley of Ladakh, Spiti, and Lahaul regions. *H. tibetana* and *H. salicifolia* berries were collected from Spiti and Lahaul regions, respectively. Berries were collected in triplicate from three different locations (3 × 3) of the same geographic area and labeled as Lahaul 1, Lahaul 2, and Lahaul 3. Berries were collected during June to November of 2003 at the stage of commercial maturity, as judged by juiciness and appearance. Berries were collected and identified with the help of experts from the Field Research Laboratory (DRDO, India), Leh, India and Himachal Pradesh Agricultural University, Kullu, India. Berries were stored in polyethylene bags and ferried to the NIIST Trivandrum by air under cold conditions and stored at −20°C until analysis.

Berries were separated into berry pulp, seed coat and kernel. Seeds were separated from the berries just before analysis. Frozen berries were taken from the freezer (−20°C), thawed at 25°C for 1 h, and crushed gently so that the seeds remained intact and the seeds were then separated manually from the pulp. Seeds were coarse powdered and seed coat was separated from the kernel by winnowing.

2.2. Chemicals and Reagents.

Biochemical standards such as β-carotene, fatty acids, β-sitosterol, stigmasterol, campesterol, quercetin, kaempherol, ascorbic acid, malic acid, citric acid, gallic acid, protocatechuic acid, parahydroxybenzoic acid, vanillic acid, salicylic acid, cinnamic acid, *p*-coumaric acid, ferulic acid, caffeic acid, XO and allopurinol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tocopherols and tocotrienols were purchased from Calbiochem (Merck Ltd., Mumbai, India). Folin-Ciocalteau's (F-C) reagent, and HPLC

grade hexane, isopropyl alcohol, methanol, water, acetic acid and formic acid were obtained from Merck Ltd (Mumbai, India). Other chemicals and reagents were of analytical grade. Isorhamnetin and quinic acid used were isolated from SB berries and characterized by spectroscopic studies.

2.3. Proximate Composition of Berries.

AOAC methods were used for the determination of moisture (No. 930.15), protein (No. 988.01) and ash content (No. 942.02) (201). Moisture was determined by oven dry method. Total fat and sugar were respectively estimated by soxhlet and anthrone method. Analysis was carried out in triplicate and mean value and standard deviation were determined**.**

Total protein: Most of the nitrogen in the plants occurs as proteins and amino acids. Total amount of nitrogen in plant material is therefore indicating the amount of total proteins and amino acids. Nitrogen content was estimated by the Kjeldahl method. About 1g sample and 0.5g of digestion mixture (2.5g SeO_2 + 100g K_2SO_4 + 20g $CuSO_4$) were weighed into a Kjeldahl's flask. 10 mL conc. H_2S0_4 was added and heated until color of the solution was changed to clear blue green. This clear solution was made up to 100 mL under cold conditions. The Kjeldahl apparatus was set up for protein estimation with 20 ml boric acid and 1 mL mixed indicator (bromocresol green + methyl red). 5 mL of digested sample with 20 ml 40% NaOH and 10 ml water were taken in the apparatus connected to the steam line. Steam was bubbled through the mixture and the NH_3 evolved was stripped with steam and trapped in boric acid. Upon ammonia evolution, color of boric acid changes to blue and the steam bubbling is continued for 20 minutes. The resulted boric acid was titrated with 0.1N HCl till blue color of the solution disappears. Amount of nitrogen in the samples was calculated by the following relation

$$\% \text{ of nitrogen} = \frac{14 \times 100 \times N_{Hcl} \times 100}{w \times 5 \times 1000}$$

Where, 'N_{Hcl}' and 'w' represents the normality of HCl used and weight of the sample respectively. The amount of protein was expressed in terms of glycine.

Total sugars – Anthrone method: In this method the total carbohydrate was estimated by the dehydration of carbohydrate to furfural and its subsequent condensation reaction with anthrone (9,10–dihydro–9–oxoanthracene) to yield a blue green colored product. 1 g of substance was dried in air oven, defatted with hexane and refluxed with 80% ethanol for 6 hours. Filtered and made up to 25 mL. 0.2 to 1 mL of glucose solution (0.1 mg/mL) and 0.2 to 0.8 mL of sample solution were pipetted in test tubes and made up to 1ml with distilled water. 4 mL of anthrone reagent (0.5 g anthrone and 10 g thiourea in 1L of 66% v/v of H_2SO_4 and stored at 4 ^oC) was added to the test tubes and warmed to 80 to 90 ^oC for 15'. Cooled under tap water and absorbance was measured at 620 nm in uv–visible spectrophotometer (UV – 2001, Shimadzu, Japan). The amount of carbohydrates in the sample was determined by plotting the calibration curve using authentic standard of glucose.

2.4. Extraction of Lipids-Christies Method:

1 g of berries was homogenized in 10 mL methanol for 2 min in a blender, then 20 mL chloroform was added and homogenization continued for 5 min more. The mixture was centrifuged at 1500 × *g* and filtered. Solid residue was resuspended in chloroform methanol (2:1 vol/vol, 30 mL) for 5 min. The mixture was centrifuged and filtered, and the residue was washed with a chloroform/methanol mixture (1:1, v/v, 30 mL). The filtrates and washings were combined, and one-fourth of the total volume 0.88% aqueous potassium chloride solution was added, shaken well, and allowed to settle. The lower layer was separated off and washed with methanol/water (1:1 v/v). The purified lipid layer was filtered and dried over anhydrous Na_2SO_4, the

solvent was removed in a rotary film evaporator, and the amount of lipids was noted. Lipids were stored in chloroform at −20°C for further analysis (171).

2.5. GC Profiling of Fatty Acids

Fatty acid methyl esters (FAME) of berry lipids were prepared according to the IUPAC method (202). FAME were analyzed using a Hewlett-Packard 5890 series II model gas chromatograph (Avondale, PA) equipped with a flame ionization detector. The column used was an HP-FFA (cross-linked FFAP: 30 m × 0.5 mm × 1 μm; Hewlett-Packard, Avondale, PA). Injector and detector port temperatures were 250 and 300°C, respectively. The column temperature was maintained at 100°C for 1 min and then increased to 180°C at 5°C/min and maintained at that temperature for 15 min. Nitrogen was used as the carrier gas at 20 mL/min. FAME components in the samples were determined by comparing the retention time (T_R) with that of standard FAME run under the same conditions. FA composition was expressed as weight percentage of the total FA.

2.6. Total Carotenoids

Aliquots of lipids in hexane (0.1g/mL) was added to 0.5 mL of 5 g/L NaCl, vortexed for 30 s and centrifuged at 1500 × *g* for 10 min (Cooling Centrifuge C-30, REMI, India). The supernatants were diluted with hexane and the absorbance at 460 nm was measured. The amount of carotenoids was calculated by plotting a calibration curve with authentic standard of *β*-carotene (1–10 μg/mL) and expressed in terms of *β*-carotene (158). The mean ± standard deviation of three replications was recorded.

2.7. HPLC Profiling of Tocopherols/Tocotrienols

Lipids were dissolved in hexane (1 mg/mL) and filtered through 0.45 μM PTFE membrane. A Shimadzu HPLC binary system (LC- 10A) with LC-10AD pumps, a Rheodyne injector fitted with a 20 μL sample loop, a SPD-

10A UV-vis detector, and a CR 7Ae data processor for data acquisition, analysis, and display was used for the analysis. A Phenomenex NH_2 column (5 µm, 4.6 X 250 mm; Luna) was used as a stationary phase with a mobile phase of *n*-hexane and isopropanol (96:4 v/v) at a flow rate of 1 mL/min. The UV detector was set at 297 nm. The HPLC conditions were standardized using individual standards and their various mixtures (203). The mean ± standard deviation of three replications was calculated.

2.8. HPLC Profiling of Sterols

Sterols in the lipid samples were hydrolyzed and analyzed as their aglycones. Approximately 1 g of sample was refluxed with 6 mL methanol and 1 mL 50% KOH (wt/v) for 3 h in a water bath. The unsaponifiable matter was extracted with petroleum ether and desolventised in a thin film evaporator under reduced pressure below 50 ^{0}C. The residue was redissolved in chloroform, made up to a known volume with methanol and analyzed in the same HPLC binary system used for tocopherol analysis, using a reverse phase C-18 Zorabax column (4.6 × 250 mm) (Rockland Technologies, Newport, USA) with methanol/water (96.5:3.5 v/v) at 1.5 mL/min as mobile phase. The UV detector was set at 206 nm and a calibration curve was plotted with authentic sterol standards (1–5 µg/mL) (204). The mean ± standard deviation of three replications was calculated.

2.9 HPLC Quantification of Vitamin C

1 g of sample was homogenized repeatedly with 0.01 mol/L metaphosphoric acid (6 X 20 mL) and centrifuged at 2200 × *g* for 5 min (Cooling Centrifuge C-30, REMI). The supernatant was filtered through a 0.45 µm filter and analyzed immediately. Juice samples were also treated with metaphosphoric acid, centrifuged and filtered. A 20 µL sample was injected into a Shimadzu LC-8A HPLC equipped with a Phenomenex C-18 ODS-2 column (5 µm, 250 mm × 4.60 mm; Luna) and a PDA detector (SPD-M 10A

VP) set at 246 nm, and 3.7 mmol/L phosphate buffer (pH 4) at a flow rate of 1mL/min was used as mobile phase. Authentic standards of ascorbic acid (0.5–5 μg/mL) were used for optimizing the HPLC conditions. Results are reported as the mean ± standard deviation of three independently extracted samples (205).

2.10. Organic Acid Analysis

2.10.1 Isolation of quinic acid: 100g freeze dried and defatted berry pulp was extracted with water (3 X 250 mL) and dried under reduced pressure below 65^{o} C. 10 g of this water extract was subjected to silica column chromatography (silica 230-300 mesh) with a gradient mixture of ethyl acetate and methanol. White crystalline powder obtained from polar fractions was identified as quinic acid by spectral techniques.

Quinic acid:

Colourless needles (methanol) with melting point 162-163°C;

UV λ_{max}: 217 nm;

^{1}H NMR (300 MHz, d_4-MeOH): 1.81 (m), 1.99 (m), 3.93 (m), 3.41 (dd, J = 3, 9 Hz), 4.06 (q, J = 3, 6 Hz), 1.95 (m), 2.05 (m);

^{13}C NMR :(75 MHz, d_4-MeOH) 177.6 (C-7), 75.5 (C-1), 74.7 (C-3), 69.8 (C-4), 66.2 (C-5), 36.6 (C-6).

2.10.2. HPLC profiling of organic acids: 1 g of sample was homogenized repeatedly with HPLC-grade water (6 X 25 mL), clarified by centrifugation at 2000 × *g* for 15 min and filtered through a 0.45 μm filter. The juice was further diluted with water, centrifuged and filtered. HPLC analysis was carried out isocratically using phosphoric acid solution at pH 2.4 as mobile phase at a flow rate of 0.7mL/ min. A Shimadzu LC-8A HPLC equipped with a Phenomenex C-18 ODS-2 column (5 μm, 250 × 4.6mm; Luna)

thermostatted at 25^0 C (CTO-10 AC VP Column Oven), a 20 µL loop and a PDA detector (SPD-M 10A VP) was used for the analysis. Authentic standards of quinic acid (1–10 µg/mL), malic acid (1–10 µg/mL), citric acid (1–10 µg/mL), maleic acid (0.5–10µg/mL) and fumaric acid (0.5–10µg/mL) were used to plot the calibration curve. Peaks in the samples were identified by comparing their T_R and uv spectra with those of reference compounds and were quantified at 215 nm. The mean ± standard deviation of three replications was determined.

2.11. Total Phenolics

1g of the sample was extracted (5 X 20 mL) with 70% methanol, filtered, desolventised and made up to 25 mL with methanol. Juice was clarified by centrifugation and supernatant was used for the analysis. Aliquots of the extracts (0.5 mL) were oxidized with 0.5 mL of F-C reagent and the reaction was neutralized with the addition of 1 mL 20% Sodium carbonate (Na_2CO_3). The mixture was incubated at room temperature for 90 min. and the absorbance of the resulting solution (blue color) was measured spectrophotometrically at 760 nm. Calibration plot was obtained with gallic acid and total phenolic content was expressed as milligram gallic acid equivalents (GAE) per gram of extract/sample (206).

2.12. Flavonoids

2.12.1. Extraction of flavonoids: 10 g of the parts of berries was homogenized with ethanol (6 x 50 mL) in presence of 250 mg of ascorbic acid under nitrogen atmosphere at room temperature. The juice was clarified by centrifugation at 1500 × *g* and the supernatant was used for analysis.

2.12.2. Total flavonoids: The amount of total flavonols was estimated by the aluminum chloride method suggested by Chang et al **(207)**. Quercetin, which

is having a moderate absorbance, was used as standard. 10 mg of quercetin was dissolved in 80% ethanol and then diluted to 25, 50 and 100 µg/mL. The diluted standard solutions (0.5 mL) were separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Similarly, 0.5 mL of extracts were reacted with aluminum chloride for determination of flavonoid content as described above

2.12.3. Isolation of isorhamnetin. Freeze dried seeds (500 g) were powdered and defatted with n-hexane (1L) and homogenized with methanol (4 X 1 L) at room temperature. The extracts were desolventised under reduced pressure at ≤ 50 ^{o}C, to methanol extract (140 g). 100g extract was refluxed with methanol (500 mL), deionised water (200 mL) and HCl (80 mL) for 2.25 h at 85 ^{o}C. Methanol was removed and 500 mL water was added. Then the mixture was neutralized with $NaHCO_3$ and extracted with ethyl acetate (6 X 100 mL) and the solvent was dried under reduced pressure at ≤ 50^{o} C, to give aglycone mixture (10 g). Ethyl acetate extract (5 g) was subjected to liquid chromatography on silica gel (230-400 mesh) eluting with gradient mixtures of n-hexane, ethyl acetate and methanol. Fractions (50 ml) were collected and pooled according to their similarity on TLC to give four fractions (A1-A4). Further repeated purifications of sub fractions A2 on liquid chromatography afforded 20 mg compound pure flavonoid. This was subjected to spectral analysis and identified as isorhamnetin.

Isorhamnetin: yellow crystals; m.p. 205-206° C;
EI-MS 316 (M^+, 82.5), 301 (M^+-15, 6.6), 208 (0.42), 108 (10.8), 123 (17.5), 85 (10), 151 (13.7), 57 (33.3), 40 (100);

UV λ_{max} (MeOH): 254, 368; (+NaOMe) 268, 326, 414; (+$AlCl_3$) 262, 425; (+$AlCl_3$/HCl) 260, 423; (+NaOAc) 273, 322, 387; (+NaOAc/H_3BO_3) 256, 363, 428 nm;

^{1}H NMR (300 MHz, d_5-py): 8.31 (1H, d, *J* = 1.7Hz, H-2'), 8.21 (1H, dd, *J* = 1.9, 8.4 Hz, H-6'), 7.37 (1H, d, *J* = 8.5 Hz, H-5'), 6.87 (1H,d, *J*=1.8 Hz, H-8), 6.76 (1H,d, *J* = 1.8 Hz, H-6), 3.84 (3H, s, OCH_3).

Isorhamnetin was recrystalised from ethyl acetate-methanol mixture and used as reference for the optimization of HPLC analytical conditions.

2.12.4. HPLC analysis of flavonoids: Flavonoids in the samples were extracted with cold ethanol. The juice was clarified by centrifugation at 1500 × *g* and the supernatant was used for analysis. The berry extract and juice were hydrolysed to obtain corresponding aglycones following the method described by Stricher (208). About 20 mL of the juice or 1g of extracts was refluxed with methanol (25 mL), deionised water (10 mL) and HCl (4 mL) for 2.25 h at 85^0 C. The solution was filtered, concentrated under reduced pressure, made up to a known volume with methanol and stored at 4^0 C until analysed. This homogenate was subsequently filtered through a 0.45 μm (PTFE) filter and analysed in a Shimadzu LC-8A HPLC (Shimadzu Corporation, Kyoto, Japan) fitted with a Rheodyne 7725 injector with a 20 μL sample loop and a reverse phase C-18 ODS-2 column (Phenomenex, Torrance, CA, USA) (5 μm, 250 mm × 4.6 mm; Luna) using methanol and 0.5% phosphoric acid (50:50 v/v) as mobile phase (1.5 mL/min).

2.12.5. HPLC-DAD-MS/MS analysis of flavonoid glycosides: HPLC-DAD-MS/MS analytical conditions for the flavonoid glycoside profiling were optimized. MS/MS conditions were optimized using rutin. Quantification of the flavonoid glycosides were performed in DAD using the calibration plot drawn with quercetin, kaempherol and isorhamnetin.

Instrument : Agilent 1200 HPLC with 6310 ion trap

Column: C-18, ODS-2, (5µ, 250X4.6 mm, Phenomenex, Luna)

Mobile Phase: Gradient of water, methanol and 10% acetic acid

Flow rate 0.8 mL/min

Time (min.)	**A (%,v/v) (Water)**	**B (%,v/v) (Methanol)**	**C (%,v/v) (10% Acetic acid)**
0	98	0	2
8	98	0	2
15	83	15	2
60	63	35	2
70	48	50	2
80	0	98	2
90	0	98	2

MS/MS Conditions

Capillary voltage	2500 V
Corona current	1000 nA
Nebuliser pressure	50 psi
Dry gas	5L/min
Drying temperature	350 ^{o}C
Vaporization temperature	250 ^{o}C
Ionization mode	APCI+ESI
Polarity	-ve

2.13. Standardization of HPLC-DAD Analytical Method for Phenolic Acids

2.13.1 Preparation of extracts: Five grams of the powdered berry parts were homogenized at 200 rpm with methanol-water (60% for SB pulp and seed coat, and 70% for kernel) mixtures (6 X 25 mL) at room temperature for 30 min. in nitrogen atmosphere. The mixture was centrifuged at 2000 X g for 15 min and supernatants were collected, combined and dried under vacuum at ≤50 ◦C and kept at −20 ◦C until analyzed.

2.13.2. Fractionation of phenolic acids: Free and bound phenolic acids in the extracts were fractionated according to the procedure described by Kozłowska et al and Zadernowski et al (209, 170). Crude phenolic extracts were dissolved in 25 mL of water, acidified with 6N HCl to pH 2 and filtered to remove the precipitated phospholipids. It was then extracted with diethyl ether (5 X 25 mL) at room temperature. The diethyl ether extracts were combined and evaporated under vacuum at ≤40^{o} C and referred as free phenolic acid fraction. The water phase was neutralized with 2M NaOH and evaporated under vacuum at ≤50 ^{o}C almost to dryness. The residue was treated with 20 mL 4N NaOH in nitrogen atmosphere for 4 h at room temperature. The reaction mixture was then acidified to pH 2 with 6N HCl and extracted with diethyl ether as before and analyzed as phenolic acids liberated from esters. The water phase was then neutralized and dried as before. The residue was hydrolyzed with 50 mL 2MHCl for 30 min at 95 ^{o}C. The mixture was cooled, adjusted to pH 2 and extracted with diethyl ether as before. This extract was referred to as the phenolic acids liberated from their glycosides. The residual fatty material present in phenolic acid fractions thus obtained were removed by dissolving in 20 mL 5% $NaHCO_3$ solution and extracting with diethyl ether (5 X 20 mL). Then the aqueous phase was acidified with 6N HCl to pH 2 and extracted with diethyl ether as before. The

extracts were dried and redissolved in 5mL methanol and kept at −20 ^{o}C till analyzed.

2.13.3. Preparation of standard solutions: Standard solutions of nine phenolic acids at a concentration of 0.5 mg/mL in methanol were prepared and several dilutions in mobile phase were made. Solutions were filtered through 0.45 μm PTFE filters, and injected directly.

2.13.4. Chromatographic conditions : A Shimadzu LC-8A HPLC system (Shimadzu, Japan) with a binary solvent delivery system (LC-8A), a column temperature controller (CTO-10AV), a Rheodyne injector with 20 μL sample loop and a DAD detector (SPD-M 10 A) coupled with Class VP analytical software were used for analysis. A reverse phase (RP) column (Phenomenex C-18, ODS-2, 5μm, 250 X 4.6 mm) with an extended guard column was used as stationary phase and column temperature was maintained at 35 ^{o}C. The mobile phase was a gradient elution of water containing 2% acetic acid (solvent A) and methanol (solvent B) at a flow rate of 1 mL/min.

The gradient program of mobile phase was as follows.

Time (min.)	**A (%, v/v) (2% acetic acid)**	**B (%, v/v) (Methanol)**
0	100	0
5	100	0
10	90	10
15	90	10
20	80	20
25	80	20
25	40	60
30	0	100

2.14 Analysis of PAs

2.14.1. Total PAs - Butanol–HCl Assay: 0.25mL of each extract (1 mg/mL) was added to 3 mL of a 95% solution of *n*-butanol/HCl (95:5 v/v) in a stoppered test tube, followed by 0.1mL of a solution of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 2M HCl. The tubes were incubated for 40 min at 95 ^oC. The absorbance of the red coloration was read at 550 nm. Results were expressed in terms of cyanidin chloride equivalents, as percentage of PAs on dry wt of the extract (210).

2.14.2. Total PAs-Vanillin –HCl Assay: The content of PAs in extracts and fractions was determined using modified vanillin assay and the results were expressed as catechin equivalents (211). To 1 mL of catechin solution (0-300 µg/mL) or sample solution (200-300 µg/mL). 2.5 mL methanol (control) or 1% vanillin in methanol and 2.5 mL 9 mol/L HCl in methanol were added. The reaction mixture was incubated for 20' at room temperature and absorbance at 500 nm was measured. The amount of PA in samples was estimated from the calibration curve plotted with reference catechin (211).

2.14.3. Extraction of PA. Pulp, kernel, and seed coat were finally powdered to an average particle size of 0.5 mm. Powdered samples (2 g) were extracted with 20 mL methanol in water (100, 90, 80, 70, 60, 50, 25 & 0%) and acetone in water (100, 90, 80.70, 60, 50, 25, & 0%). Similar methanol- water and acetone -water mixtures containing 1% acetic acid were also used as extraction solvents. For the extraction, the samples were homogenized at 200 rpm with solvent mixtures for 10' and subsequently centrifuged at 2000 X g (C-30, Remi, Mumbai India) and supernatants were collected. The residues were resuspended in their respective solvent mixtures and the procedure was repeated 2 more times. The supernatants were combined and dried under reduced pressure at 40^o C and redissolved in 25 mL methanol. The amounts of

PA in these extracts were estimated by vanillin-HCl assay (211) and expressed as catechin equivalents.

The solvent mixtures, as optimized in the previous step were used as the extraction media for the preparation of crude phenolic extracts of SB berries and leaves for further studies. 20 g of the powdered samples of SB pulp, seed coat and kernel were extracted (5 X 100 mL), desolventised, dissolved in methanol and stored at -20^{o} C. Total phenolics and PA in these extracts were estimated using F-C reagent (203) and vanillin-HCl assay (207) respectively.

2.14.4. Purification of PA extracts. The crude phenolic extracts of SB berries were purified with sephadex LH-20 column chromatography. Samples (500 mg) of crude extracts were suspended in 95% (v/v) ethanol and applied on to chromatographic column packed with sephadex LH-20 (2.3 X 40 cm) and equilibrated with 95 % ethanol (v/v). The column was exhaustively washed with 95% ethanol (v/v) and eluted with 25% acetone. Acetone elutes were combined and dried under vacuum at 40 ^{o}C. The PA fractions thus obtained were lyophilized and kept under refrigeration.

2.14.5. Average degree of polymerization (ADP). The ADP was estimated using the purified PA fractions as the ratio of absorbance at 510 nm obtained from butanol/ HCl reaction to the absorbance at 500 nm obtained from the reaction with vanillin in acetic acid (212). Butanol/HCl assay was performed as discussed in section 2.14.1. For vanillin assay vanillin reagent was prepared by dissolving 0.5% vanillin in glacial acetic acid containing 4% HCl. Extracts were dissolved in minimum quantity of methanol and diluted with acetic acid. Incubated for 5' and absorbance at 500 nm was measured. ADP was calculated as the ratio of absorbance of butanol/HCl and vanillin assay mixtures for equal quantities of samples.

2.14.6. Depolymerisation of PAs in presence of phloroglucinol: A solution of 0.1N HCl in MeOH, containing 50g/L phloroglucinol and 10g/L ascorbic acid was prepared. 1 mL of purified PA extract in methanol (5g/L) was treated with 10 mL of this solution for 20 min. at 50 ^{o}C and then combined with 5 volumes of 40 mM aqueous sodium acetate to stop the reaction.

2.14.7. HPLC-DAD-MS/MS analysis of depolymerisation reaction products.

Instrument : Agilent 1200 HPLC with 6310 ion trap

HPLC conditions:
Column: C-18, ODS-2, (5µ, 250X4.6 mm, Phenomenex, Luna)
Mobile Phase-Gradient of 1% acetic acid in water
Flow rate : 1 mL/min

Time (min.)	**A (%,v/v) (1% Acetic acid)**	**B (%,v/v) (Methanol)**
0	95	5
10	95	5
20	80	20
25	60	40
35	10	90

Calibration curves were plotted with catechin and epicatechin and the reaction products were quantified in terms of catechin and epicatechin.

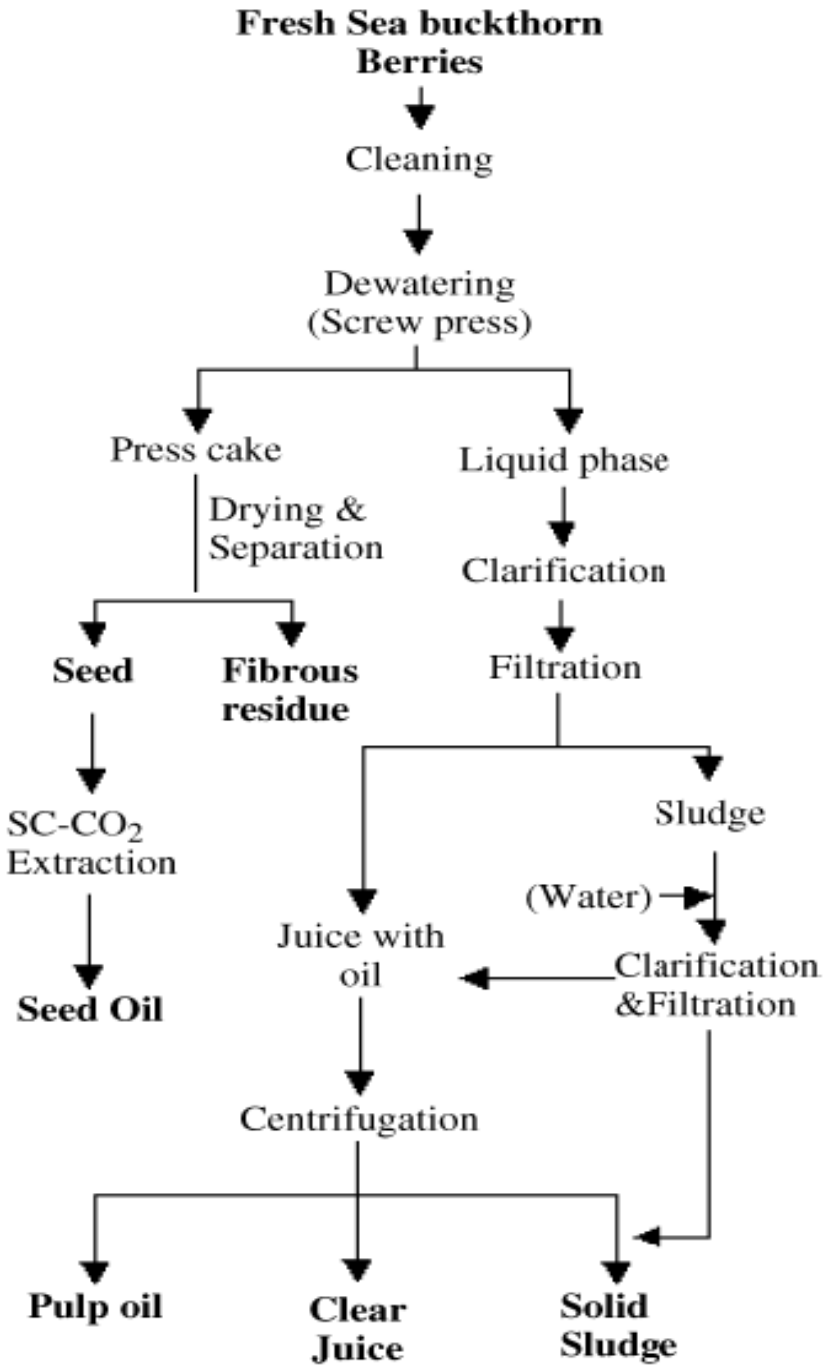
MS/MS Conditions
MS ionization conditions were optimized by using catechin.

Capillary voltage	2500 V
Corona current	1000 nA
Nebuliser pressure	50 psi
Dry gas	5L/min
Drying temperature	350 ^{o}C
Vaporization temperature	250 ^{o}C
Ionization mode	ESI
Polarity	-ve

2.15. Processing of Fresh SB berries.

A schematic diagram of the patented method for processing fresh SB berries is shown in Fig. 2.1. Batches of 90, 35 and 47 kg of fresh berries with a moisture content of 70–75% were processed. Fresh SB berries taken from cold storage were first cleaned by spraying with water to remove dirt and other impurities and then blanched at 80^{o} C to inactivate enzymes and to facilitate the extraction of juice and oil in subsequent steps. The cleaned and blanched berries were subsequently fed into a mechanical screw press of 100 kg/h capacity for dewatering. The compression ratio of the press was about 10 and the pressure was adjusted using the back-pressure control to separate about 80% of the total water present in the fresh berries along with oil and suspended solids. Fibrous press cake with unbroken seeds was obtained as a residue. To achieve higher yields, three stages of extraction were performed, the first with fresh berries and the second and third with the addition of 25% by weight of warm water (60^{o} C) to the residue. The juice obtained from all three pressings was pooled and subjected to clarification. The pooled juice was heated to 80^{o} C with stirring in a stainless steel steam-jacketed kettle for 60 min. During clarification the pulp oil of orange/red colour was found to float on the surface, while the suspended solids settled at the bottom. The clarified juice was filtered while hot by passing it through a muslin cloth to separate the debris and solids as sludge. The sludge was again mixed with hot water (1:2) and the liquid phase containing residual oil was added to the previous juice. Thus the liquid fraction was separated into juice with pulp oil and solid sludge. The juice with pulp oil was then centrifuged (5000 × *g*, 60^{o} C) using a continuous centrifuge (RTA 1-01-025,GEA Westfalia Separator, Oelde, Germany) to separate the fractions (clear juice, orange/red pulp oil and residual solids as sludge). The fibrous cake with seed was dried in a crossflow drier and separated into fibrous residue and seeds manually.

Figure 2.1: Processing of fresh sea buckthorn berries.



2.16. SC-CO_2 extraction of seed oil

SB seeds separated from the dried residue were ground in a laboratory grinder (LB-105S, REMI, Mumbai, India) to an average particle size of 0.3 mm. The ground seeds were extracted using a pilot-model supercritical fluid extraction unit (SFE-2L, Thar Designs, Inc., Pittsburgh, PA, USA) at a temperature of 60^{o} C, a pressure of 4.5 × 107 Pa and a gas flow rate of 60 g/min for 3 h. The seed oil was collected in the cyclone separator of the extraction unit.

2.17. Activity Guided Fractionation of SB berries.

Phase I: Freeze dried berries were thawed at room temperature for 20 min. and manually separated into seeds and pulp. Seeds were coarse powdered and seed coat was separated from the seed kernel by winnowing. Pulp, seed kernel and seed coat were finally powdered to an average particle size of 0.5 mm. Powdered samples (100 g) were independently extracted with 1L (5 X 200 mL) hexane, ethyl acetate, methanol and water. For extraction, the samples were homogenized at 200 rpm with solvents for 30 min in nitrogen atmosphere and subsequently centrifuged at 2000 X *g* and supernatants were collected. The residues were resuspended in their respective solvents and the procedure was repeated 5 more times. The supernatants were combined, dried under reduced pressure at ≤ 50 ^{o}C, and redissolved in 50 mL absolute methanol. Extraction scheme is given in Fig-2.2.

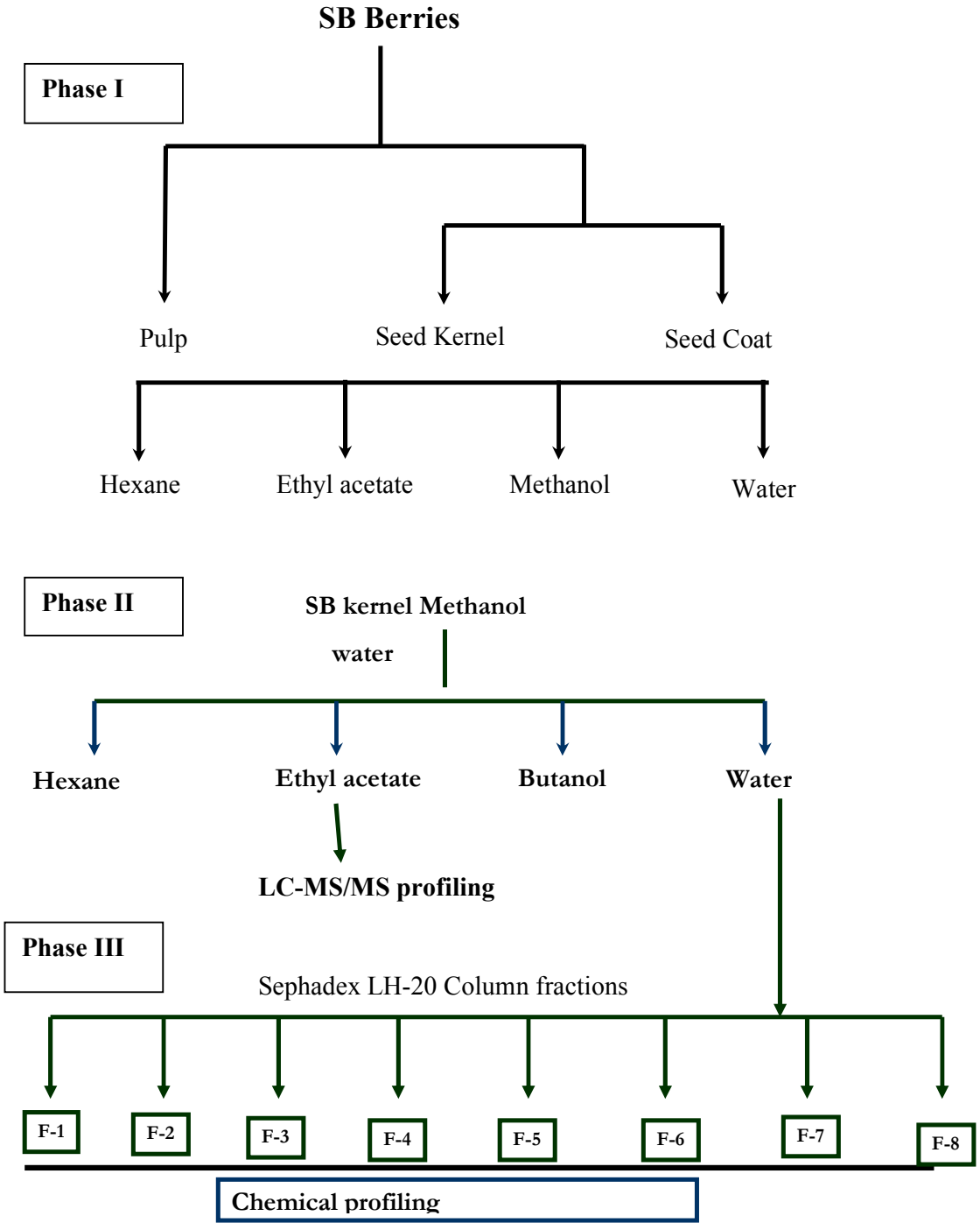
Phase II. Kernel methanol extract with highest in vitro AO activity was further fractionated between hexane, ethyl acetate, butanol and water according to the scheme given in Fig -2.2.

5 g kernel methanol extract was made free from methanol, and resuspended in 100 mL water. Then it was partitioned between the solvents (5 X 100 mL) in their increasing polarity order hexane, ethyl acetate, butanol

and water. Fractions thus obtained were desolventised under reduced pressure and dissolved in 25 mL methanol. Yield and proximate composition of fractions were noted and each fraction was evaluated for their in-vitro AO activities. Ethyl acetate and water fractions of kernel methanol extract were found to have high activity.

Phase III. Water fraction was further fractionated using sephadex gel LH-20 column chromatography. A dual pump flash chromatographic system (BUCHI C-615.) equipped with a chromatographic column packed with sephadex LH-20 (2.3 X 40 cm) at a flow rate of 10 mL/min was used for the fractionation. 500 mg of water fraction was suspended in 95% (v/v) ethanol and applied to the column and equilibrated with 95 % ethanol (v/v). The column was washed with 95% ethanol (v/v) for 30 min and eluent was collected as fraction-1. Then a gradient of acetone (Pump A) and water (Pump B) was used for elution (Table 2.1). 100, 90, 80, 70, 60, 50 and 25% of acetone were used for the elution, each solvent mixture was run for 30 min and fractions 2 to 8 were collected. Fractions were dried under vacuum at 40^{o} - C, lyophilized and kept under refrigeration. Schematic presentation of the fractionation is given in Fig-2.2

Figure 2.2: Activity guided extraction scheme of SB berries



2.18. In vitro AO Capacity Assays.

2.18.1. DPPH radical scavenging capacity: DPPH radical scavenging activity of SB samples and reference compounds were measured according to the method reported by Singh, et al; with slight modifications (213). Assay was performed in 3mL reaction mixture containing 2.9 mL of 0.1 mM DPPH solution in methanol and 0.1 mL of methanol (as control) or sample in methanol (as test). The mixture was shaken vigorously and left to stand for 20 minutes at room temperature in the dark. The optical density (OD) of the resulting solution was measured spectrophotometrically at 517 nm. The capability to scavenge the DPPH radical was calculated using the following formula:

$$\text{Scavenging Activity (\%)} = [1- (\text{OD of sample} / \text{OD of control})] \times 100$$

2.18.2. ABTS radical scavenging capacity: ABTS radical cation scavenging assay was carried out by the method of Re et al (214). ABTS radical cation was generated by adding 7 mM ABTS to 2.4 mM potassium persulphate and the mixture was allowed to stand for overnight in the dark at room temperature. This solution was diluted to obtain an absorbance of 0.7 ± 0.05 with ethanol at 734 nm (Schimadzu UV-Vis spectrophotometer model 2450) and was added to various concentrations of samples and reference compounds (2-100 μg). The solution was shaken thoroughly and the absorbance was measured after 7 min. The capacity to scavenge the ABTS radical cation was calculated using the formula,

$$\text{ABTS radical cation scavenging capacity (\%)} = [(A_1-A_2)/ A_1] \text{ X100}$$

where A_1 is the absorbance of ABTS solution without test sample and A_2 is the absorbance of ABTS solution with test sample. Trolox was used as

reference compound and the ABTS radical scavenging activity was expressed as trolox equivalent antioxidant capacity (TEAC).

2.18.3. Fe(III) reducing power: The reducing powers of SB samples and reference compounds were determined according to the method reported by Yen and Chen (215). Different concentrations of samples/standards were added to 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) followed by the addition of 2.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The reaction mixture was incubated at 50^o C for 30 min. at the end of which 2.5 mL of 10% trichloroacetic acid were added, and the mixture was centrifuged at 5000 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.1%, 0.5 mL), and the OD was measured at 700 nm using spectrophotometer (Shimadzu-2450). Increased absorbance of the reaction mixture indicated increased reducing power.

2.18.4. Fe(II) chelation capacity: The Fe(II) chelating potential of SB samples and reference ccompounds was investigated according to the method of Dinis et al with certain modifications (216). Briefly the reaction mixture containing different concentrations of samples/standards (50 - 2500µg/mL), 0.25 mL $FeCl_2$ (1mM) and 1mL ferrozine (5mM) was adjusted to a total volume of 2mL with methanol, shaken well and incubated for 10 minutes at room temperature. The absorbance of the mixture was measured at 562 nm against blank. EDTA was used as a positive control. Percentage of iron chelation was calculated using the formula;

Chelation effect (%) = [1- (Absorbance of Sample /absorbance of Control)] x 100.

2.19.5. Hydroxyl radical scavenging activity. 0.1 mL EDTA (1 mM), 0.01 mL FeCl3 (10mM), 0.1 mL H_2O_2 (10 mM), 0.36 mL of deoxyribose (10 mM), 1 mL of extract concentrations (10-100 µg/mL), 0.33 mL phosphate buffer (50 mM, pH=7.4) and 0.1 mL of ascorbic acid (1 mM) were added in sequence. The mixture was incubated at 37^{o} C for 1 hr. 1 mL of the incubated mixture was mixed with 1 mL of 10% trichloroacetic acid and 1 mL of thiobarbituric acid, 0.025 M NaOH and heated for 1 hr on water bath at 80^{0} C and a pink chromogen developed, which was measured at 523 nm (217).

2.17.3. Superoxide radical ion scavenging (SOS) activity: The superoxide radicals were generated. The scavenging activities of SB samples and reference compounds were determined by xanthine-XO system coupled with nitro-blue tetrazolium (NBT) reduction (218). In this method superoxide radical reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. The capacity of the extracts and standard compounds to scavenge superoxide radical was assayed as follows: the reaction mixture contained 50 µl of 10 mM xanthine in 0.1 N NaOH, 790 µL of Tris buffer (pH 7.4) containing 0.1 mM EDTA, 20 µL of 2.5 mM NBT in Tris buffer, and 0.1 mL extract or standard compound (as test) or 0.1 mL tris buffer (as control). The reaction was initiated by the addition of 40 µL of XO (0.04 U/ml) in Tris buffer (pH 7.4) and incubated at 25 ^{o}C for 20 minutes. Optical density of the resulting solution was measured at 560 nm using spectrophotometer (Shimadzu-2450). The ability to scavenge superoxide radicals was calculated using the following equation:

Scavenging Activity (%) = [1- (OD of Sample /OD of Control)] x 100.

2.19.4. XO inhibitory activity: XO inhibitory activity was measured spectrophotometrically by measuring uric acid formation at 290nm with xanthine as substrate (219). The assay system consisted of 1mL reaction mixture containing 0.1U/mL XO with 0.2 mL xanthine (0.26 μM) in 50 mM phosphate buffer (pH=7.4). The reaction was initiated by adding the enzyme with or without inhibitors and the change in absorbance of the mixture at 290 nm for 10' was measured against a blank prepared in the same way but replacing XO with phosphate buffer. Another reaction mixture was prepared (control) without test compounds in order to have a maximum uric acid formation. Various concentrations of allopurinol were used as a positive control. The substrate concentration was kept less than 1μM to avoid the substrate inhibition. The % inhibition of uric acid formation was calculated by comparison with control and expressed as

$$\text{XO inhibition (\%)} = [(A_1-A_2)/ A_1] \text{ X100},$$

where A_1 is the change in absorbance per minute without the sample and A_2 is the change in absorbance per minute with the sample.

2.20. Statistical analysis

SD and ANOVA calculations were performed using Microsoft Office Excel 2003. Graphical calculations were performed using Microcal Origin 6.0 Professional.

Chapter 3.0

RESULTS AND DISCUSSION

If I have seen further (than certain other man)
it is by standing upon the shoulders of giants

.....Newton

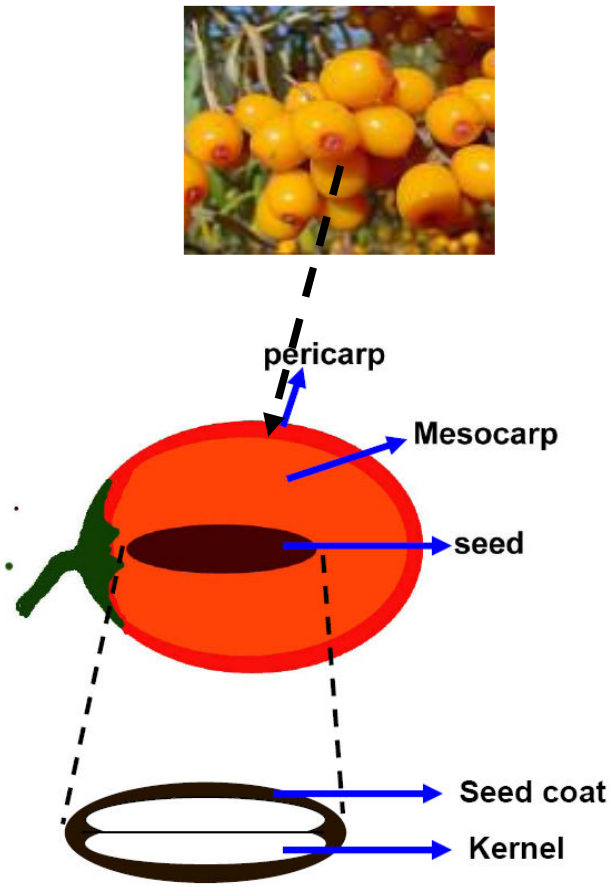
3.1. Phytochemical Profiling of *H rhamnoides* Berries of Trans-Himalayan Origin

Phytochemical profile of plants largely depends on the agricultural practices and geo-climatic conditions. This demands detailed profiling of each plant with respect to their genetic variations and geo-climatic area of cultivation. Sea buckthorn (SB) grows in cold regions of Asia, Europe, and North America. Indian Himalayas host world's second or third largest area under SB (30,000 – 40,000 ha). In India it grows in the cold and arid regions of Trans-Himalayas at an altitude from 2500 m to 4500 m. In Ladakh it grows approximately 11,500 hector area and covers major part of the forest area in the region. It has been found growing abundantly in such adverse conditions where temperature varies between -30 to 30^{o} C and having annual precipitation only 9 cm. It also grows wild in cold deserts such as Lahaul and Spiti in Chamba and Kinnaur districts of Himachal Pradesh. SB in Trans-Himalayan region of India is represented by three species viz; *H. rhamnoides var. turkestanica, H. salcifolia* and *H tibetana.* 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. In these cold deserts plants are under extreme climatic stress and are expected to have a distinct phytochemical profile. There were no systematic studies to record the phytochemical profile of SB berries of Trans-Himalayan origin. In this chapter detailed chemical profiling of SB berries belonging to the most common species in India i.e. *H. rhamnoides* from Ladakh region was attempted.

H. rhamnoides berries from Ladakh region was orange red in color. The oval shaped berry had a hard seed enveloped with juicy pulp. The berries were

4-6 mm in diameter and 20-40 mg in weight. The berries were separated into pulp, seed coat and kernel and analyzed for their chemical constituents in detail. They were characterized in terms of their proximate composition, lipid profile, phenolic and organic acids composition.

Figure 3.1: Anatomy of Sea Buckthorn berries



3.1.1. Proximate Composition:

Proximate composition of pulp and seed of *H. rhamnoides* berries was determined following the standard methods and the results are presented in Table 3.1. Pulp and seed showed considerable variation in terms of their

proximate composition. Fresh berry pulp had 81.3% moisture, 4.5% fatty matter, 2.0% protein, 8.5% carbohydrate and 1.0% minerals. Seed contained 64.0% moisture, 7.2% fatty matter, 2.3% protein, 18.2% carbohydrate and 3.1% minerals. Fiber content in the berries was also estimated (Table 3.1). Pulp and seed respectively had 1.2 and 0.7% fiber.

Reports on the proximate composition of *H. rhamnoides* berries of Indian Trans-Himalayan origin are fragmented. Present study showed that both pulp and seed of Indian *H. rhamnoides* berries are good source of medicinal oil. The oil content obtained in the present study was in accordance with the previous reports on *H. rhamnoides* berries of different origin. From the previous reports it is clear that the oil contents in the pulp and thus in the whole berries varied considerably with origins and other factors (129,139,147,148,154,171,220). In fresh berries of *H. rhamnoides*, the oil level ranges from 1.4% in sub species (*ssp*) *sinensis* from China up to 13.7% in *subsp. turkestanica* from the Western Pamirs (154).Within a single population, the oil content in the berries correlates with morphological characteristics such as size and color of the berries, which may lead to a difference up to 2-fold (129,154). In addition, harvesting time influences oil content in the berries (129,148,154). The oil content in seeds of *H. rhamnoides* is commonly ~10%, although higher values (up to 15–16%) have been reported in some cultivars and wild berries from the Altai, Czech Republic and Tajikistan (129, 139,148, 220,171).

Proximate analysis showed that *H. rhamnoides* berry was not a good source of protein. Carbohydrate content in the seed was much higher than that in pulp. Glucose, fructose, mannitol, sorbitol, xylose and xylitol are the main sugars reported in *H. rhamnoides* berries of Chinese origin (127,147). There are no reports on the detailed profiling of carbohydrates in the seeds

Table 3.1. Proximate composition of *H. rhamnoides* berry pulp and seed (on fresh wt., Mean % ± SD)

Characteristics (%)	Pulp	seeds
Moisture	81.3 ± 1.6	64.0 ± 0.9
Fatty Matter	4.5 ± 0.4	7.2 ± 0.6
Protein	2.0 ± 0.2	2.3 ± 0.4
Sugar	8.5 ± 0.7	18.2 ± 1.1
Minerals	1.0 ± 0.2	3.1 ± 0.2
Crude Fiber	1.2 ± 0.3	0.7 ± 0.2

3.1.2. Fatty Acid Composition of Pulp and Seed Oils

Pulp and seed oils, extracted by Christies' method were subjected to FA composition analysis. Methyl esters of the FA (FAME) were prepared and subsequently analyzed using GC. The individual FAs were identified by comparing their retention time with that of reference FAME. The FA compositions of pulp oil and seed oil are summarized in Table- 3.2. In the oil from berry pulp, the dominating FAs were palmitoleic (48.2%), palmitic (32.5%), and oleic (12.9%) acids. 3.5% linoleic, 0.5% stearic and 0.5% linolenic acids were also obtained. FA profile of seed oil was different from that of pulp oil. Seed oil contained linoleic (26.2%), linolenic (18.1%) palmitic (17.7%) and oleic (12.9%) acid as major FA. Small amounts of palmitoleic (3.5%) and stearic (3.1%) acids were also found in seed oil. While 16:1 acid (48.2%) was the most predominant FA in pulp oil, its content in seed oil was as low as 3.5%

Table 3.2. FA composition of pulp and seed oils of *H. rhamnoides* (wt % ± SD)

	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Pulp oil	32.5 ± 1.4	48.2 ± 3.1	0.5 ± 0.9	12.9 ± 0.1	3.5 ± 0.6	0.5 ± 2.5
Seed oil	17.7 ± 1.2	3.5 ± 1.1	3.1 ± 0.2	29.5 ± 2.3	26.2 ± 2.2	18.1 ± 1.9

$C_{16:0}$ - Palmitic acid $C_{16:1}$ - Palmitoleic acid $C_{18:0}$ - Stearic acid
$C_{18:1}$ - Oleic acid $C_{18:2}$ - Linoleic acid $C_{18:3}$ - Linolenic acid

The FA profile obtained here for pulp oil is in accordance with the previous reports on SB of Chinese and European origin. Palmitoleic (16–54%), palmitic (17–47%) and oleic (2–35%) acids are reported as the dominating FA in the soft parts of *H. rhamnoides* berries (139,145,148,220). 12.1 – 39.0% palmitoleic acid is reported in the pulp oil of berries of Chinese and Finnish origin (129). Vereshchagin et al; reported that the Central Asian and Baltic forms contained higher proportion of palmitoleic acid in the TAG of berry pulp (55.0 and 42.0%, respectively) and Caucasian form had 16.0% (150). The FA composition of the pulp oil of a Siberian form reported by Ozerinina et al; is similar to that of the Central Asian and the Baltic form (149). Cakir reported palmitoleic (47.9 %) and palmitic (25.0%) acids were the major FAs in mesocarp oil of *H. rhamnoides* from Turkey (173). Genetic factors (i.e. differences between species and subspecies) play a major role in the variations of FA composition (148). In ripe berries, the FA composition in the soft parts varies with harvesting time. In berries of same origin, the FA compositions of seeds and soft parts remain rather constant among different harvesting years (148). The variation in the FA composition, especially in the soft parts of the berries, provides a great potential for industrial application and plant breeding when seeking extreme sources of certain FAs.

The occurrance of palmitoleic acid in large proportion as shown in this study is unique among vegetable oil as no other oil of plant origin is reported to contain palmitoleic acid in substantial amount. Palmitoleic acid is reported to facilitate cell membrane fluidity in a manner similar to that of PUFA but with low susceptibility to oxidation. An increase in the level of dietary palmitoleic acid has been suggested to improve the metabolism of vascular smooth muscle cells (221). Hypocholesterolemic and hypoglyceridemic activities comparable to that of linoleic and linolenic acids also have been reported for palmitoleic acid (222).

Seed oil had oleic acid as the major FA (29.5%) followed by linoleic acid (26.2%) and α-linolenic acid (ALA) (18.1%). The proportions of linoleic acid and ALA reported in seed oil are in the range of 30–40 and 20–35%, respectively. Other major FAs reported in seeds are oleic (13–30%), palmitic (15–20%), stearic (2–5%), and vaccenic (18:1 n-7, 2–4%) acids (139,145,220). Inspite of the differences in some morphological characteristics and in growth conditions, *subsp. sinensis* (from China), *mongolica* (from Russia) and *rhamnoides* (from Finland) have almost identical FA composition in seeds (145, 148,154). Present study showed that *H rhamnoides* seeds of Indian origin had slightly different FA profile with oleic acid as the major FA followed by linoleic and ALA. ALA is ubiquitous in plant leaf lipids and is present in some commodity seed oils: 8 to 10% in soybean and canola, >50% in linseed oil, and 65 to 75% of perilla oil. The seed oils of many *Labiatae* species have >50% ALA (222). Linoleic and linolenic acids are considered as essential FAs. These FAs are having significant role in neural and visual development and are precursors for biologically active metabolites, the eicosanoids. As membrane components, they have significant role in maintaining the fluidity and permeability of biological membranes (223).

3.1.3. Carotenoids in Pulp and Seed Oils

More than 600 carotenoid compounds have been isolated and characterized from natural sources. In plants carotenoids are involved in photo protection or light collection. Most carotenoids contain an extended array of double bonds which are responsible for their AO activity. Among the carotenoids only α-carotene, β-carotene, γ-carotene and β-cryptoxanthine can be converted into vitamin A. Nutritional significance of carotenoids in prevention of cancer, heart disease, and age related macular degeneration (AMD) has been suggested (224-226).

Total carotenoids in the pulp and seed oils was quantified and expressed in terms of β-carotene, (Table-3.3). Pulp oil was found to have significantly higher amount of carotenoids (2548 mg/Kg). Seed oil contained moderate amount of carotenoids (389 mg/Kg). Berry pulp and seed respectively contained 115 and 28 mg/kg (Fresh wt) of carotenoids

The carotenoid content in the berries are subjected to extreme variation; differences up to 10-fold has been reported even within the same natural population and subspecies. Total carotenoids (calculated as β-carotene) from 1 to 120 mg/100 g in fresh berries have been reported (129). Both raw material and methods of oil isolation influence the content of carotenoids in the oils. Common levels of carotenoids are 1000–5000 mg/kg in pulp oil and 200–1000 mg/kg in seed oil (129). The concentration in seeds is typically 1/20–1/5 of that in soft parts (129). β-Carotene constitutes 15–55% of total carotenoids, depending on the origin (129). α-carotene, γ-carotene, dihydroxy-β-carotene, lycopene, zeaxanthin and canthaxanthin have been reported to as the other carotenoids in SB berries (129).

Epidemiological studies suggest β-carotene as an important dietary anticarcinogen as well as a protective factor against heart disease. β-Carotene

and other carotenoids are important AOs in plants. Carotenoids scavenge singlet oxygen generated by the interaction of light with chlorophyll, before the singlet O_2 damages the plant cells. Carotenoids inhibit lipid peroxidation and also quench many other free radicals. The free radical scavenging activities of carotenoids can be attributed to the stability of carotenoid radicals by the delocalization of unpaired electrons over the carotenoid conjugate polyene system (227). Many studies have demonstrated that β-carotene inhibits auto oxidation of lipids in biological tissues and food products. Peroxyl radicals in particular, have been shown either to add to the long chain of conjugated double bonds present in carotenoids, or to take part in electron transfer reactions giving rise to carbon centered β-Carotenyl free radicals (228):

β-Carotene + $ROO^{\bullet}$ ---------- [ROO- β-Carotene],

or [ROO^{-} + β-Carotene $^{\bullet}$]

Table. 3.3. Carotenoid and tocol content of pulp and seed oils of *H .rhamnoides* berries (mg/kg of oil, Mean ± SD)

	Carotenoids (mg/kg)	**Tocopherols** (mg/kg).						
		αT_1	**αT_3**	**β T**	**Γ T**	**γT_3 +δ T**	**δT_3**	**Total**
Pulp Oil	2548 ± 29	954 ±73	95 ±15	27 ±6	126 ±5	187 ±11	14 ±2	1394 ±87
Seed Oil	389 ± 17	608 ±45	10 ±1	21 ±1	433 ±37	496 ±12	tr	1193 ±51

- **T_1 = tocopherol, T_3 = tocotrienol**

3.1.4. Tocopherols and tocotrienols (tocols) in pulp and seed oils

Compounds showing vitamin E activities are termed as tocopherols. In nature eight chemical constituents have been found to have vitamin E activity, they are d-α, d-β, d-γ and d-δ tocopherols and d-α, d-β, d-γ and d-δ tocotrienols (Fig 3.2). These are fat-soluble in nature and also act as major radical scavengers in membranes. Vegetable oils such as wheat germ oil, palm oil and rice bran oil provide the best source of these bioactive phytochemicals essential in human nutrition.

Tocols in the pulp and seed oils of *H rhamnoides* were profiled in RP-HPLC (Figure 3.3). Individual tocols in the samples were identified by comparing their T_R (retention time) with that of reference compounds. The amount of tocols was calculated from the calibration curve plotted with reference compounds. HPLC analysis showed that both pulp and seed oils were significantly rich in tocols (Table-3.3). Pulp oil had 1394 mg/kg of tocols and α-tocopherol (954 mg/kg) contributed 68.4% of total tocol. γ-tocopherol (126 mg/kg) and α-tocotrienol (95 mg/kg) were the other major tocols in the pulp oil. Under the chromatographic conditions used here, the resolution between γ-tocotrienol and δ- tocopherol was poor, therefore they were calculated together. γ-tocotrienol and δ- tocopherol together contributed 187 mg/kg. Presence of β-tocopherol (27 mg/kg) and δ- tocotrienol (14 mg/kg) were also detected. 1193 mg/kg of tocols was obtained for seed oil. Seed oil also had α-tocopherol (608 mg/kg) as the major tocol, which contributed 51% of total tocols. In contrast to the pulp oil, seed oil had high amount of γ-tocopherol (433 mg/kg). Presence of α-tocotrienol (10 mg/kg), β-tocopherol (21 mg/kg) and δ- tocotrienol were also observed in seed oil. γ-tocotrienol and δ- tocopherol together contributed 496 mg/kg.

This is the first detailed report on the tocol composition of SB berries of Himalayan origin. Both pulp and seed oil had significantly higher amount of tocols than those in common vegetable oils like rice bran (980 mg/kg), soya bean (960 mg/kg), palm (1180 mg/kg), sesame (290 mg/kg), sun flower (550 mg/kg) and safflower (800 mg/kg) oils (229). According to the available reports on SB berries belonging to other regions the total tocol content vary within the range 100–300 mg/kg in seeds and 10–150 mg/kg in fresh berries (145,171,129). In the pulp of *H rhamnoides* berries of Chinese origin α-tocopherol alone constitutes up to 90% of the total tocols, while both α-and γ-isomers (each representing 30–50% of total) are the major ones in seeds. α, β, and γ-tocotrienols each accounts for 0.5–5% of total tocopherols and tocotrienols in soft parts, whereas in seeds the β isomer (2–8%) clearly dominates accompanied by only trace amounts of α and γ-isomers (145,171,129). The tocol profile obtained for the SB berries of Indian origin are matching with the reports available on berries of Chinese and Polish origin. A tocopherol profile of 75–89% of α-tocopherol, 4–11% of γ-tocopherol, 2.4–12.2% of β-tocopherol, 0.3–2.4% of δ-tocopherol, 0.4–4.8% of β-tocotrienol, 0.4–3.2% of α-tocotrienol, and 0.6–2.5% of δ-tocotrienol was reported for the pulp of berries of the *sinesis* and *mongolica* subspecies of *H. rhamnoides* from China (171). Zadernowski *et al;* reported 101–128 mg/100 g of tocopherols in pulp oil of *H. rhamnoides* berries of Polish origin with a profile of 62.5–67.9% of α-tocotrienol, traces of γ-tocotrienol, and 30–40% of δ-tocopherol. The amount and composition of tocols in the berries largely depend on the species, geo-climatic variations and maturity (157).

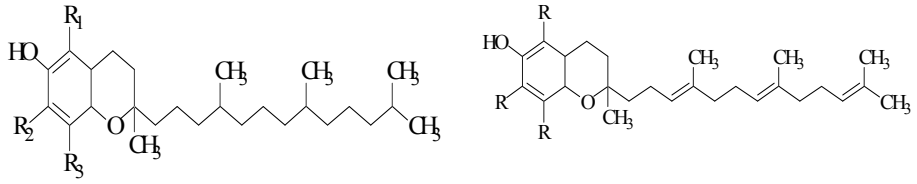
The beneficial effects of vitamin E compounds include AO activity, lipid lowering effect, nutrient function, prevention of degenerative diseases like CVD, cataract, arthritis and cancer, and immune system maintenance (230,231). Quireshi et al; demonstrated that tocotrienols inhibited the activity of 3-hydroxy-3-methyl glutaryl coenzyme A (HMGCoA) reductase, a rate

limiting enzyme in cholesterol synthesis (232). Tocopherols and tocotrienols seem to be the most efficient lipid AOs in nature. Tocopherols inhibit lipid peroxidation by scavenging lipid peroxyl radicals much faster than these radicals can react with adjacent FA side chains or with membrane proteins.

$$\alpha\text{–Tocopherol} + \text{Lipid-}O_2 \longrightarrow \alpha\text{-Tocopherol}^{\cdot} + \text{Lipid–}O_2H$$

The –OH group of the tocopherol gives up its hydrogen atom to the peroxyl radical and quench it. This leaves a tocopheryl radical. The tocopheryl radical is stabilized by the delocalization of unpaired electron over the conjugated polyene or aromatic system. The tocopheryl radical is terminated by the polymerization reaction or by some regenerating mechanism in the body. Tocotrienols exerts greater AO than tocopherols (233).

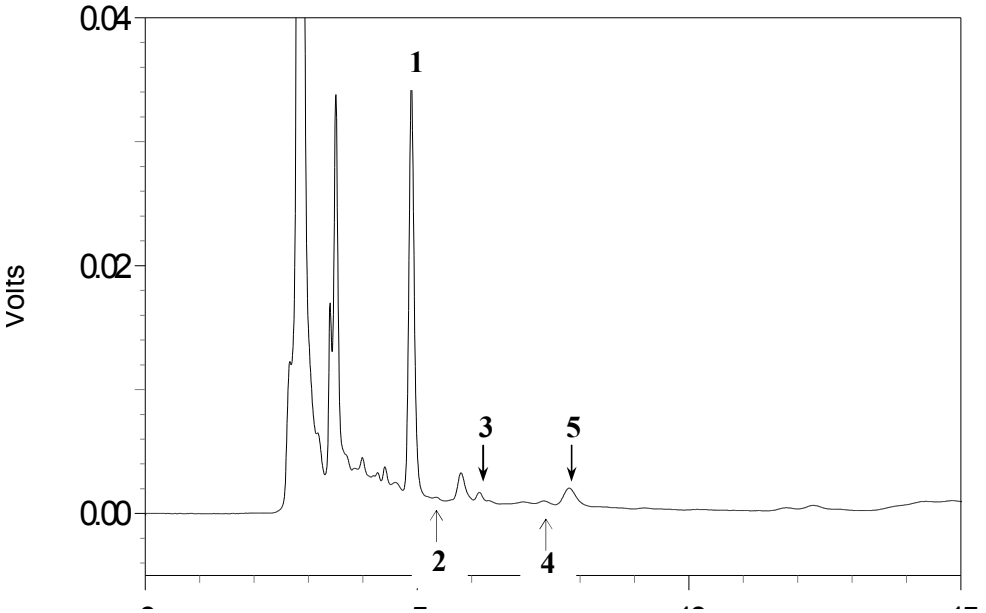
Figure 3.2. Tocopherols and tocotrienols



(A) Tocopherols **(B) Tocotrienols**

R_1	R_2	R_3	(A) Tocopherols	(B) Tocotrienols
CH_3	CH_3	CH_3	α	α
CH_3	H	CH_3	β	β
H	CH_3	CH_3	γ	γ
H	H	CH_3	δ	δ

Figure 3.3 HPLC profile of tocols in *H rhamnoides* pulp oil at 295 nm.



1: α-Tocopherol, 2: α-Tocotrienol, 3:β-Tocopherol,4: γ- Tocopherol, 5: γ- Tocopherol + δ- Tocopherol

3.1.5. Sterols in Pulp and Seed oils

In plants sterols are usually found as their glycosides. Steroidal glycoside in pulp and seed oil were hydrolyzed to their aglycones and subsequently analyzed in HPLC (Figure 3.5). Sterols in the samples were identified by comparing the retention time with that of reference compounds. Amounts of individual sterols were determined from the calibration curves and are given in Table 3.4.

The β-sitosterol (3034 mg/kg) and stigma sterol (1062 mg/kg) were found to be the major sterols in the pulp oil. In comparison to pulp oil, seed oil was found to be a richer source of phytosterols with 16200 mg/kg of sterols. β-sitosterol was found to be the major sterol along with stigmasterol and campesterol in SB seed (Figure 3.4).

According to the previous reports the sterol content in SB berries is in the range of 0.02–0.04% in the soft parts and 0.1–0.2% in seeds (both based on fresh weight) (154). In fresh berries of *subsp. sinensis* and *rhamnoides* the sterol content reported to fall in the range of 350–500 mg/kg, of which 70–80% is in the soft parts (154). Typical values for sterol content are 1–2% in seed oil and 1–3% in oil from the soft parts (154,234). Sitosterol constitutes 60–70% of seed sterols and up to 80% of those in soft parts. Isofucosterol is another major sterol representing 10–20% of seed sterols and 2 –5% of sterols of the soft parts. Campesterol, stigmastanol, citrostadienol, avenasterol, cycloartenol, 24-methylenecycloartanol, obtusifoliol, each represents 1–5% of total sterols of both fractions (154,234). The amounts of identified sterols in pulp and seed oils obtained in the present study are in accordance with the previous reports. Some minor peaks in HPLC profiles of both pulp and seed oils could be identified as sterols, which might have further contributed to the total sterol content.

Table 3.4. Sterols in *H. rhamnoides* pulp and seed oils (mg/kg ± SD)

Sterols	Pulp Oil	Seed Oil
β-sitosterol	3034 ± 84	13305 ± 132
Stigmasterol	1062 ± 67	1125 ± 111
Campesterol	Tr	1770 ± 50
Total	4096 ± 98	16200 ± 260

Figure 3.4 HPLC profile of sterols in *H rhamnoides* seed oil.

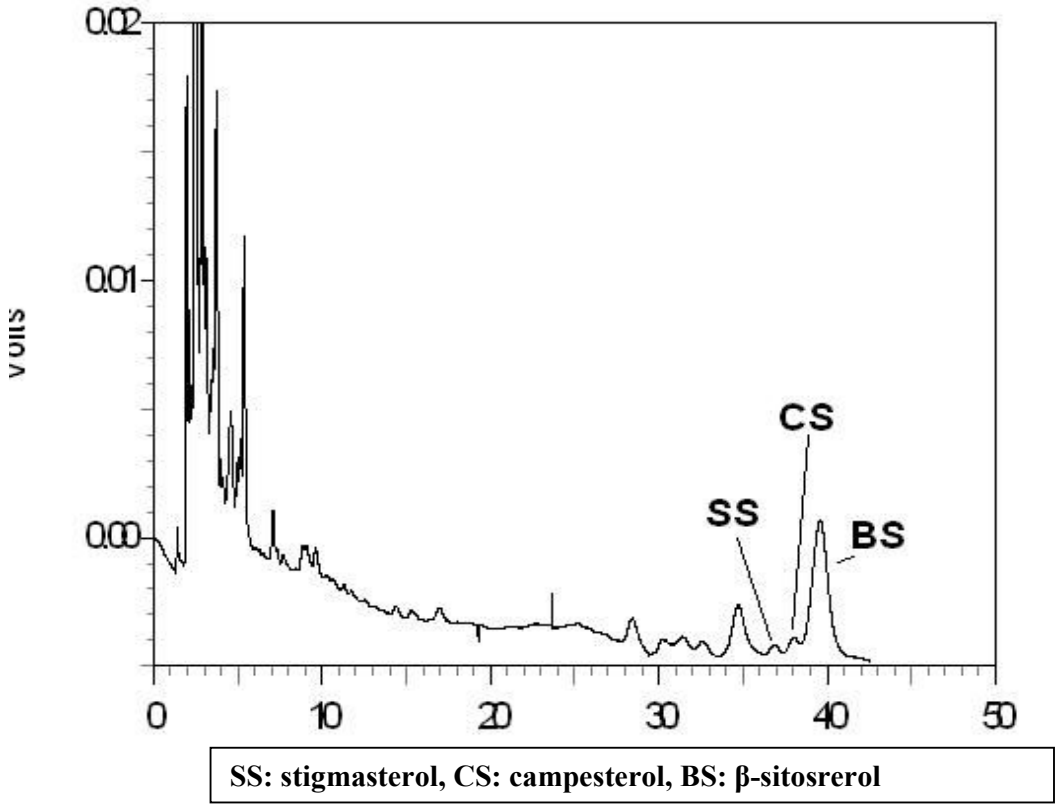
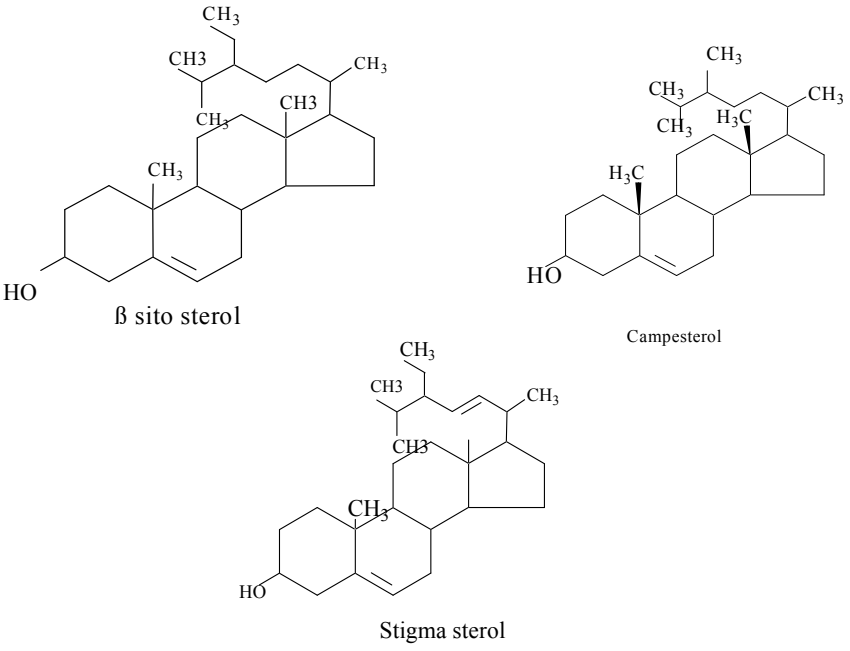


Figure 3.5 Structures of major sterols in SB berries.



3.1.6. Vitamin C Content in the Berries:

Like tocopherol, vitamin C (ascorbic acid) has been defined as an essential nutrient. Vitamin C is common in a variety of fruits and vegetables. Being a biologically essential phytochemical for human, vitamin C content in *H. rhamnoides* berries was analyzed. It was revealed that soft parts (fresh) of *rhamnoides* berries contained significantly higher amount of vitamin C (2232 mg/kg) (Table 3.5). Several authors have reported the high vitamin C content in Chinese berries (3600-25000mg/kg) (1*27*), however the reports about the nutritional qualities of Indian berries are rather limited. SB berries are well known for its extraordinarily high levels of vitamin C, and therefore considered as an important quality parameter of the SB juice. Vitamin C content in the berries were significantly higher than the common vitamin C sources like blackcurrant (1550 mg/kg), lemon (650 mg/kg), orange (500 mg/kg), grape (400 mg/kg), mango (250 mg/kg) and apple (150 mg/kg**)** (235).

Ascorbic acid in aqueous phase of plasma have significant role in AO mechanism of body. It has many cellular activities that may be directly or indirectly related to its AO properties. It has strong reducing property in solution of pH> 4. In vivo most of the ascorbic acid is maintained in a reduced state by other endogenous reductants. It has been demonstrated to be an effective scavenger of superoxide, hydroxyl, and peroxyl radicals as well as hydrogen peroxide and singlet oxygen. Ascorbic acid can directly act as AO by reacting with aqueous radicals or indirectly by restoring the AO activities of other AOs, like α-tocopherol. In studies with human plasma lipids, ascorbic acid was found to be far more effective in inhibiting lipid peroxidation initiated by peroxyl radical initiator than other AO components such as protein thiols, urate, bilirubin and α-tocopherol (236).

3.1.7. Organic acid Composition of berries

SB Berries are highly acidic in nature. Total titrable acids in the berries were found to be 3.6% in terms of malic acid. SB berry juice had a pH of 3.1. Therefore the berries were analyzed in detail for their organic acid content.

Isolation of quinic acid: Quinic acid, a tetrahydroxy cyclohexanoic acid (Figure 3.6) was isolated from the water extract of pulp by silica column chromatography and identified by spectral techniques. Quinic acid thus obtained was used as a reference compound for the HPLC analysis. Organic acids along with high vitamin C content imparted considerable acidity to SB berries. Detailed profiling of organic acids was attempted in this study.

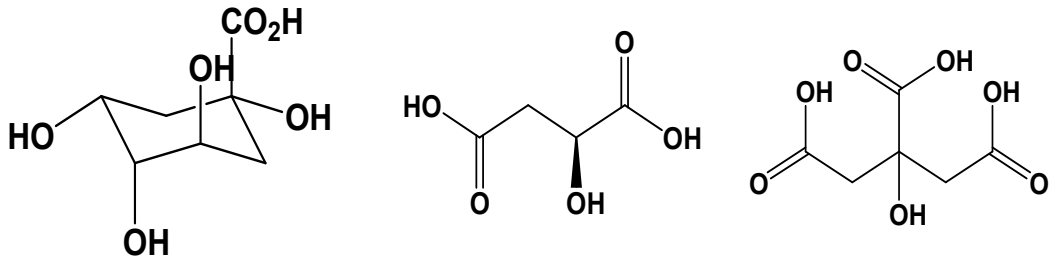
HPLC profiling of organic acids: RP-HPLC-DAD analytical conditions for the profiling of organic acids in SB berries were optimized for the first time. Since the organic acids are polar in nature a RP C_{18} column was selected as stationary phase. Phosphoric acid solution at pH 2.4 was used as mobile phase. Low pH of the mobile phase is reported to lower the ionization of hydroxyl and carboxyl protons and hence the mobility in non-polar stationary phase. Highly acidic mobile phase inversely affect the life of column materials. Therefore the acidity of mobile phase was selected so as to balance these two opposite factors. HPLC equipped with DAD was used for the analysis. DAD enables the recording of uv-visible spectra of analytes during the chromatographic run. Spectro-chromatograms thus obtained is highly useful for the identification of compounds and for assessing the purity of peaks. Chromatographic conditions were optimized using the reference compounds and the T_R and uv spectra were recorded. Individual organic acids in the samples were identified by comparing their T_R and uv spectra with those of reference compounds (Figure 3.7).

The composition of low molecular acids in the SB berries is given in Table 3.5. The berries had 2.80% quinic acid in soft parts, along with malic acid (1.60%), citric acid (0.16%) (Figure 3.6) and traces of fumaric and maleic acids. The HPLC analysis showed quinic acid as the major organic acid in SB berries. This finding is different from earlier reports of malic acid as the major organic acid in SB berries (127). However, Beveridge et al; reported the presence of quinic acid in processed SB juice. Higher amount of quinic acid a biologically important organic acid signified the nutraceutical value of SB berries (147). Sheng et al; identified quinic acid and its analogues as the active molecules in the Cat's claw extract, an effective medicinal preparation against the chronic inflammations, and G-I dysfunctions like ulcer, tumors and infections through in-vitro and in-vivo studies (236). SB berries are recommended against G-I disorders in traditional medicinal systems. G-I protective properties of SB berries might be attributed to the high content of quinic acid. Detailed studies in this regard are required. Presence of reactive hydroxyl and carboxylic acid groups makes quinic acid a promising starting material for stereo specific cyclic organic compounds in synthetic chemistry.

Table 3.5. Vitamin C and organic acids content in *H. rhamnoides* fresh berries (80% moisture) mean % ± SD.

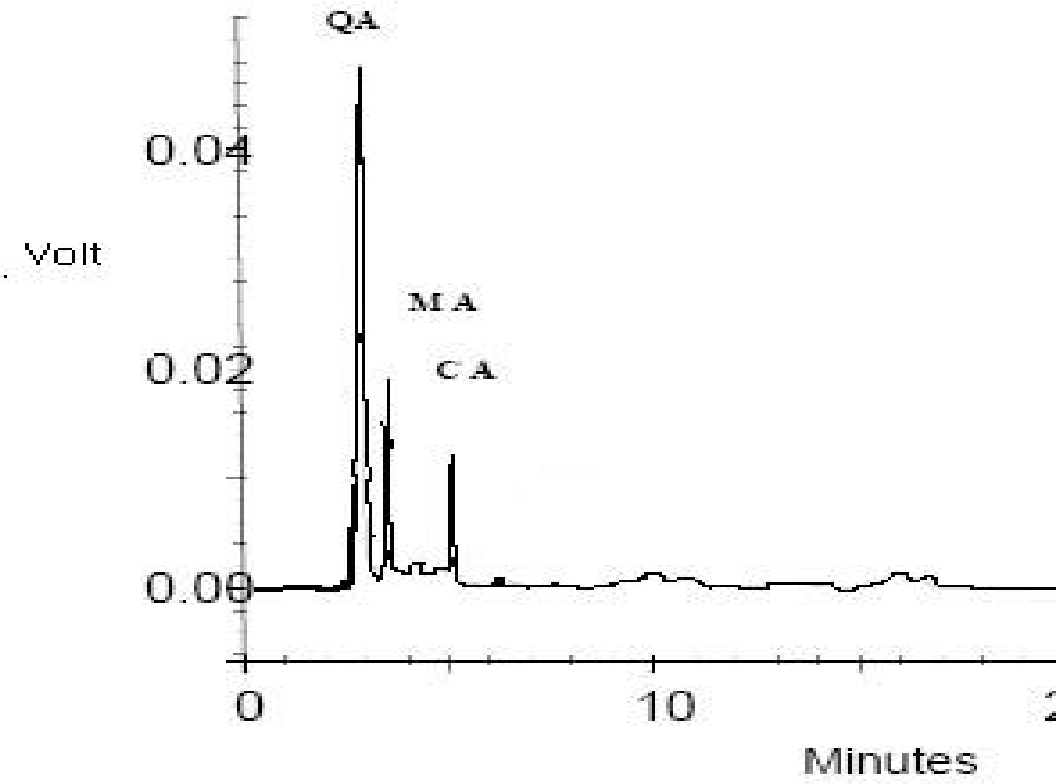
Composition	Amount
Vitamin C (mg/kg)	0.22 ± 0.02
Organic acids (%)	
Quinic acid	2.80 ± 0.12
Malic acid	1.60 ± 0.09
Citric acid	0.16 ± 0.03
Total organic acids	4.56 ± 0.12

Figure. 3.6 Structures of major organic acids in SB berries



Quinic acid **Malic acid** **Citric acid**

Figure 3.7 HPLC Chromatogram of organic acids in SB berry pulp.



QA: quinic acid, MA; malic acid, CA: Citric acid

3.1.8 Polyphenols

Phenolic compounds or polyphenols constitute one of the most numerous and ubiquitously distributed group of plant secondary metabolites with more than 8000 phenolic structures currently known. Natural polyphenols can range from simple molecules (phenolic acids, phenyl propanoids, flavonoids etc) to highly polymerised compounds (lignans, melanins, tannins etc). These compounds play an important role in growth and reproduction, provide protection against pathogens and predators and contribute towards colour and sensory characters of plants. Polyphenols exhibit a wide range of biological effects including anti-allergic, antimicrobial, anti-inflammatory, anti-atherogenic, anticancer, hepatoprotective, cardioprotective and vasodilatory actions (61,69,70,84,80). The varying chemical and biological properties requisite the detailed chemical finger printing of this class for the better understanding of phytochemistry of medicinal plants.

Previous reports suggest SB berries and leaves as a good source of polyphenols and larger portion of their biological activities are attributed to their polyphenol content. Reports on the detailed profiling of SB polyphenols are rather limited. SB berries of Indian origin are particularly least studied on this context. Therefore detailed profiling of polyphenols in the berry parts are attempted in the present study.

Amount of polyphenols, in anatomical parts of SB berries were quantified by using F-C reagent. F–C reagent method is widely used for the quantification of phenolics in plant sources. In this method, a mixture of molybdate and tungstanate in highly basic medium is used as the test mixture. Reduction of molybdotungsatanate reagent to a blue product by phenolics is the key step for this reaction.

Mo(VI) (yellow) $\longrightarrow$ Mo(V) (blue)

Calibration plot was obtained with gallic acid and results are expressed as GAE (Table 3.6).

Pulp had 2.73% of phenolics in dry weight. Seed coat and kernel respectively contained 3.95% and 8.58% phenolics. Amount of total phenolics in the berries and seeds were deduced from these results. The whole berries contained 3.62% phenolics whereas the seeds had 7.15% phenolics. The values obtained for the phenolics in these berries are in accordance with the previous reports on Chinese and Polish berries (158, 170).

Table 3.6. Total polyphenols, flavonols and PAs in *H. rhamnoides* berries (dry mass). (Mean ± SD (n = 5).

	Polyphenols (%)	**Flavonols (%)**	**Proanthocyanidins (%)**
Pulp	2.73 ± 0.12	0.21 ± 0.05	1.26 ± 0.14
Seeds Coat	3.95 ± 0.04	0.02 ± 0.00	0.87 ± 0.10
Kernel	8.58 ± 0.32	0.53 ± 0.01	4.40 ± 0.22
Berries	3.62	0.24	1.67
Seeds	7.15	0.35	3.30

3.1.8.1 Flavonols

Flavonols are important class of polyphenols with significant and well-documented biological properties. The amount of total flavonols, a major class of flavonoids in the SB berries and leaves were quantified using aluminum chloride reagent and summarized in Table 3.6. Aluminum chloride forms chelating complexes with flavonols in quantitative manner resulting in a bathochromic shift in band II absorption of flavonols (207). SB berry pulp,

seed coat and kernel were found to contain 0.21. 0.02 and 0.53% of flavonols. Berries and seeds respectively had 0.24 and 0.35% flavonols.

According to the results of aluminum chloride quantification of flavonols, flavonols contributed approximately 10% of total phenolics in SB berries. Therefore detailed profiling of SB flavonols was attempted in this study. Flavonoids are mainly occurring as their glycosides and esters in plants. The variations in number, position and nature of glycosidic bonds and nature of glycoside part result in great diversity in flavonoid glycosides. In order to minimize the complexity, the SB flavonoid glycosides were hydrolyzed and analyzed.

Repeated chromatographic separation of hydrosylate of kernel methanol extract yielded two yellow colored solids with characteristics of flavonoids, one of which was identified as quercetin by comparison with reference sample and spectral data. The other was identified as isorhamnetin (Figure 3.8) by spectroscopic studies. The compound showed uv-visible spectra of flavonols with characteristic bathochromic shifts with NaOMe, $AlCl_3$ and H_3BO_3. IR showed characteristic absorption for hydroxyl groups. Mass spectra gave molecular weight of 316 with characteristic flavonol fragmentation pattern. ^{1}H-NMR spectra showed 5 aromatic protons at δ 8.31 (H-2'), 8.21 (H-6'), 7.37 (H-5'), 6.87 (H-8), and 6.76 (H-6) and one methoxy group at δ 3.84. The mass spectra (m/z 301, 151, 123, 108, 85, 57, 40) further confirmed the structure of isorhamnetin.

RP-HPLC-DAD analysis of flavonols; RP-HPLC-DAD conditions for the quantitative profiling of flavonoids were optimized using reference samples of quercetin, isorhamnetin and kaempherol. Since the flavonoids are medium polar in nature, RP C-18 column with an isocratic solution of methanol-water mixture was used for the resolution of flavonoids. The individual SB

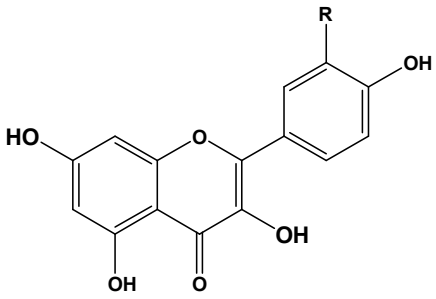
flavonoids were identified from their retention and spectral data and quantified (Figure 3.9).

Quercetin, kaempherol and isorhamnetin (Figure 3.8) were identified as the major flavonoids in the SB berries. Kernel (553 mg/100g) had the highest amount of flavonoid followed by pulp (288 mg/100g) and seed coat (18 mg/100g) (Table 3.7). Both pulp and seeds had isorhamnetin as the major flavonol followed by quercetin and kaempherol. 72-80% of the total flavonols in the pulp and seeds was contributed by isorhamnetin, whereas quercetin contributed 19-23%. The amounts of flavonols obtained for the present study were in accordance with the previous reports (164,166, 167).

Table 3.7. Flavonoids in *H. rhamnoides* berries (mg/100g of dry weight).

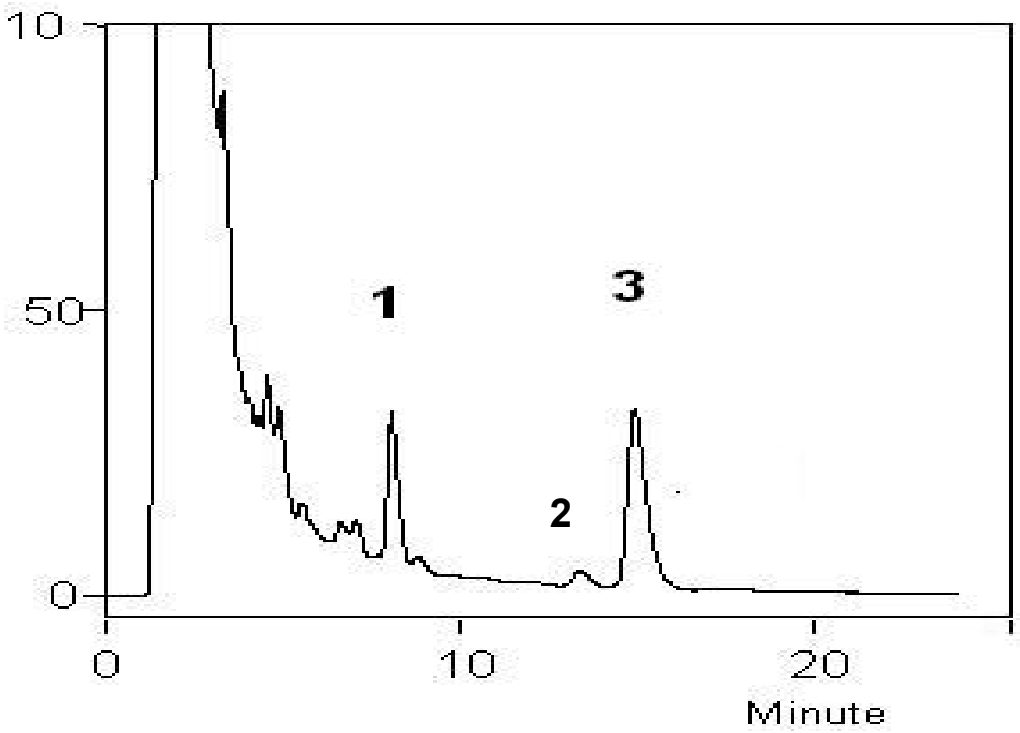
	Pulp	**Seed Coat**	**Kernel**
Quercetin	54.5 ± 8	4.2 ± 3	97.7 ± 11
Kaempherol	19.0 ± 2	0.8 ± 1	14.0 ± 8
Isorhamnetin	214.1 ± 8	13.4 ± 4	441.2 ± 7
Total	287.6 ± 17	18.4 ± 5	552.9 ± 13

Figure 3.8: Structures of major flavonols in SB berries



Flavonol	**R**
Quercetin	OH
Kaempherol	H
Isorhamnetin	OMe

Figure 3.9 HPLC-DAD chromatogram of flavonols in *H. rhamnoides* berry pulp at 270 nm



1: quercetin, 2: Kaempherol, 3: Isorhamnetin

3.1.8.2 Proanthocyanidins (PA).

Tannins, formed by the condensation of simple phenolics, are another important class of phenolics. Tannins are generally classified into hydrolysable tannins and condensed tannins or proanthocyanidins (PA). Oligomeric and polymeric flavon-3-ols are better known as PAs. The existence of PAs in common foods including fruits, vegetables, cereals and wines, affect their texture color and taste and may result beneficial and adverse nutritional effects. Plant PAs posses a variety of physiological activities such as AO, cardioprotection, anticancer and antihypertensive (168, 237-230). The greater parts of these activities are governed by the chemical structure and degree of polymerization of PAs.

Extraction of PA. The extraction efficiency of PA from plant materials largely depends on their chemical nature, and solvent systems and extraction conditions employed (240) which demand the optimization of extraction conditions for the analysis of plant PA. PA comprises a wide range of oligomeric and polymeric compounds with different sensitivity towards the reagents used for their estimation (240). The diversity in PA and the presence of other low molecular weight polyphenols like phenolic acids and different classes of flavonoids make the selection of appropriate methods difficult for the estimation of PA. In this study vanillin-HCl assay, a commonly used method for the quantification of PA in plant extracts due to its specificity towards flavonols and dihydrochalcones (240) was chosen. Since the reaction of vanillin towards the polymeric PA is more specific and sensitive in acidified methanol, the vanillin reagent was prepared in 5% HCl-methanol and reactions were also carried out in the same solvent system (241).

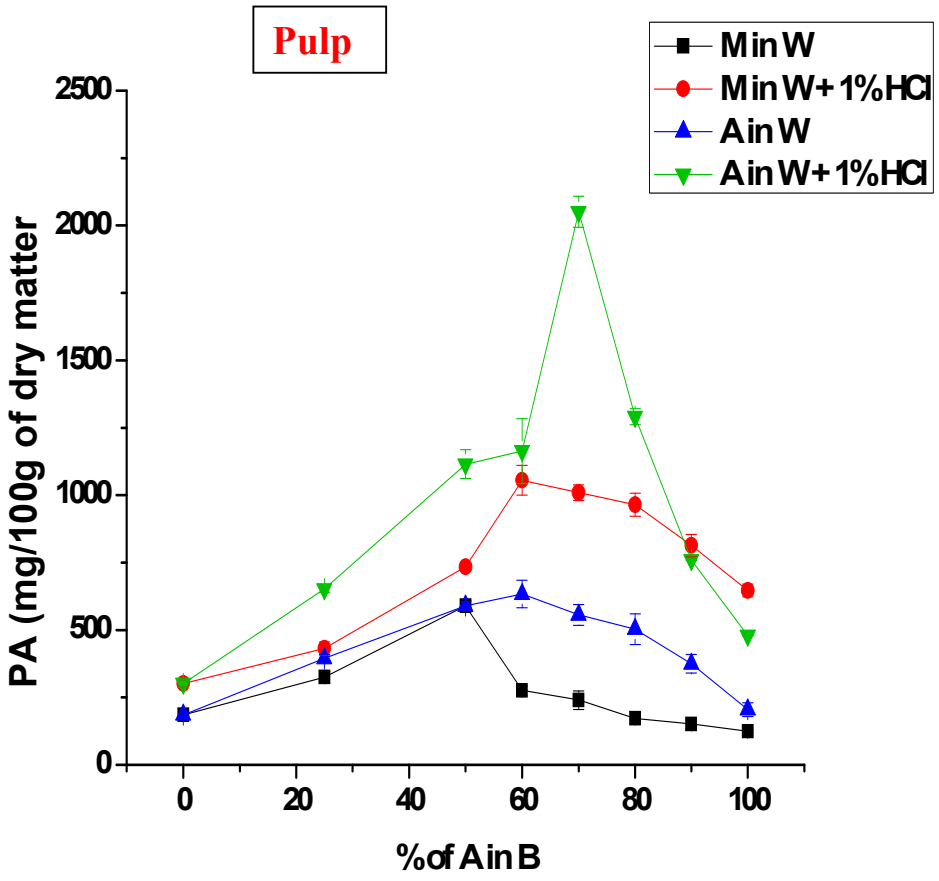
The extractability of PA of SB berry pulp and seeds with methanol-water and acetone-water mixtures and the effect of acidification were studied and the results are presented in Figure 3.10. Introduction of water to methanol and acetone resulted in considerable increase in the extractability of polyphenols, particularly PA. It is obvious that, the permeability of plant tissues is increased by the presence of water in extracting solvents, which enables the better mass transport by diffusion. Acetone water mixtures showed higher extractability, than corresponding methanol water mixtures ($P > 0.05$). Acidification of solvent mixtures enhanced the extraction efficiencies substantially in all samples studied here. 70% acetone water mixture containing 1% acid was found as most effective solvent for the extraction of PA from berry pulp, seed coat and kernel. The increased extractability of polyphenols with the acidified solvent mixtures has been previously demonstrated for beach pea (242). Higher extractability of polyphenols with

acidified solvent mixtures can be attributed to the acid catalyzed dissociation of their bonds with polar fibrous matrices (243).

Crude PA extracts of SB berries for further studies were prepared using the solvent mixtures as optimized. Crude PA extract of SB berry pulp, prepared with 70% acetone water mixture containing 1% acid was analyzed for its total phenolics and PA and the results are summarized in Table 3.8. The extract had 6.75% phenolics and 3.24% PA. Based on these analyses, the amount of PA in SB pulp was 1.17% (in dry matter). These values indicated that nearly 50% of soluble phenolics in the berry pulp were contributed by PA. Comparative evaluation of the results on PA from this study was not attempted due to the lack of similar reports for SB berries of different origin. Total PA content in the SB berry pulp (293 mg/100g) reported here was comparable with that of cranberry (418 mg/100g), blue berry (256 mg/100g) and blackcurrent (148 mg/100g), peach (67 mg/100g), green grapes (81 mg/100g) and black berry (27 mg/100g)**.** SB berries could therefore be a richer source for bioactive PA as compared to other edible fruits.

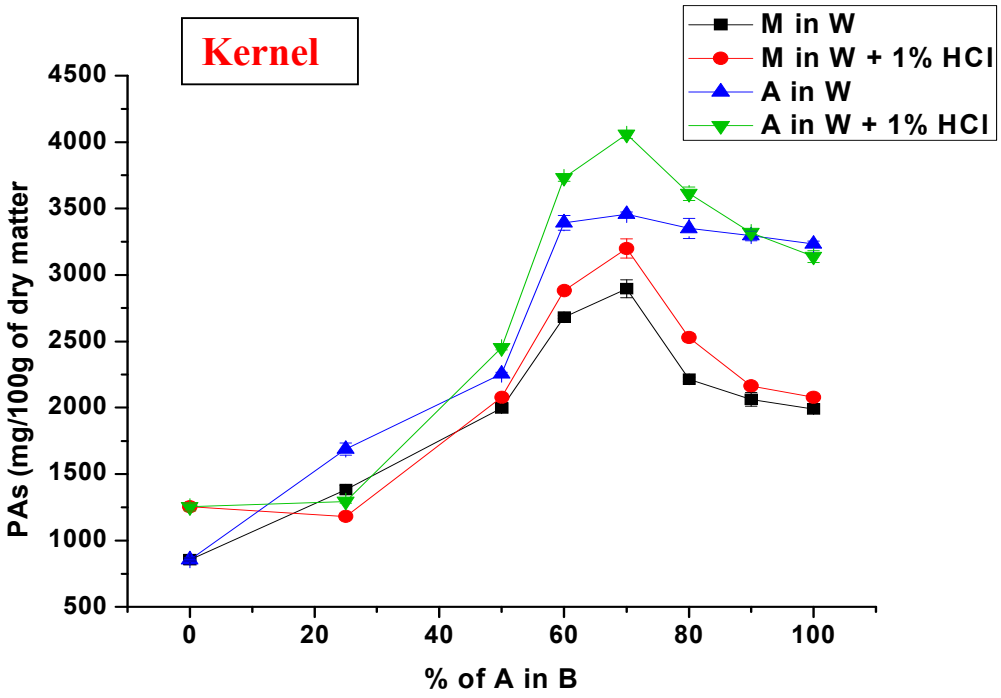
The seeds of SB berries were separated into seed coat and kernel, and extracted with 70% acetone containing 1% acid. Crude PA extract of the kernel contained significantly ($P > 0.05$) higher amount of polyphenols (22.6%) and PA (11.6%) than berry pulp (Table 3.8). Total soluble phenolics and PA in kernel were respectively 8.58 and 4.40%. Nearly half of the soluble phenolics in kernel were also contributed by PA. The seed coat PA extract had 30.9% polyphenols with 8.71% PA. The amount of soluble PA in seed coat was respectively 3.95 and 0.87% respectively. Although the seed coat extract contained higher amount of polyphenols than that in pulp and kernel extracts ($P > 0.05$), the PA content was lower than that in those extracts.

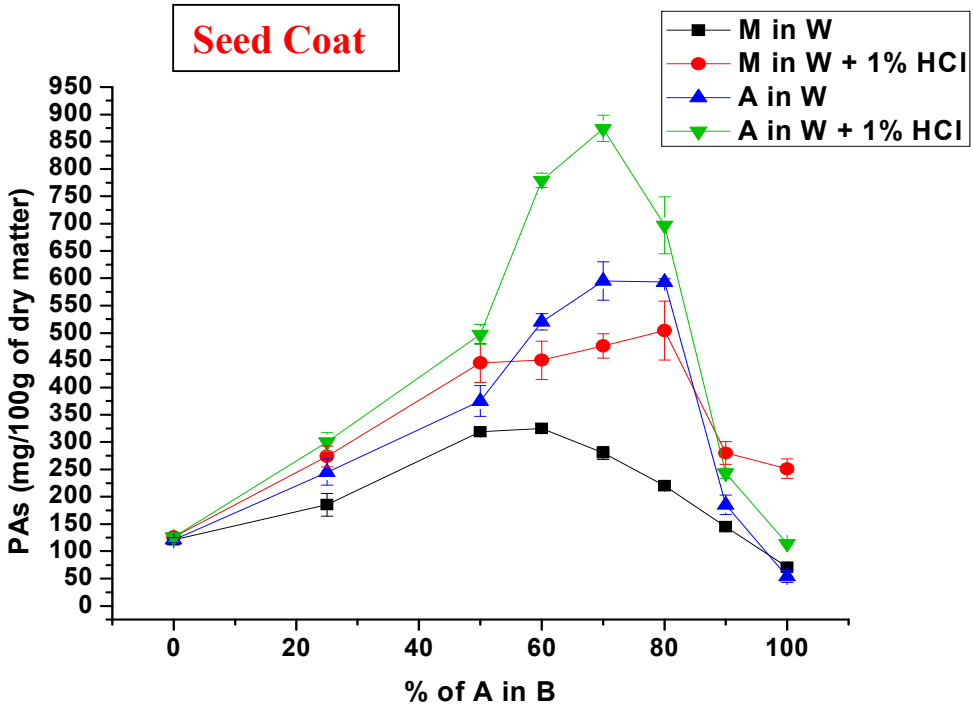
Figure 3.10. Effect of different solvents on the extraction of PAs from SB berry pulp, kernel, and seed coat.



M : methanol, **W:** water, **A:** acetone

Figure 3.10. Continuing





M : methanol, **W:** water, **A:** acetone

Purification of PA extracts: Since the PAs constitute a major fraction of polyphenols in SB berries, PAs were further purified and analysed. The crude PA extracts were purified using sephadex (LH-20) gel column chromatography. The yield of these purified fractions varied from 0.42 – 3.79% (Table 3.8). The total phenolic and PA in these purified extracts varied from 56-69% and 59 - 69% respectively. 30 - 60% of total PA present in crude extracts was found as recovered in purified extracts. These low recovery values could be attributed to the possible elution of low molecular weight PA with 95% ethanol (244). These purified extracts were used for the evaluation of degree of polymerization.

Average degree of polymerization: PA are mixtures of oligomers and polymers composed of flavan-3-ols mainly formed through $C_4 \longrightarrow C_8$ and/or $C_4 \longrightarrow C_6$ (B type) or linked through an ether bond between $C_2 \longrightarrow C_7$ (A type) and size of PA molecules is governed by their degree of polymerization. Butler et al; suggests that the reaction of vanillin with PA in acetic acid occurred only at the end groups without the depolymerisation of PA and the ADP can be calculated as the ratio of absorbance of the anthocyanins formed by the oxidative depolymerisation of PA to the absorbance of vanillin - PA adduct (212).

The values obtained for ADP of SB PA as estimated from monomer to polymer absorbance ratio showed seed coat PA showed the highest ADP of 8.2 followed by, berry pulp (7.4), and kernel (5.6). The ADP of kernel obtained was lower than the value (DP = 12) reported by Fan et al 2007, by the method of thiolysis, for the berries of Chinese origin (168). The difference could be due to the difference in methodologies adopted or due to the varietal or maturity differences of plant materials used. Lack of reports on the amount and nature of PA in SB berries make the comparison of the results obtained here difficult.

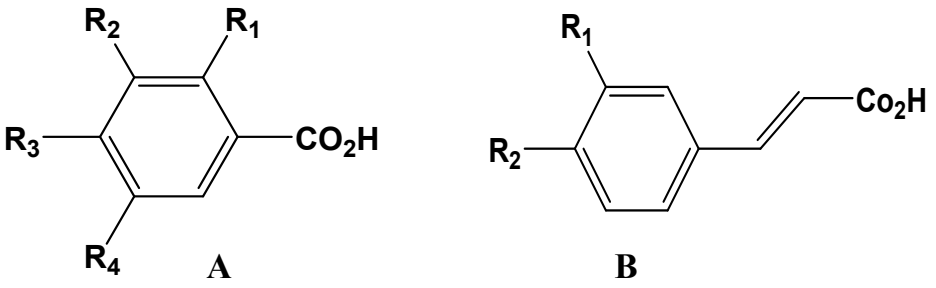
Table 3.8. Yield, total polyphenols and PAs in crude and purified PA extracts and ADP of PAs in SB berries (mean ± SD, n=5).

	Yield (%)[a]	Total polyphenolics (%)[b]	Proantho cyanidins (PA) (%)[c]	ADP of PAs
Pulp				
Crude PA Extract	36.2	6.75 ± 0.11	3.24 ± 0.54	-
Purified PA Extract	1.04	65.2 ± 0.54	68.04 ± 0.43	7.4 ± 0.6
Kernel				-
Crude PA Extract	38.3	22.6 ± 0.22	11.6 ± 0.91	-
Purified PA Extract	3.79	57.5 ± 1.31	69.8 ± 3.21	5.6 ± 0.8
Seed Coat				
Crude PA Extract	9.7	30.92 ± 2.31	8.71 ± 0.98	-
Purified PA Extract	0.61	56.05 ± 0.56	61.6 ± 2.38	8.2 ± 0.7

3.1.8.3 Phenolic Acids

Phenolic acids represent a major class of non-flavonoid plant phenolics. Phenolic acids include two main groups namely, hydroxybenzoic acid and hydroxycinnamic acid derivatives with different number and position of hydroxylation and methoxylation in aromatic ring (Figure 3.11). Phenolic acids are distributed as their free and bound forms in nature, more often bound forms occur as their esters and glycosides. High molecular weight phenolic acid glycosides constitute the class of hydrolysable tannins. There is no comprehensive report on the phenolic acid composition in SB berries. Therefore RP-HPLC-DAD analytical conditions for the profiling of SB phenolic acids were optimized and anatomical parts of the berries were analyzed in this study.

Figure 3.11. Phenolic acids



Hydroxybenzoic acids (A)					Hydroxycinnamic acids (B)		
R_1	**R_2**	**R_3**	**R_4**		**R_1**	**R_2**	
OH	H	H	H	**salicylic acid**	H	H	**cinnamic acid**
H	H	OH	H	**p-hydroxybenzoic acid**	H	OH	**p-coumaric acid**
H	OH	OH	H	**3,4-dihydroxybenzoic acid**	OH	OH	**caffeic acid**
H	OH	OMe	H	**vanillic acid**	OH	OMe	**ferulic acid**

Standardization and validation of HPLC-DAD method

Chromatographic conditions: RP-HPLC-DAD conditions for the qualitative and quantitative profiling of nine major phenolic acids viz; gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, salicylic acid, caffeic acid, p-coumaric acid, ferulic acid and cinnamic acid in SB berries were optimized for the first time. Phenolic acids are polar in nature; therefore different proportions of methanol and water were tried as mobile phase with reverse stationary phase in this study. Variations in pH of mobile phase are reported to have significant effect on the resolution and tailing of polar compounds in reverse stationary phase. Presence of acids in mobile phase can achieve better separation for phenolic acids, since it reduces the ionization of phenol, phenolic hydroxyl and carboxylic acid groups (246, 247). Higher concentration of acetic acid in mobile phase may result better separation, which in turn would shorten the life of reverse phase column. A gradient of

2% acetic acid in water and methanol was optimized as mobile phase and T_R of reference phenolic acids were obtained and the results are presented in Figure 3.12 and Table 3.9.

DAD is capable of monitoring several wavelengths simultaneously, so as to ensure that all uv-visible absorbing components are detected. HPLC-DAD can therefore be used for recording uv-visible spectro-chromatograms of compounds. Chromatograms at different wave lengths as obtained by HPLC-DAD under the optimized conditions are given in Fig 3.12. The wavelengths at which the compounds had maximum absorbance or maximum peak/noise ratio were selected as their detection wavelength and are summarized in Table 3.9. Vanillic acid and caffeic acid were found to be eluted closely, but the differences in their absorption maxima could be utilized for identifying them from each other. Vanillic acid had highest absorbance at 270 nm and that was considerably lower at 329 nm, the detection wavelength of caffeic acid. Therefore, these compounds could be differentiated by comparing their chromatograms recorded at 270 nm and 329 nm (Fig 3.12).

System suitability tests: Regression equations were obtained by the external standard method. A series of standard solutions of each of phenolic acids were prepared and linearity between peak area and concentration was studied. The linear range, regression equation and correlation coefficient of each analytes were summarized in Table 3.9. All the components showed good linearity ($R \geq 0.9893$) in a relatively wide concentration range. The LOD and LOQ of the method were evaluated and the values suggested that sensitivity was satisfactory (Table 3.9).

` A solution mixture containing 50 µg/mL of each phenolic acids was analyzed 5 times within a day to determine the intra day precision of the T_R

and peak area by the method. The inter day precisions were determined by 6 analysis over two days for each phenolic acids (50 µg/mL). The relative standard deviation (RSD) was taken as a measure of precision and the intra day and inter day precisions for the T_R and peak areas by the method are summarized in Table 3.10. The RSD for the T_R (< 1.8) and peak area (< 2.2) were found to be low and inter day and intra day precisions were satisfactory for all analytes. A reliable accuracy (97.4 – 103.9%) was also shown by this method (Table 3.10). Reference phenolic acids were added to the phenolic acid fractions and analyzed so as to determine the recovery and it was found that the recovery of all phenolic acids was between 94.3 – 101.9% (Table 3.10). For stability test, the same sample solutions kept at 4^0 C was analyzed every 12 h and the analytes were found to be rather stable with in 48 h.

Phenolic acid composition of *H. rhamnoides* berries: SB berries as pulp, kernel and seed coat were analyzed for their free and bound phenolic acids content. Phenolic acids in the berries and leaves were separated into three fractions, viz; phenolic acids present in free form, phenolic acid liberated from esters and phenolic acid liberated from glycosides and profiled by HPLC for their phenolic acid composition. Peaks in the chromatograms were identified by comparing with the T_R and uv-visible spectra of reference compounds and by spiking experiments. The amount of each analyte was calculated from the corresponding calibration curve and represented as their mean ± SD of three analyses (Table 3.11, Figure 3.13).

Composition of free and bound phenolic acids in the dry matter of berry pulp is summarized in Table 3.11. Berry pulp contained 1068 mg/kg of total phenolic acids, of which 58.8% was derived from phenolic glycosides. Free and phenolic acids bound to esters respectively constituted 20.0 and 21.2 % of total phenolic acids. Gallic acid was identified as the predominant phenolic acid both in free and bound forms, which accounted for 66.0% of

total phenolic acids in pulp. Considerable amounts of protocatechuic acid (136 mg/kg), ferulic acid (69 mg/kg), salicylic acid (54 mg/kg), p-hydroxybenzoic acid (40 mg/kg) and p-coumaric acid (37 mg/kg) were found to be present in pulp. Presence of vanillic acid (7 mg/kg), cinnamic acid (12 mg/kg) and caffeic acid (8 mg/kg) were also detected in pulp.

Seeds were separated into kernel and seed coat and analyzed for their free and bound phenolic acids composition and the values on dry matter are summarized in Table 3.11. The total phenolic acid content in kernel (5741 mg/kg) was higher than those in berry pulp and seed coat (Table 3.11). Phenolic acids liberated from soluble esters (57.3% of total phenolic acids) were the most abundant phenolic acid fraction in kernel. Free and bound phenolic acids liberated from glycosidic bonds respectively contributed 8.4% and 34.3% of total phenolic acids. The total phenolic acid content in the kernel was two times higher than those reported for sesame and cotton seed, and slightly lower than the values reported for rapeseed and canola meals, and flax seed. Gallic acid (3441 mg/kg), protocatechuic acid (1330 mg/kg), p-hydroxybenzoic acid (265 mg/kg), vanillic acid (368 mg/kg) and ferulic acid (156 mg/kg) were the major phenolic acids present in kernel. Both free and bound phenolic acid profile contained gallic acid as the predominant phenolic acid, which contributed 59.9% of total phenolic acids in kernel. In addition to this presence of cinnamic acid (82 mg/kg), p-coumaric acid (74 mg/kg) and caffeic acid (25 mg/kg) were also detected in kernel.

The total soluble phenolic acids content in seed coat (448 mg/kg) was lower than that in kernel and pulp (Table 3.11). Proportion of free phenolic acids in total phenolic acids in seed coat was higher than that in kernel and pulp. Phenolic acids bound to esters and glycosides respectively contributed 49.1% and 20.3% of total phenolic acids in seed coat. Gallic acid (230 mg/kg), protocatechuic acid (82 mg/kg), cinnamic acid (44 mg/kg), vanillic

acid (39 mg/kg), p-hydroxybenzoic acid (26 mg/kg), ferulic acid (17 mg/kg) and caffeic acid (10 mg/kg) were identified in seed coat. Protocatechuic acid (43 mg/kg) was present as the major phenolic acid of the free phenolic acid fraction, whereas gallic acid (200 mg/kg) was the major in bound phenolic acid fractions in seed coat. Gallic and protocatechuic acids respectively accounted for 51.3% and 18.3% of total phenolic acids in the seed coat.

Detailed reports on the composition of phenolic acids in anatomical parts of SB berries are limited. Rosch et al 2003; reported the amount of gallic acid (1.-2.6 mg/mL) and protocatechuic acid (2.1-2.9 mg/mL) in SB berry juice (*H. rhamnoides,* collected from Germany) and their contribution to its total AO capacity (164). Hakinen et al; reported the proportion of p-coumaric acid, ferulic acid, p-hydroxybenzoic acid and ellagic acid in total phenolic content of SB (*H. rhamnoides*) berries from Finland (248). In another report on SB berries (*H. rhamnoides*) of Polish origin, salicylic acid was identified as the predominant phenolic acid both in free and bound forms by GC-MS analysis (170). Presence of gallic acid in SB leaves is previously reported (249). In these reports, the SB berries were analyzed as whole for the phenolic acid composition and their distribution of phenolic acids among the anatomical parts of berries as done in the present study was not discussed in detail. In our study free and bound phenolic acid composition of pulp, seed coat and kernel of SB berries were separately studied for the first time. The major fraction (Approx. 70%) of phenolic acids in SB berries was found to be concentrated in the seeds. The report of salicylic acid as the predominant phenolic acid in SB berries from Poland (170) is contrary to the present study, where gallic acid was found as the major phenolic acid both in pulp and seeds of berries of Indian origin. The berry pulp in this study contained only 54 mg/kg of salicylic acid, which contributed 5.06% of total phenolic acids in pulp. These results warrant the detailed studies on the phenolic composition of SB berries from different varieties and geo-climatic regions.

Table 3.9. Detection wave length, T_R and linear regression data of phenolic acids.

Compound	Detection wavelength (nm)	T_R (Min)	Regression equation[a]	Linear range (µg mL^{-1})	Correlation coefficient	LOD[b] (µg mL^{-1})
Gallic acid	280	8.4	$Y = 1.298 \times 10^5 x + 187$	10.8– 150.2	0.9992	1.20
Protocatechuic acid	260	16.8	$Y = 0.991 \times 10^5 x - 1101$	8.0–220.4	0.9995	0.72
p-hydroxybenzoic acid	255	25.6	$Y = 1.894 \times 10^5 x - 548$	12.5 - 175	0.9924	2.78
Vanillic acid	260	31.8	$Y = 1.374 \times 10^5 x + 413$	13.2 - 150	0.9893	1.97
Salicylic acid	300	42.5	$Y = 0.844 \times 10^5 x + 1332$	12.4–175.2	0.9961	2.87
Cinnamic acid	275	49.8	$Y = 1.282 \times 10^5 x + 990$	10.1–166.8	0.9971	1.77
Caffeic acid	325	32.4	$Y = 2.307 \times 10^5 x + 14489$	6.6–208.5	0.9939	1.04
Ferulic acid	310	39.2	$Y = 2.357 \times 10^5 x - 15307$	5.0–220.0	0.9981	1.65
p-Coumaric acid	325	40.8	$Y = 2.443 \times 10^5 x + 1495$	10.4–240.8	0.9903	0.65

[a] x : peak area, y : concentration (mg/mL).
[b] Detection was expressed as LOD = 3.3s/a, where a is the slope, and s is residual standard deviation of regression line

Table 3.10. Precision, accuracy and recovery of phenolic acids.

Compounds	Precision (RSD %)[a]				Accuracy (%)[d]	Recovery[e] (%)
	Intra day[b]		Inter day[c]			
	RT	peak area	RT	peak area		
Gallic acid	0.92	1.62	1.12	2.05	99.6 ± 0.8	92.5 ± 1.2
Protocatechuic acid	0.72	1.71	1.33	1.98	102.3 ± 1.0	94.3 ± 2.1
p-hydroxybenzoic acid	0.85	1.25	1.28	1.79	98.1 ± 0.9	102.5 ± 1.9
Vanillic acid	1.01	1.38	1.78	2.23	101.7 ± 2.1	98.2 ± 1.8
Salicylic acid	1.09	1.72	1.12	1.87	103.9 ± 0.4	95.9 ± 1.1
Cinnamic acid	1.13	1.41	1.36	1.08	98.9 ± 0.8	98.7 ± 1.6
Caffeic acid	0.97	0.82	1.61	0.85	98.8 ± 1.9	98.1 ± 1.9
Ferulic acid	0.78	1.66	1.19	0.97	100.6 ± 1.4	96.6 ± 2.2
p-Coumaric acid	1.09	1.34	1.06	1.28	97.4 ± 0.6	97.2 ± 1.8

[a] RSD% = ((SD/mean) X 100), [b] n = 5, [c] n = 6. [d] Mean (Found/nominal)X100% (n=6) ± SD, at medium analyte concentration of 50 μg mL^{-1}. [e] Mean ± SD (n = 3).

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Table 3.11. Free and bound phenolic acids in berry pulp, kernel and seed coat (mg/kg of dry matter) (mean ± SD (n = 3).

Phenolic acids			Hydroxybenzoic acid derivatives					Hydroxycinnamic Acid Derivatives				Total
			GA	Pro-CA	p-HBA	V A	S A	CA	P-CA	FA	Caf. A	
Pulp	Free		88 ± 5.7	33 ± 2.5	8 ± 1.8	5 ± 0.4	46 ± 1.2	7 ± 0.2	11 ± 1.8	13 ± 2.9	3 ± 0.4	214 ± 9.6
	Bound	Esters	152 ± 1.0	23 ± 0.6	8 ± 3.2	1 ± 0.4	5 ± 0.8	5 ± 0.2	12 ± 0.3	18 ± 0.1	2 ± 0.3	226 ± 4.3
		glycosides	465 ± 0.3	80 ± 0.1	24 ± 1.0	1 ± 0.1	3 ± 0.3	-	14 ± 4.8	38 ± 1.9	3 ± 0.1	628 ± 2.5
	Total		705	136	40	7	54	12	37	69	8	1068
Kernel	Free		329 ± 16.2	99 ± 12.1	28 ± 3.9	5 ± 0.2	ND	- 5 ± 0.3	5 ± 0.4	10 ± 0.8	3 ± 0.3	484 ± 21.6
	Bound	Esters	2198 ± 221.4	690 ± 77.7	144 ± 5.7	47 ± 7.5	ND	52 ± 3.2	65 ± 12.7	81 ± 4.6	12 ± 0.9	3289 ± 236.9
		glycosides	914 ± 18.0	541 ± 12.1	93 ± 6.7	316 ± 34.3	ND	25 ± 1.0	4 ± 0.0	65 ± 2.2	10 ± 9.4	1968 ± 123.3
	Total		3441	1330	265	368	ND	82	74	156	25	5741
Seed coat	Free		30 ± 5.3	43 ± 10.8	17 ± 4.7	12 ± 4.5	ND	19 ± 1.2	ND	15 ± 2.2	1 ± 0.4	137 ± 5.6
	Bound	Esters	164 ± 8.3	27 ± 0.6	7 ± 0.8	5 ± 0.8	ND	11 ± 0.1	ND	2 ± 0.2	4 ± 0.1	220 ± 5.6.
		glycosides	36 ± 2.4	12 ± 0.7	2 ± 0.3	22 ± 0.3	ND	14 ± 0.7	ND	-	5 ± 0.2	91 ± 2.5
	Total		230	82	26	39	ND	44	ND	17	10	448
Leaves	Free		689 ± 13.6	16 ± 1.8	144 ± 6.6	17 ± 1.7	ND	122 ± 0.5	1 ± 0.0	44 ± 2.9	18 ± 1.9	1051 ± 30.2
	Bound	Esters	2899 ± 23.2	19 ± 1.6	36 ± 4.3	6 ± 0.0	ND	54 ± 5.2	ND	119 ± 1.6	ND	3133 ± 17.7
		glycosides	634 ± 0.2	15 ± 0.4	67 ± 0.7	14 ± 0.9	ND	62 ± 1.8	ND	12 ± 0.5	ND	804 ± 5.9
	Total		4222	50	247	37	ND	238	1	175	18	4988

Figure 3.12 HPLC-DAD chromatograms of phenolic acids at different wavelengths.

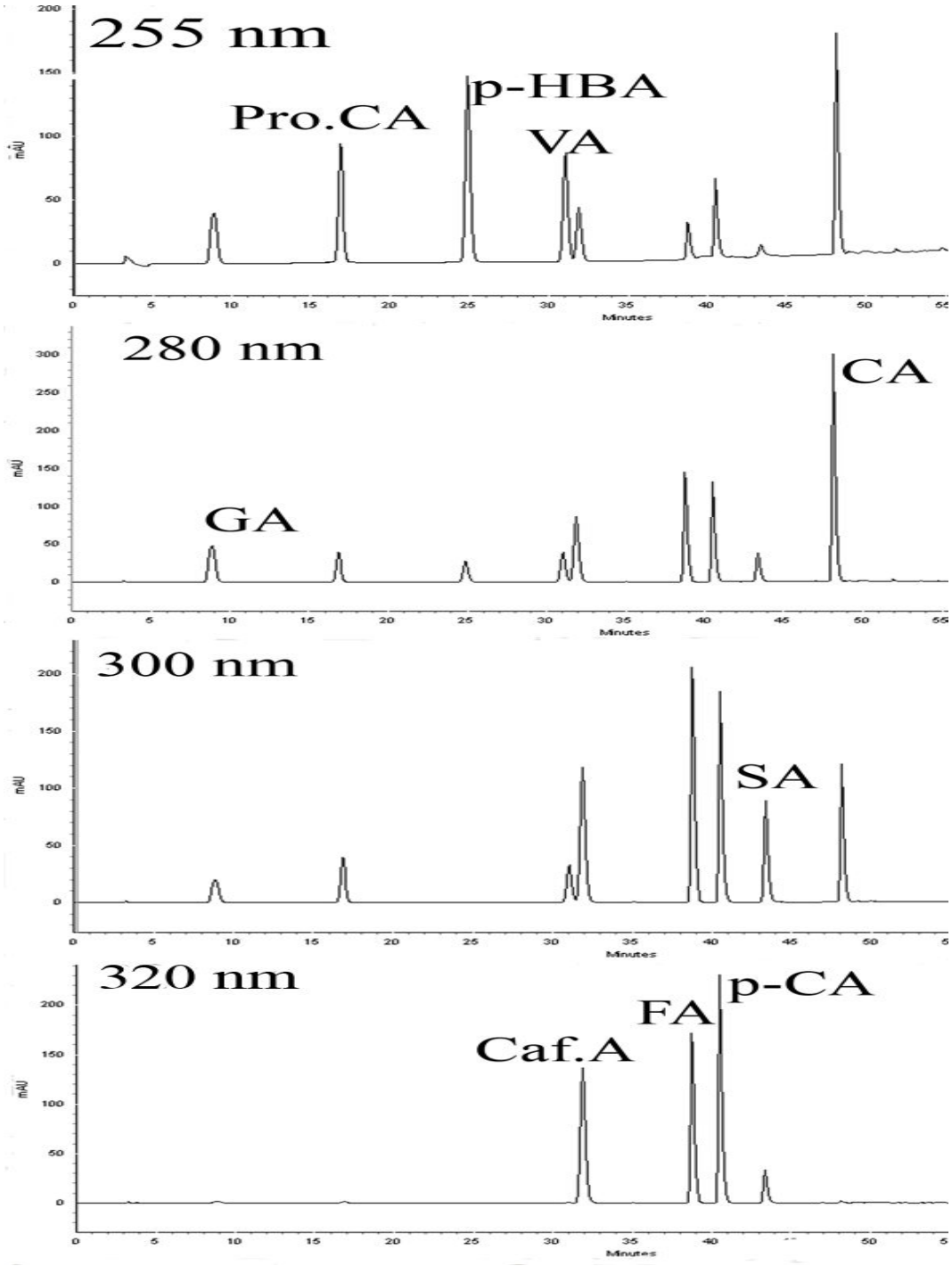

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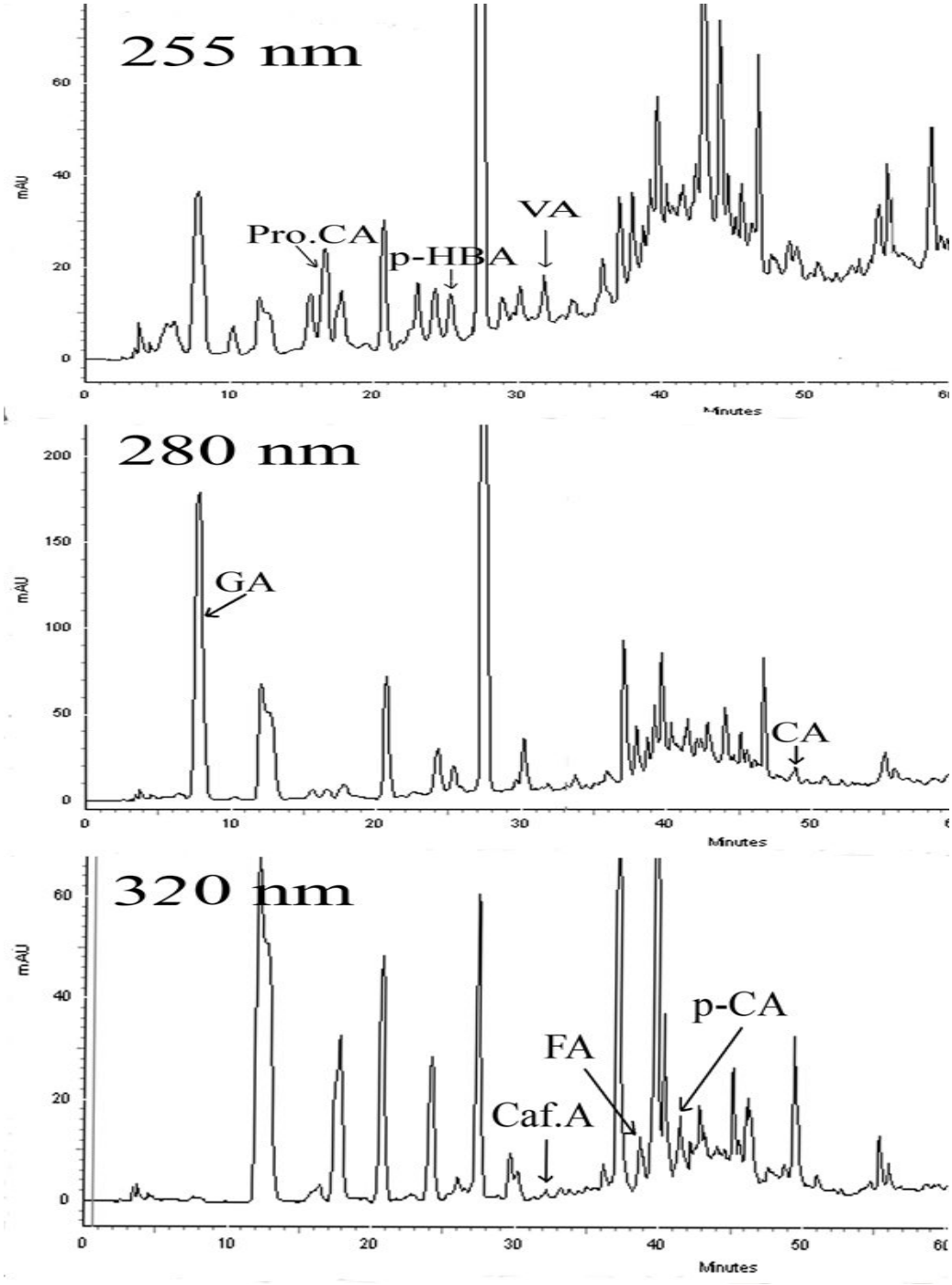
Figure 3.13. HPLC-DAD chromatograms of phenolic acids in *H. rhamnoides* kernel extract at different wavelengths.



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This is the first comprehensive report on the chemical evaluation of *H rhamnoides* berries of Indian origin. Detailed studies with large number of samples of different species and from different geo-climatic region are required for the complete chemical finger printing of Indian SB. Comparison of berries belonging to major species of SB in India is attempted in section 3.2

3.2. Phytochemical Profiling of Berries of Major SB Species in Indian Trans-Himalayas

In India, SB is abundantly grown in the Trans-Himalayan cold deserts such as Ladakh, Lahaul, and Spiti at altitudes from 2500 to 4500 m. The Ladakh region extends between 32° 15'–36° N latitude and 75° 15'–80° 15' E longitude; Spiti and Lahaul lie between 31° 44'–32° 59' N latitude and 76° 46'–78° 41' E longitude. *H. rhamnoides, H. salicifolia*, and *H. tibetana* are the predominant SB species found in Indian Himalayas. *H. rhamnoides* is widely distributed in the Trans-Himalayan region, but *H. salicifolia* and *H. tibetana* have limited distribution and are observed only in the Lahaul and Spiti regions, respectively (130,138,250). The SB-growing areas in Trans-Himalayas host unique geoclimatic conditions of high altitude coupled with extreme temperature variations (–40 to 30°C), low precipitation, and low oxygen in air. In these cold deserts plants are under extreme climatic stress and are expected to have a distinct phytochemical profile. No systematic studies have been carried out from this perspective on Himalayan SB berries, particularly on *H. salicifolia* and *H. tibetana*, which are exclusive to high altitudes of Himalayas. This chapter deals with the chemical composition of berries belonging to three major SB species (*H. rhamnoides, H. salicifolia*, and *H. tibetana*) from different locations in Indian Trans-Himalayan region. Nutritional quality of these berries was compared in terms of their proximate composition, polyphenol, flavonoid, and vitamin C contents and lipid profile.

Table 3.12; Seed content and proximate composition of SB berries of *H .rhamnoides, H. salcifolia and H. tibetana* species from India (Mean ± S D, n=3).

SB species	Geographic location	Seed (%)	Moisture (%)	Pulp oil (%)	Ash (%)	Protein (%)
H. rhamnoides	Lahaul-1	10.4	74.4±0.5	3.08±0.22	1.05±0.02	2.25±0.62
	Lahaul-2	8.1	74.4±0.4	2.99±0.32	0.79±0.14	1.86±0.59
	Lahaul-3	9.7	72.7±0.2	3.04±0.26	0.93±0.19	2.11±0.45
H. rhamnoides	Spiti-1	7.9	76.9±0.7	3.38±0.45	0.60±0.29	2.17±0.49
	Spiti-2	7.4	74.6±0.5	3.14±0.23	0.73±0.09	2.15±0.34
	Spiti-3	9.3	67.2±1.3	3.38±0.17	0.99±0.14	2.44±0.19
H. rhamnoides	Ladakh-1	9.8	72.3±0.9	3.60±0.15	0.89±0.12	2.12±0.25
	Ladakh-2	8.5	72.1±0.5	3.52±0.31	0.79±0.11	1.99±0.23
	Ladakh-3	8.6	73.4±0.4	3.61±0.25	0.92±0.09	2.34±0.39
H. salicifolia	Lahaul-1	3.4	85.8±1.0	1.49±0.20	0.26±0.06	1.13±0.29
	Lahaul-2	3.7	82.3±0.9	1.53±0.15	0.40±0.08	1.54±0.38
	Lahaul-3	4.1	83.5±0.6	1.65±0.28	0.39±0.02	1.62±0.32
H. tibetana	Spiti-1	9.0	74.5±0.4	2.25±0.56	1.02±0.10	3.09±0.13
	Spiti-2	8.8	73.2±1.1	2.45±0.32	0.96±0.09	2.98±0.22
	Spiti-3	8.7	72.9±0.9	2.50±0.19	0.87±0.12	2.96±0.29

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3.2.1 Proximate composition of berries.

The proximate composition of fresh berries of the three different species is summarized in Table 3.12. The moisture content of fresh berries of *H. tibetana* and *H. rhamnoides* was in the range of 67–77%, and in *H. salicifolia* was 82–86%. Seeds constituted 7.4–10% of the fresh berries in *H. tibetana* and *H.* rhamnoides and 3.4–4.1% in *H. salicifolia.* This showed *H. salicifolia* had higher proportion of juicy pulp than the other two species. In these berry samples, the oil content of the pulp of *H. rhamnoides* contained higher amounts of oil (3.0–3.6% of fresh berries) than for *H. tibetana* (2.2–2.5%) and *H. salicifolia* (1.5–1.6%). The ash content of *H. rhamnoides* and *H. tibetana* (0.60 – 1.05% of berries) was also higher than in *H. salicifolia* (0.26 – 0.40%). Total protein content in the pulp of the berries was found to be higher for *H. tibetana* (2.96–3.09% of fresh berries) than for *H. rhamnoides* (1.86–2.44%) and *H. salicifolia* (1.13–1.62% of berries). In general, proximate composition of SB berries varied from species to species. Among the studied samples only *H. rhamnoides* was obtained from different geographical locations. Not much variation was observed in the proximate composition among *H. rhamnoides* berries collected from different geographical locations.

3.2.2 Vitamin C content in berries

SB berries are well known for their extraordinarily high levels of vitamin C, which is considered as an important quality parameter of SB berries. Several authors have reported high vitamin C content in SB berries (3600 – 25000 mg/kg) (127). The studied berries of Indian origin were also found to contain high amount of vitamin C with wide range (1444 – 29840 mg/kg) (Table 3.13). Vitamin C content in the berries of *H. salicifolia* (22,979–29,840 mg/kg) and *H. tibetana* (8,789–9,279 mg/kg) were much higher than that in the common species

H. rhamnoides (1,444–4,877 mg/kg) (Table 3.13). Vitamin C content in the *H. salicifolia* was particularly interesting due to its significantly high value.

Extensive variations in vitamin C content in *H. rhamnoide*s berries have been reported among individuals, populations and subspecies. The vitamin C concentration ranging from 28 to 310 mg/100g of berries has been reported in the European ssp. *rhamnoides* (251). Wide ranges of vitamin C content have been reported for berries of Russian cultivars belonging to ssp. *mongolic*a (40 to 300 mg/100g of berries) and ssp. *fluviatilis* (460 to 1330 mg/100g of berries) (252). Chinese cultivars of *ssp. sinesnsis* have a vitamin C content of 200 to 2500 mg/100g of berries (253-255).

3.2.3. Polyphenol content in berries:

Total phenolics in the berries was determined using F-C reagent method and tabulated in Table 3.13. The amount of the phenolics in pulp of the berries ranged from 3950 to 6719 mg/kg of fresh berries. Among the studied samples *H. salcifolia* berry pulp contained highest amount of phenolics (5913 – 6719 mg/kg) followed by *H. tibetana* (5719 – 5893 mg/kg) and *H. rhamnoides* (3956 – 5728 mg/kg). *H. rhamnoides* berries from Spiti region had lower amount of phenolics in their pulp than those from Ladakh and Lahaul regions. The values obtained for the phenolics content in Indian SB berries was in accordance with the *H. rhamnoides* berries of Chinese and Polish origin (158, 170). There are no reports on the phenolics content of *H. salcifolia* and *H. tibetana* berries.

Table 3.13. Vitamin C, polyphenol and flavonol content of SB berries of *H.rhamnoides, H. salcifolia and H. tibetana* Species from India (Mean ± S D, n=3).

SB species	Geographic location	Vitamin C (mg/kg)	Polyphenols (mg/kg)	Flavonols (mg/kg)
H. rhamnoides	Lahaul-1	2924 ± 36	5646 ± 16	308 ± 12
	Lahaul-2	2305 ± 29	5213 ± 32	285 ± 21
	Lahaul-3	4877 ± 19	5138 ± 19	212 ± 49
H. rhamnoides	Spiti-1	1866 ± 17	3956 ± 21	122 ± 20
	Spiti-2	1444 ± 36	4132 ± 39	156 ± 17
	Spiti-3	1666 ± 23	4018 ± 17	180 ± 9
	Ladakh-1	2145 ± 53	5319 ± 51	251 ± 25
H. rhamnoides	Ladakh-2	2568 ± 42	5728 ± 19	291 ± 17
	Ladakh-3	2425 ± 36	5219 ± 72	261 ± 22
	Lahaul-1	27692 ± 91	6019 ± 35	428 ± 51
H. salicifolia	Lahaul-2	29840 ± 185	5913 ± 42	353 ± 16
	Lahaul-3	22979 ± 78	6719 ± 33	398 ± 9
	Spiti-1	9279 ± 79	5893 ± 47	392 ± 12
H. tibetana	Spiti-2	8789 ± 65	5719 ± 35	401 ± 15
	Spiti-3	89845 ± 45	5811 ± 27	342 ± 14

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3.2.1. Flavonoids: Aluminium chloride forms complexes with flavonols in a quantitative manner resulting a bathchromic shift in band II absorption of flavonols (207). This property is widely used for the high through put quantification of flavonols. Flavonols contribute a major fraction of polyphenols in berry pulp. Total flavonol content in the berry pulp was determined using aluminium chloride method and tabulated in Table 3.13**.** *H. salicifolia* (353 – 428 mg/kg) and *H. tibetana* (342 – 401 mg/kg) had slightly higher amount of flavonols than in *H. rhamnoides* (122 – 308 mg/kg). *H. rhamnoides* collected from Spiti region had slightly lower amount of flavonols than those from Lahaul and Ladakh. The amount of flavonols (122–428 mg/kg) in the berries of these three species was also found to be slightly higher than the reported amounts of flavonols in cranberry (100–263 mg/kg), bog whortleberry (184 mg/kg), lingonberry (74–146 mg/kg), blackcurrant (115 mg/kg), and crowberry (102 mg/kg).

The results presented here indicate that the chemical profile of H. salicifolia, with a lower seed content, lower levels of fatty matter, and high levels of vitamin C and flavonoids, differed perceptibly from the other two. The chemical properties of H. salicifolia and H. tibetana have not been well documented until now.

3.2.4. Composition of pulp oil.

Nutritional quality of the SB pulp oil is attributed to its high carotenoid and tocopherol content along with unique fatty acid profile. Nutritional quality of the pulp oil from the three species of SB was evaluated in terms of their chemical composition.

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Table: 3.14. FA composition of pulp oils of SB berries from *H.rhamnoides, H. salcifolia and H. tibetana* Species from India (Mean ± S D, n=3).

S.B species	Geographic location	FA (wt%)						
		14:0	16:0	16:1	18:0	18:1	18:2	18:3
H. rhamnoides	Lahaul-1	0.5 ± 0.1	28.4 ± 1.1	50.3 ± 1.2	0.6 ± 0.0	11.3 ± 1.7	8.1 ± 0.3	1.3 ± 0.9
	Lahaul-2	0.6 ± 0.0	29.4 ± 0.7	50.1 ± 1.1	0.7 ± 0.1	9.7 ± 0.6	7.9 ± 0.2	1.6 ± 0.7
	Lahaul-3	0.5 ± 0.0	28.3 ± 1.1	49.7 ± 0.9	0.5 ± 0.0	11.5 ± 1.6	8.5 ± 0.4	1.7 ± 0.8
H. rhamnoides	Spiti-1	0.6 ± 0.2	35.8 ± 1.2	45.6 ± 2.3	0.5 ± 0.1	9.8 ± 1.5	8.2 ± 0.2	0.8 ± 0.1
	Spiti-2	0.3 ± 0.1	35.8 ± 1.1	45.6 ± 1.9	Tr	9.8 ± 1.3	8.2 ± 0.8	0.8 ± 0.1
	Spiti-3	0.6 ± 0.0	30.7 ± 2.6	51.8 ± 0.9	Tr	12.3 ± 0.6	5.9 ± 1.0	0.4 ± 0.0
H. rhamnoides	Ladakh-1	0.4 ± 0.0	33.8 ± 1.5	46.4 ± 2.0	1.0 ± 0.1	13.4 ± 0.94	5.4 ± 1.3	0.4 ±1.3
	Ladakh-2	0.9 ± 0.1	31.2 ± 1.4	49.1 ± 3.1	0.8 ± 0.0	13.9 ± 3.49	4.6 ± 0.6	Tr
	Ladakh-3	0.8 ± 0.1	30.9 ± 1.3	45.6 ± 2.6	0.9 ± 0.0	16.8 ± 1.73	5.8 ± 0.4	0.8 ± 0.4
H. salicifolia	Lahaul-1	0.3 ± 0.1	29.0 ± 3.1	32.9 ± 0.5	0.1 ± 0.0	17.6 ± 0.5	16.1 ± 0.9	0.6 ± 0.1
	Lahaul-2	0.1 ± 0.0	28.9 ± 0.8	41.2 ± 1.2	0.7 ± 0.1	12.6 ± 1.1	14.3 ± 1.8	1.0 ± 0.0
	Lahaul-3	0.2 ± 0.1	28.0 ± 1.9	35.4 ± 3.0	0.6 ± 0.2	13.1 ± 1.3	15.0 ± 1.3	0.8 ± 0.2
H. tibetana	Spiti-1	1.1 ± 0.2	25.7 ± 0.5	32.1 ± 4.2	0.5 ±0.1	26.0 ± 1.9	9.3 ± 1.1	5.2 ± 1.0
	Spiti-2	0.9 ± 0.3	28.5 ± 2.4	34.1 ± 2.3	0.4 ± 0.0	22.2 ± 2.8	9.2 ± 0.5	3.5 ± 0.6
	Spiti-3	0.8 ± 0.1	26 ± 1.8	36.0 ± 1.4	0.4 ± 0.2	23.5 ± 2.4	8.3 ± 0.8	5.6 ± 0.9

3.2.4.1. FA composition. The FA compositions of pulp oil of the three species of berries are summarized in Table 3.14. In the pulp oil, the dominating FAs were palmitoleic, palmitic, oleic, linoleic, and linolenic acids. The highest variations among the three species were observed in the proportion of palmitoleic acid (32–50%). *H. rhamnoides* contained the highest proportion of this rare FA in the pulp oil (45–50%). Variation in the next highest FA, palmitic acid, was in the range of 25–36% in the pulp oil from the three species. The proportion of oleic acid (22–26%) in pulp oil of *H. tibetana* was higher than that of *H. salicifolia* (13-18%) and *H. rhamnoides* (10–17%). The proportion of palmitoleic acid in the pulp oils of the studied berries was found to have a negative correlation with that of oleic acid. The average amount of palmitoleic acid was highest in *H. rhamnoides* (48.2%) followed by *H. salicifolia* (36.5%) and *H. tibetana* (34.0%) pulp oils and the amount of oleic acid followed the reverse order (11.7, 14.4 and 23.6% respectively). The proportion of palmitoleic acid correlates negatively with that of oleic acid. The highest level of palmitoleic acid (up to 54%), and the lowest level of oleic acid (as low as 2%) are reported for *subsp. mongolica* (145,148). In the present study the proportion of ALA was found to be slightly higher in *H. tibetana* (3.5–5.6%) than that in the other two species. Traces (>1%) of myristic and stearic acids were also present in all three species. Trace amounts of arachidic acid (20:0) were observed in pulp oil of *H. salicifolia* (data not shown).

This is the first report on the FA profile of SB berries of Indian origin. The variations in the FA profile of pulp oils from the berries belonging to different species and geographical regions are previously reported by many authors (148,150,173,256,257,). Large variations were observed in the proportions of palmitoleic, palmitic and oleic acids. For example Kallio et al; reported that *H. rhamnoides* berries of ssp. *mongolica* contained less oleic acid

(4.6 v/s 20.2%) and more palmitic (33.9 v/s 27.4%) and palmitoleic (32.8 v/s 21.9%) acids than those of ssp. *sinesis* (171). According to Abid et al; *H. rhamnoides* berries from Northern Pakistan contained almost equal amounts of palmitic (34.5%) and palmitoleic (33.4%) acids as predominant FA (175). The results of the present study showed palmitoleic acid as the predominant FA in the pulp oil of Indian berries with extreme variations from 32 to 50%. In considering the therapeutic value of this rare FA, these results indicate the potential of breeding and industrial application to evolve palmitoleic acid rich varieties.

3.2.4.2. Carotenoids. Carotenoids in the soft parts impart beautiful yellow orange color to SB berries. The amounts of total carotenoids in the pulp oils of berries were quantified and expressed in terms of β-carotene (Table 3.15). The amount of carotenoids in pulp oils of these species varied from 700 to 3500 mg/kg. The carotenoid content of pulp oils of *H. rhamnoides* (2350 – 3420 mg/kg) and *H. tibetana* (2693 – 3166 mg/kg) were similar, and *H. salicifolia* had the lowest amount (692 – 840 mg/kg). The greenish yellow color of *H. salicifolia* berry itself indicated its low carotenoid content. Carotenoid content in the pulp oil of *H. rhamnoides* berries from Ladakh (2350 – 2650 mg/kg) was slightly lower than that from the Lahaul (2660 – 3420 mg/kg) and Spiti (2576 – 2820 mg/kg) regions. The carotenoid content in *H. rhamnoides* was comparable with the values reported for the berries of Chinese origin (129). However, authentic reports on the carotenoid content in *H. tibetana* and *H. salicifolia* from other geographical areas are not available. β-Carotene as the major component in SB pulp oils as well as α-carotene, γ-carotene, dihydroxy carotene, lycopene, and xeaxanthin has been reported (258). Carotenoid content in the berries are subjected to extreme variation; differences up to 10-fold has been reported even with in the same natural population and subspecies. Levels of total carotenoids from 1-120 mg/kg of fresh berries have been reported in the literature.

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3.2.4.3. Tocopherols/tocotrienols.

Tocols in pulp oil were quantified by HPLC, and the results are given Table 3.15. The amount of tocols in pulp oil varied from 600 to 1800 mg/kg among the three species studied. *H. salicifolia* contained the least amount of tocols (666 – 902 mg/kg), as compared with *H. rhamnoides* (1301 – 1788 mg/kg) and *H. tibetana* (1368 – 1546 mg/kg). *H. rhamnoides* berries from the Lahaul region showed a slightly higher content of tocols than those from Spiti and Ladakh. The relative proportion of individual tocols was almost identical in the three species studied here with α-tocopherol predominating (40–60%).

Tocopherols constituted about 70 to 80% of total tocols, whereas tocotrienols accounted for 20 to 30%. The proportion of the other major isomers, α-tocotrienol and γ-tocopherol, was 5–25 and 4–16%, respectively. γ-Tocotrienol and δ-tocopherol together represented 10–25% of total tocols. Other isomers of tocols were present in trace amounts. The total amount of tocopherols in these berries, except for *H. salicifolia*, was in accordance with the values reported from China and Poland (171,157). The tocopherol profile of Indian berries differed from the values reported for the Poland varieties in the proportion of δ - tocopherol. More resemblance in tocopherol profile of *H. rhamnoides* cultivars between Indian and Chinese origin might be due to the geo-climatic closeness in growing areas. Comparison of the tocopherol profile of berries of *H. salicifolia* and *H. tibetana* species with those of other geographic areas is not attempted here due to the lack of authentic reports. SB berries from the Indian region of the Himalayas were therefore a very rich source of tocols and were comparable with those of berries from other regions in the case of *H. rhamnoides*. It is well known that, as AOs, tocols, particularly tocotrienols, modulate diseases such as atherosclerosis, diabetes, cancer, ageing, and the like

Table-3.15: Carotenoids, tocopherols and tocotrienols composition of pulp oils of SB berries of *H. rhamnoides, H. salcifolia and H. tibetana* species from India (mg/kg of pulp oil) (Mean ± S D, n=3).

SB. Species	Geographic location	Carotenoids	Tocopherols/tocotrienols						
			α T1	α T3	β T1	γ T1	γ T3+ δT1	δT3	Total
H. rhamnoides	Lahaul-1	2660 ±82	859±40	293±24	16±5	152±21	362±45	12±2	1694±61
	Lahaul-2	3420 ±102	1068±49	227±16	12±2	165±13	288±21	17±2	1788±41
	Lahaul-3	2940 ±42	773±28	148±28	19±4	149±28	344±21	16±4	1564±67
H. rhamnoides	Spiti-1	2576 ±108	1006±26	183±22	18±3	170±35	228±24	10±3	1615±76
	Spiti-2	2820 ±62	1046±71	164±34	9±4	120±22	117±23	16±3	1494±76
	Spiti-3	2706±35	679±54	247±28	11±4	121±20	241±14	17±1	1318±59
H. rhamnoides	Ladakh-1	2400 ±60	954±73	95±15	27±6	126±5	187±11	14±2	1394±87
	Ladakh-2	2350 ±22	858±73	120±27	22±4	82±10	201±22	15±1	1301±80
	Ladakh-3	2650 ±61	903±50	109±27	21±5	90±5	208±35	17±1	1348±93
H. salicifolia	Lahaul-1	801 ±25	354±15	46±7	5±2	94±9	291±18	8±1	799±23
	Lahaul-2	840 ±42	517±30	124±23	4±1	57±18	183±12	18±6	902±35
	Lahaul-1	692 ±52	390±22	89±12	9±2	33±6	128±35	18±1	666±62
H. tibetana	*Spiti-1*	3166 ±32	867±46	127±17	15±3	86±16	431±46	19±4	1546±47
	Spiti-2	2693 ±41	895±67	95±7	18±3	62±18	363±30	17±5	1448±109
	Spiti-3	2840 ±18	864±13	85±11	19±2	64±16	336±33	12±4	1368±25

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Chemical composition of SB berries largely depends on their origin/ssp., geo-climatic conditions and agronomic practices (131). Systematic mapping of chemical composition of SB berries of different varieties and origins are still lacking. The results of the present study showed that the pulp oil of two Indian SB berries (*H. rhamnoides* and *H. tibetana*) were rich sources of bioactive lipophilic compounds, i.e., carotenoids and tocopherols, and had a distinct FA profile, with palmitoleic acid as the major FA. The presence of palmitoleic acid as the predominant FA is unique among oils of plant origin, and this observation verified previous reports based on Chinese varieties (131,148,171).For the first time the distinct nature of the berries of *H. salicifolia*, which is only found in the Himalayan region, was revealed, including a high content of hydrophilic components such as flavonols and vitamin C and a very low content of oil and lipophilic components such as carotenoids and tocopherols. The high nutritional quality of berries of *H. tibetana*, which is found only in India, is reported here for the first time. A high content of tocopherols and carotenoids, with high amount of palmitoleic acid and other bioactive molecules, enhances the value of SB pulp oil as a health supplement. Reports on the lipophilic constituents of SB berries from China, Canada, Poland, and Russia are available. However, no authentic data on the SB berries grown in the Indian region of the Himalayas have been reported. The cold desert conditions in which Indian SB is grown may have an influence on the phytochemical profile, as shown in this preliminary study. Detailed studies with large number of samples representing different varieties and geoclimatic conditions are required for the complete finger printing of Indian SB berries.

3. 3. Processing of Fresh Sea Buckthorn Berries

Indian Himalayas hosts world's second or third largest cultivation of SB. Even though so far, no attempt has been made to utilize SB berries grown in this region. An attempt has been made here for the first time to develop a process for the production of high quality pulp oil and oil free clear juice with maximum retention of bioactive phytochemicals. The seeds obtained as a byproduct were subjected to SC-CO_2 extraction for superior quality oil. Three batches of SB berries were processed at pilot scale and process parameters were analysed. The present study also reports a detailed chemical evaluation of process streams and products from Indian SB berries for the first time.

3.3.1. Process development

The schematic diagram of the process is given in Figure 2.1. Dewatering is the most critical operation of this process, the efficiency of which determines the product yield and quality. A continuous dewatering screw press, designed in our laboratory for fresh spices with high moisture content (700–800 g/kg), was used for this operation. The fresh berries used here had an average seed/pulp ratio of 1:9 and a moisture content of about 70% of fresh weight. Dewatering was conducted three times for each batch. Whole berries were fed into the screw press and the pressure was controlled by applying back-pressure in such a way as to avoid choking. At this stage a liquid phase (~50% of fresh berry weight) with pulp oil and suspended solids was separated from a solid fibrous residue. The solid phase was then resuspended in 25% by weight of hot water and pressed again. This operation was repeated once more and the three liquid phases were pooled. It was observed that the weight of the pooled liquid phase was approximately equal to the fresh weight of berries used in each batch. About 80% of the original

water content of the berries was separated after the three operations. The pooled crude juice was then clarified as described above. At 80°C the suspended solids were found to coagulate and settle at the bottom, leaving a clear juice above with an orange/red oil layer at the top. High-speed continuous centrifugation yielded clear orange/red oil (2.7–2.8% of fresh berry weight) that accounted for 66–70% of the oil present in the soft parts. The final clear juice obtained was about 75–80% of fresh berry weight (Table 3.16). Results of the compositional analysis of process streams are shown in Table 3.17. The pulp of the fresh berries used in the process contained 44–46 g/kg of oil on a fresh weight basis. The oil content in the juice (0.3–0.8 g/kg) and sludge (64–68 g/kg) shows that about 85% of the total oil in the pulp of berries was extracted with the liquid phase by the high-pressure screw pressing employed here. The oil content in the fibrous residue (29–32 g/kg) and sludge together comprised about 25–30% of the oil present in the pulp of berries. A solvent or supercritical fluid could be employed to recover this residual oil, but that was not attempted here. The seeds in the fibrous residue were easily separated after drying followed by air classification. The dried seeds were ground and their oil was extracted using SC-CO_2. The process streams/products were characterized for their phytochemicals, which are known to possess biological activity.

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Table 3.16 Results of process development trials of fresh sea buckthorn berries.

Trial No.	Berries (kg)	Seed pulp ratio	Water Added (kg)	Liquid phase (kg)	Wet fibrous residue[a] (kg)	Wet solid sludge (kg)	Juice (kg)	Pulp Oil		
								Qty. (kg)	Yield on fresh berries (%)	Extraction efficiency (%)
1	90.0	1:9.3	16.0	89.2	19.0	6.8	73.3	2.51	2.79	68.1
2	35.0	1:9.4	7.5	34.1	8.1	3.8	27.4	0.97	2.77	69.3
3	47.0	1:9.2	9.0	45.3	10.0	6.5	35.1	1.25	2.72	66.5

[a] Mass includes seeds.

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Table 3.17. Compositional analysis of process streams (%) [a].

Trial No.	Process streams	Solids	Fat	Protein	Ash	Soluble sugars	Crude fiber
1	Pulp of Berries	27.9 ± 0.1	4.6 ± 0.1	1.8 ± 0.2	1.0 ± 0.1	9.5 ± 0.4	2.4 ± 0.1
	Juice	13.2 ± 0.4	0.1 ± 0.0	0.5 ± 0.1	0.8 ± 0.0	5.8 ± 0.2	tr [c]
	Fibrous Residue [b]	36.2 ± 0.5	3.0 ± 0.2	5.9 ± 0.3	0.7 ± 0.1	11.8 ± 0.3	9.8 ± 0.7
	Sludge	20.2 ± 0.2	6.8 ± 0.4	2.4 ± 0.1	0.8 ± 0.1	4.5 ± 0.4	1.1 ± 0.1
2	Pulp of Berries	26.2 ± 0.1	4.4 ± 0.1	2.1 ± 0.2	1.0 ± 0.0	9.2 ± 0.5	2.4 ± 0.1
	Juice	10.7 ± 0.3	0.0 ± 0.0	0.4 ± 0.0	0.6 ± 0.1	4.9 ± 0.1	tr [c]
	Fibrous Residue [b]	38.7 ± 0.1	3.2 ± 0.1	6.4 ± 0.5	0.6 ± 0.1	11.8 ± 0. 5	12.1 ± 0.5
	Sludge	19.1 ± 0.1	6.7 ± 0.2	2.0 ± 0.4	1.0 ± 0.1	4.8 ± 0.2	0.8 ± 0.1
3	Pulp of Berries	26.9 ± 0.1	4.4 ± 0.1	2.2 ± 0.2	1.0 ± 0.1	9.8 ± 0.4	2.4 ± 0.2
	Juice	10.9 ± 0.2	0.0 ± 0.0	0.5 ± 0.2	0.5 ± 0.0	4.9 ± 0.1	tr [c]
	Fibrous Residue [b]	34.9 ± 0.0	2.9 ± 0.2	6.1 ± 0.4	.8 ± 0.1	11.3 ± 0. 4	10.1 ± 0.5
	Sludge	19.5 ± 0.1	6.4 ± 0.1	2.1 ± 0.2	1.0 ± 0.0	4.7 ± 0.4	0.6 ± 0.1

[a] Values are mean ± SD, n=3.
[b] analyzed and expressed without seed.
[c] tr ≤ 1g kg $^{-1}$

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3.3.2. Chemical Evaluation of the products

Chemical composition of products was evaluated and compared with that of fresh berries used for the process. Juice, pulp oil and seed oil were analyzed in detail and retention of bioactive phytochemicals was estimated.

3.3.2.1. Juice.

The raw juice obtained from the berries contained oil droplets and granules or clumps of insoluble brown solids, which imparted a less appreciable yellowish brown color to the juice. Clarification followed by centrifugation removed most of this oil and brown colored solids and resulted in a clear juice with attractive yellow color. Juice from each batch was analyzed for its chemical constituents. The final juice contained a relatively higher amount of solids (10.7–13.2%) in spite of the addition of water, which could be attributed to high-pressure screw pressing (Table 3.17). The oil content in the juice was found to be very low (0.03–0.08%) but sufficient to provide the characteristic flavor of fresh berries (Table 3.17). The presence of more than 0.1% of oil in juice is a negative quality, since it forms an oily ring on the top during storage and leads to storage problems such as rancidity (259). The juice also contained 1–2 mg/kg of carotenoids and about 1 mg/kg of tocopherols owing to the trace amount of oil. The protein content in the juice was very low (0.4–0.5%), as most of the protein was found in the residue (5.9–6.4%) and sludge (2.0–2.4%) (Table 3.16). Total soluble sugars varied from 4.9 to 5.8% in the juice, accounting for 40–50% of total solids (Table 3.17). Present reports indicate that glucose and fructose respectively represent 49.5-62.1% and 37.3-50.4% of total sugars in the juice (147).

Table 3.18. Polyphenols and flavonoids in the pulp of berries and juice (mg/kg) [a]

Composition	Pulp of berries	Juice		
		Trial 1	Trial 2	Trial 3
Total polyphenols	5646 ± 50	2821 ± 6	2392 ± 15	2757 ± 23
		Flavonoids		
Quercetin	109 ± 8	81 ± 3	77 ± 4	77 ± 5
Kaempherol	38 ± 2	16 ± 2	12 ± 2	14 ± 2
Isorhamnetin	428 ± 8	303 ± 9	251 ± 15	310 ± 14
Total flavonoids	575 ± 17	400 ± 10	340 ± 19	401 ± 15

[a] Values are mean ± SD, n=3.

Polyphenols and flavonoids: SB berries used for the process and the juice obtained were analyzed for their polyphenol content and the results are presented in Table 3.18. Clear juice contained 2392 – 2821 mg/kg of polyphenols. 40% of the polyphenols of the berries was found to be extracted with juice. Flavonoids being an important class polyphenols were analyzed in detail. HPLC profiling of flavonoid aglycones showed isorhamnetin as the major flavonoid (428 mg/kg) in the pulp of berries, along with quercetin (109 mg/kg) and kaempherol (38 mg/kg) (Table 3.18). Like other water-soluble phytochemicals, the juice retained 50–60% of flavonoids of berries. Juice flavonoid profile included 251–310 mg/kg of isorhamnetin, 77–81 mg/kg of quercetin and 12–16 mg/kg of kaempherol (Table 3.18). Isorhamnetin contributed 75% of total flavonoids in berries and juice. Flavonoids

contributed 10 and 14% of total phenolics in berries and juice respectively. Polyphenols imparts astringency to fruit juices. PAs, catechins and phenolic acids were the other major phenolics reported in SB berries (164). The high amount of flavonoids and other polyphenols present in the juice obtained enhances this fruit's nutraceutical value.

Organic acids: The titrable acidity of the final juice was found to be in the range 3.56 – 3.69% in terms of malic acid, while the pH was between 3.1 and 3.3 (Table 3.19). A wide range (1.9 -5.1%, in terms of malic acid) of titrable acidity has been reported for SB juice of different origins (147, 260). Detailed profiling of low-molecular-weight organic acids using HPLC showed quinic acid (2.80%) and malic acid (1.60%) as the major organic acids in the pulp of SB berries along with citric acid (0.16%) and traces of fumaric acid and maleic acid (Table 3.19). The process reported here extracted about 55–70% of organic acids from the berries into the juice, with a ratio identical to that of the berries. The juices had 3.0 – 3.6% organic acids with quinic acid (1.81-1.99%), malic acid (1.17 - 1.48%) and citric acid (0.09 - 0.13%) as major constituents. Sugar acid ratio of the juices ranged from 1.33 to 1.63. A wide range (0.4 -1.9) of sugar acid ratio has been reported for SB (*H. rhamnoides*) juices (260). Sugar acid ratio is an important factor in determining the sweetness or sourness and acceptance of fruit juices. Sugar acid ratio negatively correlates with the sourness and astringency of fruit juice (260,261)

The higher amount of quinic acid (1.81–1.99%) in the juice further highlights the nutraceutical value of SB juice. G-I protective effects of quinic acid and its derivatives are well established through in-vitro and in-vivo studies (236). The organic acid profile and the amount of quinic acid can be used as a chemical marker for evaluation of the quality of commercial SB juice.

Table 3.19. pH, titrable acidity, organic acids and vitamin C in the pulp of berries and juice (mean ± SD, n=3**).**

Composition	Pulp of berries	Juice		
		Trial 1	Trial 2	Trial 3
Total titrable acids (%) [b]	-	3.56 ± 0.02	3.69 ± 0.04	3.64 ± 0.02
pH	-	3.2	3.3	3.1
Organic acids (%)				
Quinic acid	2.80 ± 0.07	1.99 ± 0.08	1.85 ± 0.03	1.81 ± 0.02
Malic acid	1.60 ± 0.05	1.48 ± 0.02	1.17 ± 0.02	1.18 ± 0.02
Citric acid	0.16 ± 0.02	0.13 ± 0.01	0.16 ± 0.01	0.09 ± 0.00
Total acids	4.56 ± 0.01	3.60 ± 0.06	3.18 ± 0.05	3.08 ± 0.03
Vitamin C (mg/kg)	2232 ± 42	1840 ± 10	1683 ± 12	1752 ± 17

[b] Expressed in terms of malic acid.

Vitamin C: SB berries are well known for their extraordinarily high levels of vitamin C, which is considered as an important quality parameter of SB juice. The pulp of the Indian berries used in the process contained 2232 mg/kg of vitamin C (Table 3.19). Approximately 75% of the vitamin C in the pulp of berries was retained in the juice in this process, resulting in 1683–1840 mg/kg of vitamin C in the final clear juice. The high vitamin C concentrations make SB berry highly suitable for the production of nutritious soft drinks. Origin, maturity and harvesting time of berries and processing techniques and storage conditions also affect the vitamin C content of the juice (127,145,147,251,252)**.** Vitamin C content ranging from 290 to 1760 mg/kg

has been reported for the juices obtained from *H rhamnoides* berries of Finnish origin (260)

3.3.2.2 Pulp oil

The pulp oil obtained by the process retained its characteristic flavor in contrast to the solvent extracted oil. The chemical profile of the pulp oil was examined in detail.

FA Composition: The FA profile of the pulp oil obtained from the process was determined using gas chromatography and is summarized in Table 3.20. The proportions of palmitoleic, palmitic and linoliec acids in pulp oil were 45.6–49.1, 30.9–33.8 and 4.6–5.8% respectively. The uniqueness of SB pulp oil in terms of the proportion of palmitoleic acid enhances its nutritional value.

Carotenoids: Total carotenoids in the pulp of berries and pulp oil were quantified and expressed in terms of *β*-carotene (Table 3.21). A carotenoid content of 128 mg/kg was found in the soft parts of berries. Variety, maturity, climatic conditions and method of oil separation influence the carotenoid content in oil. Variations in the carotenoid content in pulp oil between batches could be attributed to the relative proportion of immature berries. The pulp oil obtained by this process was significantly rich in carotenoids (2450–2810 mg/kg), making SB berries one of the richest natural sources of carotenoids.

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Table: 3.20 FA composition of pulp oil.

Trial No	FA (wt %)				
	16:0	**16:1**	**18:0**	**18:1**	**18:2**
I	33.8 ± 1.5	46.4 ± 2.0	1.0 ± 0.5	13.4 ± 0.9	5.4 ± 1.4
II	31.2 ± 1.4	49.1 ± 3.1	0.8 ± 0.1	13.9 ± 3.5	4.6 ± 0.6
III	30.9 ± 1.3	45.6 ± 2.6	0.9 ±0.4	16.8 ± 1.7	5.8 ± 0.4

16:0; palmitic acid, **16:1;** palmitoleic acid, **18:0;** stearic acid, **18:1;** oleic acid, **18:2;** linoleic acid

Table 3.21. Carotenoid, tocopherol, tocotrienol and sterol content in the pulp of berries and pulp oil (mg/kg) [a].

Composition	Berry Pulp	Pulp Oil		
		Trial 1	**Trial 2**	**Trial 3**
Carotenoids	128 ± 2	2810 ± 26	2636 ± 20	2450 ± 14
Tocopherols/tocotrienols				
α-T [b]	47 ± 2	987 ± 22	1141 ± 11	1067 ± 35
α-T_3 [c]	7 ± 1	182 ± 14	131 ± 8	94 ± 6
β-T [b]	1 ± 0	28 ± 4	22 ± 2	21 ± 2
γ-T [b]	5 ± 1	91 ± 2	134 ± 10	109 ± 12
γ-T_3 [c] **+δ-T** [b]	6 ± 1	107 ± 8	154 ± 11	134 ± 6
δ-T_3 [c]	1 ± 0	14 ± 1	17 ± 1	15 ± 1
Total	67 ± 2	1409 ± 28	1599 ± 14	1439 ± 48
Sterols				
β-sitosterol	180 ± 8	3200 ± 49	3221 ± 36	3034 ± 48
Stigmasterol	80 ± 8	1203 ± 24	1058 ± 35	1062 ± 39
Total	260 ± 15	4403 ± 35	4279 ± 30	4096 ± 57

[a] Values are mean ± SD, n=3. [b] T : Tocopherol; [c] T_3 : Tocotrienol

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Tocopherols and tocotrienols: The pulp of the berries used in the process contained 67 mg/kg of tocopherols and tocotrienols (Table 3.21). The pulp oil obtained by the process had 1409–1599 mg/kg of tocopherols and tocotrienols. α-Tocopherol, the anomer with maximum vitamin E activity among tocols, constituted 69–73% of total tocols in pulp oil. α-Tocotrienol, γ -tocopherol, δ-tocopherol and γ -tocotrienol were other tocols identified in pulp oil. Traces of β-tocopherol and δ-tocotrienol were also detected in pulp oil.

Sterols : β-Sitosterol (180 mg/kg) and stigmasterol (80mg/kg) were found to be the major sterols in the pulp of berries, with unidentified peaks indicating the presence also of minor compounds (Table 3.21). Trace amounts of campesterol, a major sterol in seed oil, were also detected in pulp oil. Pulp oil obtained by the present process showed a similar sterol profile of berries, with 3034–3200 mg/kg of β-sitosterol and 1058–1203 mg/kg of stigmasterol.

3.3.2.3. Seed oil

Seeds from Indian grown berries have not been studied in detail for their nutraceutical values. As part of the integrated approach to processing SB berries, a comparative evaluation of seed oils obtained by hexane and SC-CO_2 extraction was made with the seeds obtained from the process reported here. The yield of hexane-extracted oil was slightly higher (6.1% by wt. of seed) than that of SC-CO_2-extracted oil (5.2% by weight of seed). All phytochemical constituents (carotenoids, tocopherols/tocotrienols and sterols) were more abundant in the oil extracted with SC-CO_2 than in the oil extracted with hexane. The lower content of phytochemicals in hexane-extracted oil could be due to non-oil components such as wax and gum being extracted with hexane, resulting in a higher extract yield with a lower concentration of phytochemicals (Table 3.23). The SC-CO_2 extraction conditions employed here (60◦ C temperature, 4.5 × 107 Pa pressure and 60 g/min gas flow rate for

3 h) were able to extract seed oil with micronutrients as effectively as or better than hexane.

Seed oil differed significantly from pulp oil in its chemical characteristics. While palmitoleic acid (45–49%) was the most predominant FA in pulp oil, its content in seed oil was as low as 2.1-3.6% (Table 3.22). The major FAs in seed oil were palmitic (17-18%), oleic (29-30%), linoleic (26-28%) and ALA (16-19%). Seed oil with more than 16% ALA otherwise called omega-3-FA is rare among vegetable oils. The biological properties of ALA are well documented. SB seed oil as a vegetable oil with high levels of ALA, therefore had nutraceutical value comparable with fish oil. The higher level of carotenoids in pulp oil (2450-2810 mg/kg) than in seed oil (~400 mg/kg) was another major difference. As reported above, the total tocol content in seed oil (1012 mg/kg) was also lower than that in pulp oil (1409-1599 mg/kg) (Tables 3.21 & 3.23). The γ -tocopherol content in pulp oil was lower than that in seed oil. The results for tocols indicate that, owing to its higher total tocol content with a high proportion of α-tocopherol, pulp oil had higher vitamin E activity than seed oil. The total sterol content in seed oil (16887 mg/kg) was about four times that in pulp oil (Tables 3.20 & 3.22). β-Sitosterol (13774 mg/kg) was the major sterol in seed oil. Seed oil also contained significant amounts of campesterol (1878 mg/kg) and stigmasterol (1235 mg/kg).

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Table.3.22 FA composition (wt. %) of hexane and supercritical CO_2 extracted seed oils (mean ± SD)

	Fatty acids					
	16:0	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**
Hexane extracted	17.9±1.2	2.1±0.3	4.1±0.6	29.8±1.0	26.1±0.8	18.8±1.2
SC- CO_2 Extracted	17.2±1.2	3.6±0.2	3.8±0.4	30.0±1.2	27.7±0.8	16.7±0.7

16:0; palmitic acid, 16:1; palmitoleic acid, 18:0; stearic acid, 18:1; oleic acid, 18:2; linoleic acid

Table 3.23. Yield, Carotenoid, tocopherol, tocotrienol and sterol content in the hexane and SC- CO_2 extracted seed oils (mg/kg) (Mean ± SD).

Composition	**Seed Oil**	
	Hexane extracted	**SC-CO_2 extracted**
Carotenoids	387 ± 6	403 ± 9
	Tocopherols/tocotrienols	
α-T [b]	520 ± 14	587 ± 12
α-T_3 [c]	11 ± 1	35 ± 4
β-T [b]	19 ± 1	21 ± 2
γ-T [b]	306 ± 12	315 ± 14
γ-T_3 [c] **+δ-T** [b]	46 ± 3	39 ± 4
δ-T_3 [c]	10 ± 1	15 ± 1
Total	912 ± 20	1012 ± 26
	Sterols	
β-sitosterol	13305 ± 76	13774 ± 112
Stigmasterol	1125 ± 64	1235 ± 57
Campesterol	1770 ± 29	1878 ± 57
Total	16200 ± 150	16887 ± 87

[a] **Values are mean ± standard error, n=3.** [b] **T : Tocopherol;** [c] **T_3 : Tocotrienol**

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The reports related to SB processing are rather limited (*1*29,177*, 17*9*)* and show a fragmented approach. Beveridge *et al;* discussed the available processes in a recent review (129). According to them, the main products from SB berries are seed oil, yellow pigment and juice. Pulp oil, though very important in terms of its quantity and phytochemical profile as compared with seed oil, was not given adequate attention by the authors, perhaps owing to the limited data being available on pulp oil recovery. The general approach to processing SB involved dewatering and juice clarification as the critical process steps (129,262,263). Details of material balance, efficiency of extraction, product yield, commercial feasibility, etc. have not been discussed in quantitative terms in the published reports. Hence comparison of the data from our processing of Indian SB berries is difficult. Our approach has been to integrate process and products and maximize the yield of juice, pulp oil and seed oil from fresh berries of SB grown in the Indian Himalayas, hitherto not attempted. Most of the unit operations employed here are similar to those reported previously. A high yield of pulp oil and juice by employing a combination of high-pressure dewatering screw pressing and continuous centrifugation was achieved in this process. Approximately 80% of the water and oil and 60% of the solids in the pulp were extracted to the liquid phase using the high-pressure screw press with a compression ratio of about 10. The continuous high-speed centrifugation employed here led to efficient separation of the liquid phase into oil free clear juice, pulp oil and solid sludge. The yield of clear juice at 75–80% of berry weight with 10–13% soluble solids appeared to be high owing to effective pressing. Further, the juice obtained here also retained more than 60% of organic acids, 70% of vitamin C, 40% of polyphenols and 50% of flavonoids present in the pulp of berries. The sugar acid ratio of the juices was in higher end of the range reported in literature. It could be seen that, even after the addition of water to facilitate dewatering and consequent dilution of juice, the concentration of total solids and bioactives remained fairly high in the final juice, which could

be attributed to the extraction efficiency. Beveridge *et al*; reported the effect of processing on the composition of SB juice (129). The authors demonstrated the use of conventional rack and cloth pressing in batch mode. The juice was treated with pectinase, followed by batch centrifugation for juice clarification, and the clarified juice was examined for vitamin C, organic acids and sugars. However, the authors did not discuss the process efficiency. The vitamin C and organic acid contents of the juice described by them are similar to those of the juice from Himalayan berries reported here. Considering the high levels of total solids and other phytochemical constituents, the final juice obtained here could be further diluted in the ratio 1:2 to formulate a ready-to drink health beverage rich in bioactive phytochemicals.

Pulp oil is another valuable co-product of this process, having an extremely high content of carotenoids, tocopherols and sterols with characteristic fatty acid 16:1. The use of vegetable oils as solvents for the extraction of pulp and seed oils has also been reported, but the efficiency of recovery was found to be poor. The present process was able to separate up to 70% of the pulp oil without using organic solvents, so maximum retention of bioactives was achieved. An important advantage of this process was that seeds could be recovered without damage in spite of the high-pressure dewatering of berries. The feasibility of extraction of high-quality seed oil using SC-CO_2 was also demonstrated.

The process reported here, for fresh SB berries grown at high altitudes in the Himalayas, constitutes an integrated approach to yield products with high efficiency and quality for nutraceutical applications. SB berries are highly perishable and have to be processed within hours of harvesting to obtain quality products. This green integrated approach would also be suitable for ecologically fragile SB growing areas. Even though SB berries from the

Himalayas have not been utilized on a commercial scale, the potential appears to be high following the process reported here.

3.4. In Vitro AO Activity Evaluation of Sea Buckthorn (*H. rhamnoides*) Berries

3.4.1 In Vitro AO Activities of *H. rhamnoides* Berry Extracts

A wide spectrum of biological activities has been attributed to SB berries. More than 300 SB medicinal preparations are mentioned in literature and several preparations have been clinically evaluated to treat radiation damage, burns, oral inflammation and gastric ulcers (191). Berries and berry products are also being used in nutraceutical and cosmaceutical applications. Apart from reports on applications and physiological properties of SB, reports describing bioactivities of SB berries in relation to their phytochemical compositions are limited. This part of the present study was aimed at the evaluation of AO properties of SB berries belonging to *H. rhamnoides* and chemical profiling of active fractions.

In plant products, biological activities are often confined to certain parts and fractions. Activity guided fractionation approach was therefore applied here. Hexane, ethyl acetate, methanol and water extracts of SB berry anatomical parts (pulp, seed coat and kernel) were prepared and subjected to AO capacity evaluation. TEAC, DPPH, superoxide and hydroxyl radical scavenging, Fe(III) reduction and Fe(II) chelation and XO inhibition capacities of berry extracts were studied using in-vitro model assay systems. The kernel methanol extract with significant yield and high AO capacity was further fractionated between hexane, ethyl acetate, n-butanol and water. Each fraction was evaluated for their AO capacity and active fractions were subjected to chemical evaluation. Activity guided fractionation scheme used for the preparation of berry samples is given in Figure 2.2

Table 3.24. Extraction efficiency in terms of yield of extract of different solvents on sequential extraction.

Berry parts	Solvents	Extraction Efficiency (in dry wt. of samples) (%)
Pulp	Hexane (PHE)	14.25
	Ethyl Acetate (PEE)	19.8
	Methanol (PME)	35.8
	Water (PWE)	24.9
	Total	94.8
Seed Coat	Hexane (SCHE)	2.3
	EtOAc (SCEE)	0.2
	Methanol (SCME)	9.2
	Water (SCWE)	6.8
	Total	18.5
Kernel	Hexane (KHE)	7.2
	Ethyl Acetate (KEE)	1.5
	Methanol (KME)	13.9
	Water (KWE)	18.9
	Total	41.5

The extractability of berry parts as affected by different solvents is summarized in Table. 3.24. Methanol and water as solvents showed high extractability of berry parts. 94.8% of the total dry matter in the pulp could be extracted with the solvents whereas total solubles in the seed coat and kernel were 18.5 and 41.5% respectively. The low soluble matter content of seed coat might be attributed to its high proportion of high molecular weight structural components.

Total phenolics by F-C Method

Phenolic compounds are one of the major classes of phytochemicals with significant AO activities. The extracts were evaluated for their total phenolics content by using F-C reagent. F-C reagent assay is also considered as a rough method for AO capacity evaluation (264)**.** Seed coat methanolic (SCME) and water (SCWE) extracts contained high amounts of phenolics (32.9 and 24.3 respectively). Phenolic concentration in kernel extracts (KME = 22.6%, KWE = 16.3%) was also high. Pulp methanol (PME) and water (PWE) extracts respectively had 6.8 and 4.4% phenolics.

3.4.1.1. DPPH radical scavenging activity

DPPH radical ($DPPH^{\bullet}$) is a commercially available stable free radical (Figure 1.6). $DPPH^{\bullet}$ can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Both hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms have been proposed for the quenching of $DPPH^{\bullet}$ by AOs (265). The quenching of $DPPH^{\bullet}$ can be monitored by tracking the decrease in its absorbance at 517nm. Compounds, which can donate electrons or $H^{\bullet}$ to quench $DPPH^{\bullet}$, are considered to have capacity to scavenge free radicals. $DPPH^{\bullet}$ is widely used as a substrate for the high through put screening of free radical scavengers.

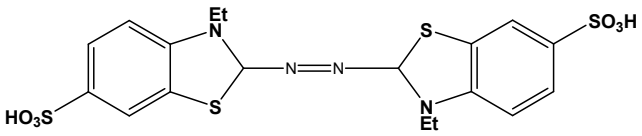
The reaction curves for the $DPPH^{\bullet}$ scavenging of berry extracts are given in Figure 3.15 and the IC_{50} values are summarized in Table 3.24. The radical scavenging capacity of extracts/compounds was compared in terms of IC_{50} values. IC_{50} value denotes the amount of extract/compounds required to scavenge 50% of $DPPH^{\bullet}$ in the reaction system (Figure 3.15). In the present study it was shown that methanol and water extracts of pulp and seed possessed high $DPPH^{\bullet}$ scavenging efficacy. The results showed that the SCME (IC_{50} = 3.0 μg), SCWE (IC_{50} = 6.6 μg), KME (IC_{50} = 6.7 μg) and KWE (IC_{50} = 19.2 μg**)** had significantly higher $DPPH^{\bullet}$ scavenging activity. Pulp

methanol and water extracts respectively showed IC_{50} values of 654.5 and 796.3 µg. The radical scavenging activities of kernel and seed coat extracts were comparable with that of standard compounds like gallic acid (IC_{50} = 1.6 µg), and catechin (IC_{50} = 5.2 µg). But the yield of SCME was very low as compared to that of KME and PME (Table 3.24). There are some recent reports on the high $DPPH^{\bullet}$ radical scavenging activity of SB whole seed extracts (128, 168). The results of the present study are in accordance with these reports.

3.4.1.2. Trolox Equivalent Antioxidant Capacity (TEAC).

ABTS cation radical is an important and popular probe used for the evaluation of AO capacity of biological fluids and phytochemicals. The method is used to monitor the decay of the radical cation ABTS, produced by the oxidation of 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) (Figure 3.14) in presence of AOs. $ABTS^{\bullet+}$ has a strong absorption at 700-750 nm. $H^{\bullet}$ donor molecules quantitatively convert $ABTS^{\bullet+}$ to colorless ABTS molecule. A mixed type mechanism (HAT and SET) has been proposed for the scavenging of $ABTS^{\bullet+}$ by AOs (265). Trolox, an important $H^{\bullet}$ donor is used as a reference in ABTS assay. The AO capacities of the compounds are represented as trolox equivalent antioxidant capacity (TEAC). TEAC of 1 mg of berry extracts is given in Table 3.25**.** SCME had the highest TEAC value (8.0 nM), followed by KME (5.8 nM), SCWE (5.6 nM) and KWE (1.1 nM). $ABTS^{\bullet+}$ scavenging activity of the berry extracts was in correlation with their phenolic content. Extracts with high phenolic content had high TEAC values. Gallic acid and catechin showed TEAC values 12.9 and 9.2 nM respectively. Due to lack of reports on the TEAC of SB berries discussion cannot be extended to previous works in this regard.

Figure 3.14. ABTS



3.4.1.3 Fe(III) reducing power

Fe(III) reduction assay is under the class of SET mediated AO assays. In this assay the presence of AOs (reductants) would bring about the reduction of the $[Fe(CN)_6]^{3-}$ to $[Fe(CN)_6]^{4-}$. Amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm with increased absorbance indicating higher reducing power.

Figure 3.16 shows the dose response curves for the reducing powers of the SBT extracts and reference compounds (gallic acid, trolox and catechin). It was found that the reducing power of all the extracts was in linear correlation with their concentration in the experimental range. The Fe(III) reducing power of 100 µg of samples is expressed as ascorbic acid equivalents (AAE) in Table 3.25**.** The reducing power values of 100 µg of KME, SCME, KWE and SCWE were 21.3, 17.9, 17.0 and 13.2 µg equivalents of ascorbic acid respectively. Pulp extracts showed very low reducing power. The reducing power of KME and SCME extracts were comparable with that of catechin and trolox. Gallic acid showed the highest reducing power (125 µg AAE) among the reference compounds used here, followed by catechin (58.4 µg) and trolox (29.6 µg). These results reveal that SB berry extracts, especially seed extracts can act as electron donors and could react with free radicals, more efficiently converting them to stable products, and terminating the radical chain reaction.

3.4.1.4. Fe(II) chelation capacity

Metals especially Cu and Fe catalyze the generation of free radicals from neutral molecules and subsequently trigger free radical reactions. Cu^{+} or Fe^{2+} mediated formations of hydroxyl radicals from hydrogen peroxide (Fenton's reactions) are typical examples. Therefore the compounds with the ability to chelate heavy metals and thus keep them unavailable for metal mediated free radical reactions are also considered as AOs. The metal chelation capacity of the berry extracts was evaluated using Fe(II)/ferrozine system, in which Fe^{2+} ions were incubated with the berry extracts/reference compounds and the free and loosely bound Fe^{2+} ions were estimated using ferrozine. The % of Fe(II) chelated by 1 mg of extracts/reference compounds are summarized in Table 3.25**.** Highest chelation capacity was shown by KME (12.4%) followed by KWE (8.4%), SCME (6.9%) and SCWE (4.8%). The reference compound EDTA chelated 89% of Fe^{2+} in the reaction systems under the experimental conditions. SB berry and its fractions had moderate metal chelation capacity.

3.4.1.5. Hydroxyl radical scavenging activity

Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to biomolecules. The scavenging effect of berry extracts against hydroxyl radical was therefore investigated using the Fenton's reaction. Only SB seed extracts showed hydroxyl radical scavenging activity and the activity increased with increasing concentration. From the IC_{50} values of the extracts (Table 3.25), it was revealed that KME (58 µg) had the highest scavenging activity followed by SCME (92 µg), KWE (109 µg) and SCWE (176 µg). Among the standard AO compounds tested, gallic acid, the most effective scavenger of DPPH and ABTS radicals, was less effective in neutralizing hydroxyl radical under the in vitro assay conditions. Catechin and trolox respectively had the IC_{50} values 125 and 141 µg.

3.4.1.6 Superoxide anion radical scavenging (SOS) activity:

Superoxide radical ($O_2^{\bullet -}$) is one of the harmful free radical found in biological systems. It attacks cellular components as a precursor of the more reactive oxygen radicals having the potential of reacting with biological macromolecules and thereby inducing tissue damage (24). In biological system, its toxic role can be eliminated by superoxide dismutase (SOD). There is an increasing interest in identifying active compounds from plant materials that can exhibit activity similar to SOD. SOS activity of SB berry extracts/reference compounds was measured using the xanthine-XO system. Xanthine-XO generated superoxide radicals reduce NBT to formazan blue (500 nm). Decreased formation of formazan blue is considered as the indication of SOS activity of samples. But in fact the reduction in blue coloration of reaction system may also be due to the reduction in the generation of superoxide by the inhibition of XO enzyme activity by samples. Therefore the inhibition of XO by the extracts/reference compounds was also studied and discussed in the next section.

The SOS activities of SB berry extracts are compared as IC_{50} values and the results are summarized in Table 3.25. Methanol and water extracts of seed coat and kernel exhibited SOS activity comparable to that of gallic acid and catechin in a dose-dependent manner. KME (69 µg) showed the highest activity among the tested samples followed by SCME (79 µg), KWE (197 µg) and SCWE (128 µg). SB pulp extracts didn't show significant scavenging activity. The results reveal that SB seed and seed coat extracts are potent scavengers of superoxide radicals or inhibitors of XO activity, or both.

3.4.1.7. Xanthine oxidase (XO) inhibition capacity.

Xanthine to uric acid conversion by XO is an important step in purine metabolism. This conversion is associated with the generation of superoxide radical anion. Hyperactivity of XO or the reduced removal of uric acid from the body leads to hyperurecimia, gout, arthritis and other inflammatory diseases. The compounds which are able to inhibit the XO activity are used to control the uric acid deposition in hyperurecemic patients. Allopurinol, a potent inhibitor of XO is used as a drug against hyperurecemic diseases like gout. This compound has reported to have many side effects. Therefore the search for potential XO inhibitory compounds from natural sources attracts special interest.

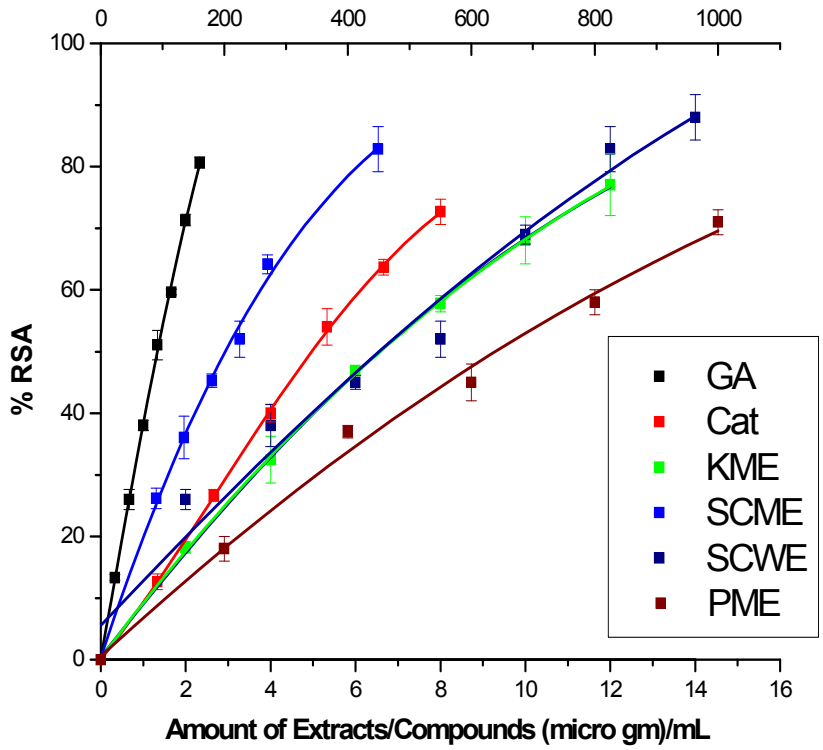
The SOS capacity of AOs evaluated using xanthine/XO NBT assay depends on both their ability to inhibit XO enzyme activity and to scavenge superoxide radicals. Therefore berry extracts were also evaluated for their XO inhibitory efficacy. The XO inhibitory efficacies of the extracts expressed as IC_{50} values are summarized in Table.3.25**.** Highest activity was shown by KME (121 µg**)**, followed by SCME (193 µg**)**, KWE (203 µg) and SCWE (291 µg) (Table 3.25). No significant XO inhibition was shown by pulp extracts. Allopurinol and catechin respectively showed IC_{50} values 14 and 39 µg under the experimental conditions. These results showed that SB seeds are good source of XO inhibiting compounds. This is the first report on the XO inhibitory capacity of SB berry extracts.

Table-3.25: TPC and in vitro antioxidant capacities of SB berry extracts and reference compounds (mean±SD. n=5).

SB extracts/ Antioxidant compounds		Total phenolics (GAE) %	DPPH* Scavenging Activity IC_{50} (µg)	TEAC (nM)	Fe(III) reducing capacity/100 µg [(AAE) (µg)]	Fe(II) Chelation capacity %/mg	Hydroxyl radical scavenging IC_{50} (µg)	Superoxide radical scavenging IC_{50} (µg)	Xanthine Oxidase inhibition IC_{50} (µg)
Pulp	**Methanol (PME)**	6.8 ± 0.3	654.5 ± 29.6	N.S	N.S	N.S	N.S	952 ± 36	N.S
	Water (PWE)	4.4 ± 0.1	796.3 ± 21.9	N.S	N.S	N.S	N.S	1654 ± 89	N.S
Kernel	**Methanol (KME)**	22.6 ± 2.1	6.7 ± 0.6	5.8 ± 0.3	21.3 ± 0.4	12.4 ± 1.9	58 ± 6	69 ± 11	122 ± 11
	Water (KWE)	16.3 ± 2.4	19.2 ± 0.9	1.1 ± 0.2	17.0 ± 0.2	8.4 ± 1.2	109 ± 8	197 ± 29	203 ± 13
Seed Coat	**Methanol** (SCME)	32.9 ± 1.9	3.0 ±0.3	8.0 ± 0.6	17.9 ± 0.6	6.9 ± 1.2	92 ± 12	79 ± 17	193 ± 21
	Water (SCWE)	24.3 ± 3.2	6.6 ± 0.8	5.6 ± 0.6	13.2 ± 1.1	4.8 ± 0.8	176 ± 22	128 ± 12	291 ± 18
Gallic acid		-	1.6 ± 0.0	12.9 ± 0.9	125.4 ± 0.1	-	N.S	35 ± 12	112 ± 09
Catechin		-	5.2 ± 0.0	9.2 ± 0.4	58.4 ± 0.2	-	125 ± 4	18 ± 4	39
Trolox		-		-	29.6 ± 0.3	-	141 ± 18	344 ± 10	-
EDTA		-	-	-	-	89.3 ± 3.4	-	-	-
Allopurinol		-	-	-	-	-	-	-	14 ± 2

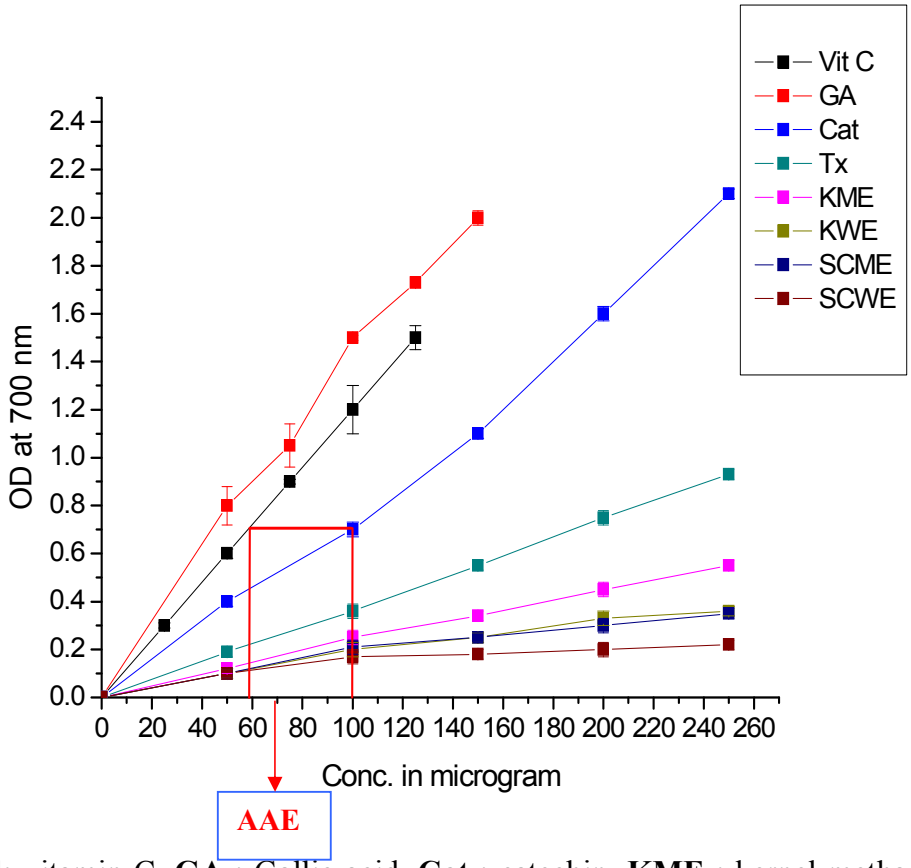
GAE : Gallic acid equivalents, AAE : Ascorbic acid equivalents, N.S : Not significant

Figure 3.15. DPPH radical scavenging efficacies of *H. rhamnoides* berry extracts and reference compounds (mean ± S D (n = 5)).



GA : Gallic acid, **Cat** : catechin, **KME** : kernel methanol extract, **KWE** : kernel water extract, **SCME** : seed coat methanol extract, **SCWE** : seed coat water extract, **PME** : pulp methanol extract.

Figure 3.16. Fe(III) reduction capacities of *H rhamnoides* berry extracts and reference compounds.



Vit C: vitamin C, **GA** : Gallic acid, **Cat** : catechin, **KME** : kernel methanol extract, **KWE** : kernel water extract, **SCME** : seed coat methanol extract, **SCWE** : seed coat water extract.

3.4.2. AO Capacity Evaluation of KME Fractions

Methanol extracts of seed coat and kernel were found to have high AO capacity and phenolic content. But the yield of seed coat extracts (~ 0.25% of dry mass of berries) was very low as compared to that of kernel extracts (~ 2.3% of dry mass of berries). Therefore kernel methanol extract (KME) was selected for further studies. KME thus was fractionated between hexane (KMHF), ethyl acetate (KMEF), butanol (KMBF) and water (KMWF). The yield of KME fractions is summarized in Table.3.26**.** Major portion of KME was resided with water fraction (50.5%). 6.9, 18.6 and 11.9% of KME were respectively resided into KMHF, KMEF and KMBF.

KME fractions thus prepared were subjected to in-vitro AO capacity studies and active fractions were evaluated for their chemical composition.

3.4.2.1 Total phenolics

The amount of phenolics of KME fractions as obtained by F-C reagent method is given in Table 3.26. Approximately 80 % of phenolics in the KME were recovered among the fractions. KMEF (35.7%) had the highest concentration of phenolics followed by KMWF (20.8%), and KMBF (18.4%). KMHF (2.9%) had the lowest phenolic concentration. Phenolic content in KMEF was enriched with respect to KME. Approximately 45% of total phenolics in the extract were found to be retained with KMWF fraction and 33% with KMEF.

3.4.2.2. In vitro AO capacities of KME fractions

AO capacity of KME fractions was evaluated by using in-vitro model assay systems and the results are tabulated in Table 3.26**.** KMEF (IC_{50} = 4.9 μg) and KMWF (IC_{50} = 4.1 μg) had higher $DPPH^{\bullet}$ scavenging activity than the parent extract KME (IC_{50}=6.7 μg). Their $DPPH^{\bullet}$ scavenging activity were comparable with that of standard compounds like gallic acid (IC_{50} = 1.6 μg),

and catechin (IC_{50} = 5.2 µg) (Table 3.25 & 3.26). Other fractions had lower activity than KME. The correlation between $DPPH^{\bullet}$ scavenging capacity and phenolic concentration of KME fractions was very poor. The KMEF with the highest phenolic content (35.7%) had scavenging capacity lower than that of KMWF with moderate phenolic content (20.8%). This indicated that phenolics in the KMWF were more potent as $DPPH^{\bullet}$ scavengers than those of KMEF.

TEAC for 1 mg of fractions of KME is given in Table 3.26**.** TEAC of KME (5.8 nM) was found to be enriched in KMEF (8.0 nM) and KMWF (8.5 nM) fractions. KMHF (0.1 nM) and KMBF (1.1 nM) were having lower activity than KME. TEAC values of extracts were also not in correlation with their phenolic content.

Fe(III) reducing power of KME fractions are shown in Table 3.26**.** KMWF (32.0 µg AAE) had the highest Fe(III) reducing power among the fractions. KMEF and KMBF respectively showed 26.9 and 19.3 µg AAE reduction power. Fe(II) chelating capacity of the KME was also enriched in its ethyl acetate and water fractions. 1 mg of KMEF and KMWF respectively chelated 16.8 and 14.6% of Fe(II) ions present in the reaction system. Chelation capacity of the extract was in line with their phenolic content (Table 3.26).

Hydroxyl radical scavenging capacity of the KME fractions was found to be distributed among the fractions. KMEF (IC_{50} = 39 µg) and KMWF (IC_{50} = 48 µg) showed slightly higher activity than KME (IC_{50} = 58 µg). KMBF (IC_{50} = 68 µg) had slightly lower activity than KME Table 3.26**.** Hydroxyl scavenging capacity of fractions showed a positive correlation with their phenolic content.

SOS activity of KME fractions are presented in Table 3.26**.** KMWF (IC_{50} = 54 μg) showed the highest activity among the fractions followed by KMEF (IC_{50} = 80 μg) and KMBF (IC_{50} = 145 μg) fractions. XO inhibitory efficacy evaluation of KME fractions showed that the activity was concentrated in KMEF (IC_{50} = 78.2 μg). KMWF ((IC_{50} = 109 μg) also showed higher activity than KME ((IC_{50} = 122 μg).

$DPPH^{\bullet}$ scavenging, TEAC, Fe(III) reducing and SOS capacities of KME fractions failed to correlate with their phenolic content. This could be due to the distinct characteristics of its phenolic constituents that warranted detailed chemical characterization of these fractions.

Table 3.26: Yield, phenolic content and in vitro AO capacities of KME fractions and reference compounds (mean±SD.

SKME Fractions/ Reference compounds	Yield (%)	Total Phenolics (%) (GAE)	DPPH* Scavenging Activity. IC_{50} µg/mL	TEAC (nM)	Fe(III) Reducing Capacity/ 100 µg [(AAE) µg]	% of Fe (II) Chelation /mg	Hydroxyl radical scavenging Capacity.	Superoxide radical ion scavenging	xanthine oxidase inhibition (µg)
Methanol Extract (KME)	-	22.6 ± 1.1	6.7 ± 0.6	5.8 ± 0.2	21.3 ± 0.4	12.4 ± 0.4	58 ± 6	69 ± 11	122 ± 9.2
Hexane (KMHF)	6.9	2.9 ± 0.6	18.9 ± 0.4	0.1 ± 0.0	0.6 ± 0.0	NS	N.S	854 ± 14	NS
Ethyl acetate (KMEF)	18.6	35.7 ± 1.8	4.9 ± 0.6	8.0 ± 0.2	26.9 ± 0.2	16.8 ± 1.1	39 ± 6	80 ± 6	78 ± 10.1
Butanol (KMBF)	11.9	18.4 ± 2.2	12.3 ± 0.4	1.1 ± 0.4	19.3 ± 0.4	8.5 ± 1.2	68 ± 4	145 ± 12	451 ± 12.6
Water (KMWF)	50.5	20.8 ± 1.9	4.1 ± 0.5	8.5 ± 0.6	32.0 ± 1.4	14.6 ± 1.5	48 ± 8	54 ± 11	109 ± 9.8

GAE : Gallic acid equivalent, AAE : Ascorbic acid equivalent.

3.4.3. Chemical profiling of active fractions

Both ethyl acetate and water fractions of KME found to have considerably high AO capacity than the parent KME. This showed that major portion of the compounds responsible for the AO activity of KME was concentrated in KMEF and KMWF. The KMEF and KMWF contributed approximately 78% of the phenolics in the KME. Therefore both KMEF and KMWF were subjected to further fractionation and detailed chemical analysis. Phenolic compounds have been reported as the major AO compounds in plants. A large number of reports on the direct correlation between in-vitro AO activities and phenolic content of plant extracts are available (128,168). Therefore phenolics as major AO compounds in this extract were subjected to detailed composition analysis.

3.4.3.1 Chemical profiling of KMEF.

KMEF was subjected to detailed chemical analysis. Phenolics contributed considerable amount (35.7%) of solids in KMEF. Total flavonols estimation using $AlCl_3$ method showed a flavonol concentration of 30.4% in KMEF which contributed 85% of phenolics. This indicated that flavonols are the major phenolics in KMEF. Therefore detailed profiling of flavonoids in KMEF was attempted. Flavonoids are usually found as their free and glycosidic forms in plant kingdom. The variations in the number and nature of glycosidic bonds and carbohydrate moiety result in large diversity of flavonoid glycosides. Structural resemblance and close elution properties with common chromatographic conditions make the isolation and identification of the flavonoid glycosides a laborious task. Recently, the development of hyphenated techniques such as LC-MS and LC-NMR considerably reduces the time and labor required for the flavonoid glycoside analysis. In this study the AO active KMEF was subjected to LC-MS/MS profiling. LC-MS is a hyphenated technique in which the resolution of compounds takes place in liquid chromatograph and the fractions are subsequently subjected to mass

analysis. Mass spectra along with HPLC retention data provides an effective tool for qualitative and quantitative analysis of phytochemicals and biomolecules.

3.4.3.1a HPLC-DAD-ESI-APCI-MS profiling of flavonoid glycosides in KMEF. HPLC equipped with both DAD and MS/MS as detectors was used for the flavonoid analysis. Under the optimized chromatographic conditions, KMEF resulted more than 20 peaks in chromatogram at 360nm (Figure 3.17). Major flavonoid glycosides were identified by examining the MS and UV spectra and by comparing with the available literature on flavonoids. Reports on the flavonoid glycosides in SB juice (164), pomace (266) and berry pulp (166) provide valuable indications for their structural characterization. The identified flavonol glycosides in the fraction were grouped into four viz. mono glucosides, mono rutinosides, 3, 7, diglycosides and 3- sophroside-7-rhamnosides (Figure 3.18).

Quercetin (Peak No.9), kaempherol (Peak No.10) and isorhamnetin (Peak No.11) were identified by comparing with reference samples (Figure 3.16). Quercetin, kaempherol and isorhamnetin were characterized by their $[M-H]^-$ and $[M-15]^-$ mass peaks (Table 3.27)**.** Calibration curves using these reference compounds were plotted and used for the quantification of corresponding glycosides.

Isorhamnetin-3-O-glucoside (Peak No. 8) was identified by comparing the characteristic mass spectral peaks obtained at m/z 477 ($[M-H]^-$) and m/z 315 ($[(M-H)-162]^-$) with previous reports (266)**.** Quercetin-3-O-rutinoside (Peak No.6) was identified by the comparison with authentic reference substance. MS-MS fragmentation of molecular ion at m/z 609 ($[M-H]^-$) produced the pseudo molecular ion at m/z 301 ($[(M-H)-208]^-$). A similar fragmentation pattern was observed for isorhamnetin-3-O-rutinoside (Peak

No.7) $[M-H]^-$ at m/z 623 and $[(M-H)-208]^-$ at m/z 315. A complete loss of disaccharide, rutinose without the formation of a fragment $[(M-H)-146]^-$ from flavonol rutinosides has been reported (266) (Figure 3.17 & 3.18).

In contrast to quercetin-3-O-rutinoside, compound 4 (Peak No. 4) resulted in abundant daughter ion at m/z 463 ($[(M-H)-146]^-$) corresponding to a loss of rhamnose. The daughter ion at m/z 447 $[M-H- 162]^-$ resulting from the loss of glucose had lower intensity than that at m/z 463. Llorach et al; demonstrated the higher sensitivity glycosidic linkage at C-7 position than C-3 position towards collision-induced fragmentation (267). Based on these assumptions compound 4 was assumed to be quercetin-3-O-glucoside-7-O-rhamnoside. Compound 5 with similar pattern of mass spectra was identified as isorhamnetin-3-O-glucoside-7-O-rhamnoside (Figure 3.17 & 3.18).

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The compound 1 showed a $[M-H]^-$ at 771, which on MS-MS fragmentation resulted in a mass peak at m/z 625 ($[M-H-146]^-$). This clearly indicated the removal of rhamnosyl moiety from hydroxyl group of more sensitive C-7. MS^3 fragmentation of 771-625 yielded ions at m/z 463 ($[M-H-146-162]^-$) and m/z 315 $[M-H-146-162-162]^-$. This fragmentation pattern was in contrast to rutinoside that showed a complete loss of the disaccharide. Previous reports describe sophroside as a disaccharide in SB pulp. Therefore compounds 2 and 3 were respectively identified as kaempherol-3-O-sophroside-7-O-rhamnoside and isorhamnetin-3-O-sophroside-7-O-rhamnoside (164,266) (Figure 3.17 & 3.18).

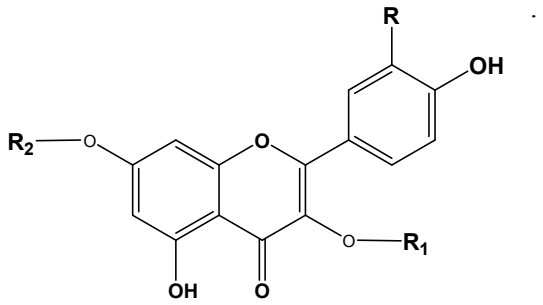
Table 3.27 HPLC-ESI-APCI profile of flavonoid glycoside in KMEF

Peak No.	Flavonoids	Characteristic mass peaks (m/z)		Amount (%)
		$[M-H]^-$	MS^2	
1	Quercetin-3-O-sophroside-7-O-rhamnoside	771	625, 463, 301	2.4 ± 0.1
2	kampherol-3-O-sophroside-7-O-rhamnoside.	755	609, 429, 285	1.3 ± 0.1
3	isorhamnetin-3-O-sophroside-7-O-rhamnoside	785	639, 459, 314	3.7 ± 0.2
4	Quercetin-3-O-glucoside-7-O-rhamnoside	609	463, 447, 301	1.1 ± 0.1
5	Isorhamnetin-3-O-glucoside-7-O-rhamnoside	625	477, 314	1.1 ± 0.0
6	Quercetin-3-O-rutinoside	609	301	5.9 ± 0.3
7	Isorhamnetin-3-O-rutinoside	623	315	4.9 ± 0.2
8	Isorhamnetin-3-O-glucoside	477	315	2.8 ± 0.1
9	Quercetin	301	286	1.1 ± 0.1
10	Kaempherol	286	271	0.6 ± 0.0
11	Isorhamnetin	315	300	2.7 ± 0,4
	Total			27.3

The amounts of identified flavonoid glycosides were quantified in terms of their aglycones using DAD and results are presented in Table 3.27. Quercetin-3-O-rutinoside (5.9%), isorhamnetin-3-O-rutinoside (4.9%) and isorhamnetin-3-O-sophroside-7-O-rhamnoside (3.7%) were found as the major flavonoid glycosides in the KMEF. Significant amounts of isorhamnetin-3-O-glucoside (2.8%) and quercetin-3-O-sophroside-7-O-

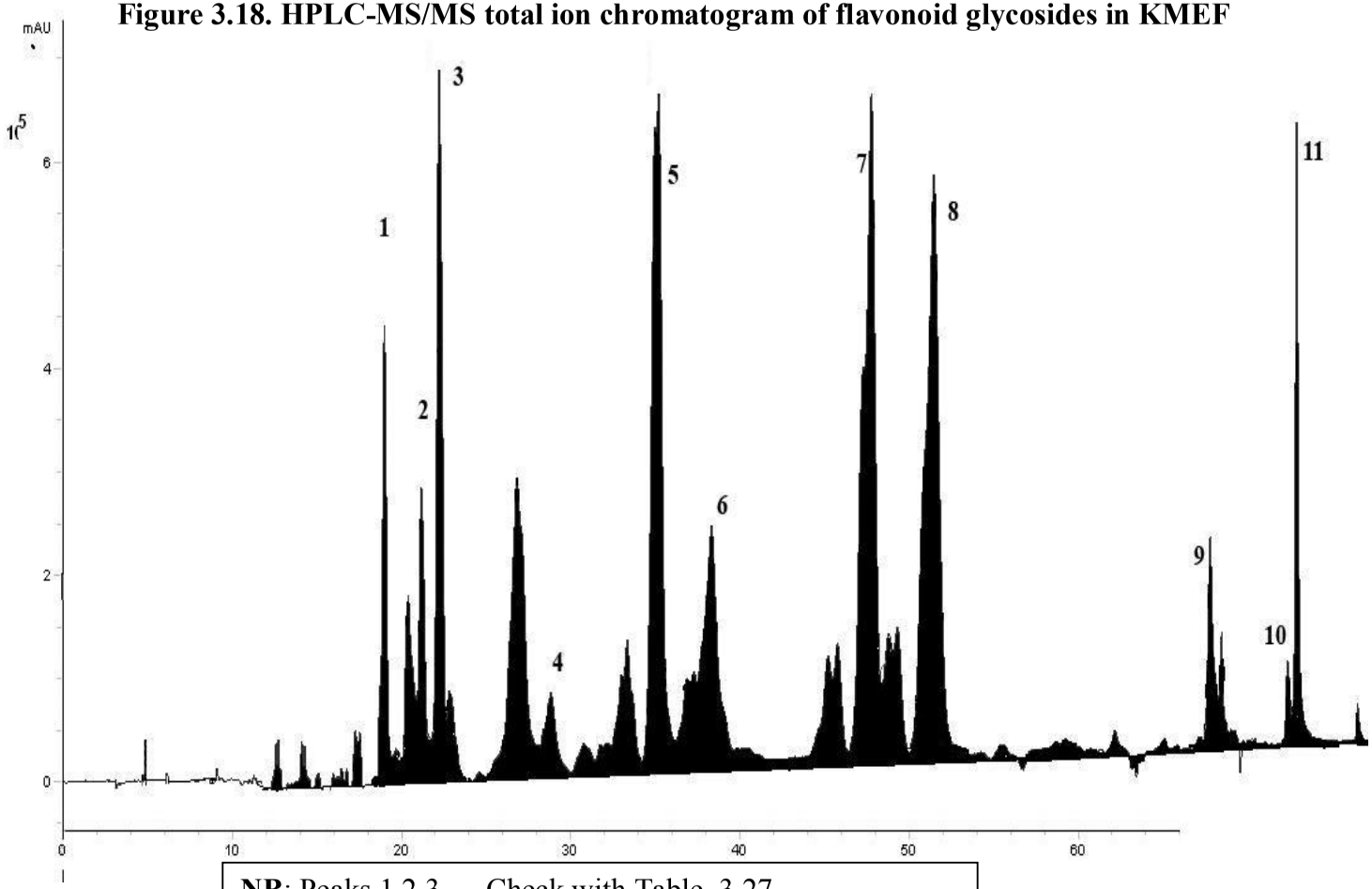
rhamnoside (2.4%) were also found in the extract. The aglycones isorhamnetin, quercetin and kaempherol respectively contributed 2.7, 1.1 and 0.6% of KMEF. The identified flavonols and their glycosides together accounted 88% (approximately) of total flavonols (determined by aluminium chloride method).

Figure 3.17. Structures of quercetin, kaempherol and isorhamnetin and their glycosides in SB seeds



Compound	R	R_1	R_2
Quercetin	OH	H	H
Quercetin-3-O-sophoroside-7-O- rhamnoside	OH	β-D-glucose-(1,2)-D-glucose	rhamnose
Quercetin-3-O-rutinoside	OH	α-L-rhamnose-(1,6)-D-glucose	H
Kaempherol	H	H	H
Kaempherol-3-O-sophroside-7-O-rhamnoside	H	β-D-glucose-(1,2)-D-glucose	rhamnose
Isorhamnetin	OMe	H	H
Isorhamnetin-3-O-sophoroside-7-O- rhamnoside	OMe	β-D-glucose-(1,2)-D-glucose	rhamnose
Isorhamnetin-3-O-glucoside-7-O- rhamnoside	OMe	glucose	rhamnose
Isorhamnetin-3-O-rutinoside	OMe	α-L-rhamnose-(1,6)-D-glucose	H
Isorhamnetin-3-O-glucoside	OMe	glucose	H

Figure 3.18. HPLC-MS/MS total ion chromatogram of flavonoid glycosides in KMEF



NB: Peaks 1,2,3…. Check with Table. 3.27

3.4.3.2 In vitro AO capacity of major flavonols in KMEF

LC MS/MS analysis showed that quercetin, kaempherol, isorhamnetin and their glycosides were the AO active compounds in the ethyl acetate fraction of kernel methanol extract. Therefore the AO activity of pure quercetin, kaempherol, isorhamnetin was evaluated and results are summarized in Table 3.28. Among the studied flavonols quercetin (IC_{50} = 12 nM) had the highest $DPPH^{\bullet}$ scavenging efficacy. Isorhamnetin and kaempherol respectively had IC_{50} values 26 and 35 nM. TEAC was also higher for quercetin (11 nM) than isorhamnetin (7 nM) and kaempherol (5 nM). Fe(III) reducing power of the flavonols was in the decreasing order of quercetin (1.2 nM), kaempherol (0.9 nM) and isorhamnetin (0.7 nM). Fe(II) chelation capacity of 5 nM of flavonols was compared. Highest Fe(II) chelation was shown by quercetin (50%) followed by isorhamnetin (39%) and kaempherol (34%). Hydroxyl radical scavenging capacity follows the order quercetin (IC_{50} = 61 nM), kaempherol (IC_{50} = 86 nM) and isorhamnetin (IC_{50} = 112 nm) IC_{50} values for SOS efficacy of quercetin, kaempherol and isorhamnetin was respectively 35, 54 and 66 nM respectively. Kaempherol (IC_{50} = 51 nm) had the highest XO inhibition capacity than isorhamnetin (IC_{50} = 59 nm) and quercetin (IC_{50} = 73 nm). Among the studied flavonols quercetin showed significantly higher AO capacity than other two. DPPH* scavenging and XO inhibition efficacies and TEAC of flavonols followed an order of quercetin > isorhamnetin > kaempherol. An activity order of quercetin > kaempherol > isorhamnetin was obtained for hydroxyl and SOS and Fe(III) reduction capacities.

The ease of oxidation and AO activity of many flavonoids and their derivatives has been demonstrated in variety of oxidation systems for many years. Radical scavenging and metal ion complexation are suggested as mechanisms of their AO action. Electron transfer from the flavonoid to the

unfilled electron deficient orbital of oxidants followed by a rapid proton transfer is suggested as the mechanism of their AO activity (268).

$$PhOH + DPPH^{\bullet} \longrightarrow PhO^{\bullet} + DPPH$$

The product, phenoxyl radical is stabilized by resonance delocalization of the unpaired electrons in the ring. Repeated studies have shown that flavonoids having greater number of hydroxyl groups or hydroxyl groups localized ortho to one another are more effective AOs. The B ring of the flavonoids is usually the initial target of oxidants as it is more electron rich than A and C rings, whose electron densities are somewhat drained away by the carbonyl group. Glycosylation of one or more of the hydroxyl groups of a flavonoid greatly reduces its AO activity. Unsaturation in C ring ($C_2 - C_3$ bond) enhances the stability of flavonoid phenoxyl radical, hence the AO activity. $C_2 - C_3$ double bond provides elongated conjugated double bond system and also results in increased planarity of molecule. Both factors favor the stability of flavonoid phenoxyl radical hence the radical scavenging (268). The free radical scavenging by flavonoids is highly dependent on the presence of a free hydroxyl group at C_3. Lower activity of flavones with similar hydroxyl configuration of flavonols demonstrates the effect of C_3 hydroxyl group. In addition to reduction in the number of hydroxyl groups, removal of the C_3 hydroxyl affects the conformation of molecule. Flavonols and flavanols with C_3 hydroxyl group are planar while the flavones and flavanones lacking this feature are slightly twisted. Planarity permits conjugation, electron dislocation and a corresponding increase in flavonoid phenoxyl radical stability. The blocking of C_3 hydroxyl group in C ring by the substitution of 3-OH by methyl and glycoside groups decreases the AO activity of flavonoids (269).

In this study significant AO activity shown by quercetin, kaempherol and isorhamnetin could be attributed to the presence of large number of hydroxyl groups including C-3 hydroxyl group along with $C_2 – C_3$ double bond. Quercetin, kaempherol and isorhamnetin are only differed in their B ring substitution pattern. Presence of orthodihydroxy groups in quercetin at 3' and 4' positions could be the reason for its significantly high AO activity over kaempherol and isorhamnetin. Slightly higher activity of isorhamnetin over kaempherol in $DPPH^{\bullet}$ scavenging and TEAC could be due to the presence of methoxyl group at 3' position. The presence of bulky methoxyl group ortho to the hydroxyl group might favored the release of $H^{\bullet}$ from hydroxyl group at 4' carbon. Lower solubility of isorhamnetin might be the reason for its slightly lower hydroxyl and SOS capacities and Fe(III) reduction power.

XO inhibitory activity of flavonoids depends on their hydroxyl group configuration and planarity of molecule. Hydroxyl groups at C-5 and C-7 and double bond between C-2 and C-3 are the important contributing factors to flavonoid's XO inhibition capacity. The $C_2 – C_3$ double bond keeps the flavonoid molecule planar. The presence of hydroxyl group at C-3 position slightly decreases the inhibition (270). Significant inhibitory activity shown by quercetin, kaempherol and isorhamnetin in this study could be attributed to their hydroxyl groups at C-5 and C-7 positions and $C_2 – C_3$ double bond. The inhibitory activity of flavonols (kaempherol > isorhamnetin > quercetin) was in confirmation with the previous results (270).

Table 3.28: In vitro AO capacities of flavonols (mean±SD).

Activity	Quercetin	Kaempherol	Isorhamnetin
DPPH* scavenging IC_{50} (nM)	12 ± 2	35 ± 6	26 ± 3
TEAC (nM)	11 ± 2	5 ± 1	7 ± 1
Fe(III) reducing /nM (AAE)	1.2 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
% of Fe (II) Chelation/5nM	50 ± 6	34 ± 3	39 ± 2
Hydroxyl radical scavenging. IC_{50} (nM)	61 ± 9	86 ± 11	112 ± 12
SOS. IC_{50} (nM)	35 ± 5	54 ± 6	66 ± 3
XO Inhibition IC_{50} (nM)	73 ± 14	51 ± 19	59 ± 22

3.4.3.3 Chemical Profiling of KMWF.

Tannins constitute greater proportions of high polar fractions of phenolics and accounts for a major class of phenolics in plant kingdom. Tannins are formed by the condensation of simple phenolics. Tannins are generally classified into hydrolysable tannins and condensed tannins or PAs. Hydrolysable tannins are high molecular weight derivatives of phenolic acids mainly gallic and ellagic acid. Gallic acid and ellagic acid derivatives are respectively called gallotannins and ellagitannins. Both gallotannins and ellagitannins are derived from pentagalloyl glucose but the latter has hexahydroxydiphenyl units and liberates ellagic acid on hydrolysis.

Tannins are the least studied class of phenolics in SB. Due to the high molecular size and complexity in structure resolution and subsequent analysis of tannins are difficult. In this study, therefore the tannin rich water fraction of kernel was hydrolyzed and phenolic acid composition was analyzed using

HPLC. PAs in the fraction was further fractionated on sephadex gel column chromatography and characterized in terms of prodelphinidin: procyanidin ratio and ADP.

3.4.3.3a Phenolic acid composition of KMWF

Phenolic acid composition of KMWF hydrolysate is presented in Table 3.29**.** The phenolic acid profile thus obtained included the phenolic acids liberated not only from the tannins but also from other low molecular weight glycosides and esters. Gallic acid was the predominant (5.39%) among the phenolic acids in KMWF. Gallic acid constituted 62.7% of total phenolic acid in KMWF, indicating that gallotannins are the major hydrolysable tannins in SB kernel. Low concentration of ellagic acid (0.39%) showed comparatively lower amount of ellagitannins. Reports on the tannin profile of SB seeds are not available hence the discussion with reference to previous report was not attempted. Isolation and utilization of SB leaf hydrolysable tannins as an antiviral drug under the trade name 'Hiporamin' have been reported (271). Detailed studies on the biological and chemical properties of seed hydrolysable tannins are warranted.

Phenolic acids other than gallic acid and ellagic acids were mainly liberated from their low molecular weight glycosides and esters. The amount of these phenolic acids in KMWF decreased in the order of protocatacheuic acid (1.25%), vanillic acid (0.90%), p-hydroxybenzoic acid (0.56%) and ferulic acid (0.39%). Small amounts of cinnamic acid, p-coumaric acid and caffeic acid were also detected in the extract (Table 3.29, Figure 3.11).

Table 3.29: Phenolic acid composition of KMWF

Composition	Amount (%)
Gallic Acid	5.39 ± 0.12
Protocatechuic Acid	1.25 ± 0.14
p-hydroxy benzoic acid	0.56 ± 0.09
Vanillic Acid	0.90 ± 0.08
Ellagic acid	0.03 ± 0.00
Cinnamic Acid	0.14 ± 0.02
P- Coumaric Acid	0.01 ± 0.00
Ferulic Acid	0.39 ± 0.05
Caffeic Acid	0.05 ± 0.01
Total	8.59

Total phenolic acid content in the extract was 8.59% accounting for 41.3% of total phenolics. This indicated that hydrolysable tannins along with other low molecular weight phenolic acid derivatives contributed to the AO capacity of KMWF. In vitro AO capacity evaluation of gallic acid in this study and previous reports confirmed the AO role of phenolic acids derivatives (268). This study also showed high $DPPH^{\bullet}$, $ABTS^{\bullet}$ scavenging and Fe(II) reduction capacity for gallic acid. Further, moderate SOS and XO inhibitory activities were demonstrated for gallic acid. Radical scavenging and metal chelation are suggested as the mechanism of AO action of phenolic acids. Number and configuration of hydroxyl groups particularly, orthodihydroxy groups and extended conjugation in side chain (eg; cinnamic acid derivatives) are the major contributing factors for their radical scavenging efficacy. Among the naturally occurring phenolic acids, gallic acid with its three hydroxyl groups is probably the most readily oxidized and

would be expected to the best AO (268). As a constituent of gallotannins, it presumably contributes significantly to the AO activity of plants.

3.4.3b. Characterization of PAs in KMWF

Total PA content in KMWF was found to be 12.8%, which constituted 60% of total phenolics in the extract. Therefore characterization of PAs in the extract was attempted. PAs are natural polymeric plant products composed of flavon-3-ols linked together by carbon-carbon bonds. PA structure varies considerably with unit; inter flavonoid bond location and branching. Modification with non -flavonoid substitutes and molecular weight distribution also contribute to this variation. Owing to this complexity and high molecular weight complete characterization of PAs still remains a difficult task in natural product chemistry. Several colorimetric methods have been proposed for their quantitative analysis (272). Depolymerisation of PAs in the presence of nucleophiles is frequently employed for the structural analysis of PAs (273). The nucleophile forms adduct with the chain extender units in the polymer, which are purified or analyzed by chromatography. Their structure once established can be used to determine the nature of monomer units within the polymer. Numerous nucleophiles such as thioacetic acid (274), benzene-p-sulphonic acid (275), benzyl mercaptan (276), mercaptanol (277) and phloroglucinol (278) have been used for the characterization of PAs. Phloroglucinol is usually preferred to the sulphur nucleophile because it is odorless (273).

Size exclusion chromatography is exclusively used for the resolution of PAs. In this study KMWF was subjected to size exclusion chromatography using sephadex gel. One fraction of KMWF PAs was purified by washing with ethanol. The purified PA fraction (KPAF) was eluted with 25% acetone (v/v) and subjected to depolymerisation in presence of a nucleophile (phloroglucinol). The reaction products were analyzed in LC-MS/MS.

Purified PA of SKWF was hydrolyzed in presence of phloroglucinol (PG) and the reaction products were analyzed in HPLC-DAD-MS/MS. Under acidic conditions, PAs become depolymerized, releasing terminal sub units as flavan-3-ol monomers and extension sub units as electrophilic flavan-3-ol intermediates. The electrophilic intermediates can be trapped by nucleophilic reagents such as phloroglucinol to generate analyzable adducts. Reaction products were identified by analyzing the uv and mass spectra and by comparing with the available reports (165,279). Amounts of individual products were quantified from the calibration plots obtained with catechin and epicatechin. ESI-MS conditions were optimized with catechin. A negative mode ESI-MS analysis was performed (Figures 3.19 & 3.20).

Peaks 1 and 2 respectively belonged to ascorbic acid and PG (Figure 3.20). Compounds 3 and 4 resulted in a similar mass spectra with a pseudo molecular ion peak at m/z 429 ($[M-H]^-$) and ions at m/z 465 ($[M+Cl]^-$). MS/MS fragmentation of m/z 429 produced daughter ions at m/z 303 ($[M-H-C_6H_6O_3]^-$) indicating lose of phloroglucinol moiety, and Retro Diels-Alder product at m/z 261 ($[M-H-C_8H_8O_4]^-$). Based on these mass spectra compounds 3 and 4 were assumed to be either gallocatechin phloroglucinol or epigallocatechin phloroglucinol. Comparing the retention data of these compounds with available reports, compound 3 and 4 were respectively assigned to be gallocatechin-(4α-2)-phloroglucinol and epigallocatechin-(4β-2)-phloroglucinol adducts (Figure 3.19) (165).

Compounds 6 and 7 (Figure 3.20) also resulted in similar mass spectra. Pseudo molecular ions at m/z 413 ($[M-H]^-$) along with m/z at 449 ($[M+Cl]^-$) indicated the presence of (epi) catechin phloroglucinol. MS/MS analysis of m/z 413 resulted in daughter ions at m/z 287 ($[M-H-C_6H_6O_3]^-$) (loss of PG) and at m/z 261 ($[M-H-C_8H_8O_4]^-$) (RDA fission) further confirmed the

assigned structure. From the retention data compounds 6 and 7 respectively assumed to be catechin-(4α -2)-phloroglucinol and epicatechin-(4α -2)-phloroglucinol (Figure 3.19) (279).

Compounds 5 and 8 (Figure 3.20) with molecular ion peaks at m/z 305 ($[M-H]^-$) and daughter ions at m/z 287 ($[M-H-H_2O]^-$) and m/z 179 ($[M-H-C_6H_6O_3]^-$) were assumed to be (epi)gallocatechin (279). Comparing with the retention data compounds 5 and 8 were respectively assigned as gallocatechin and epigallocatechin. Compounds 9 and 10 were identified as catechin and epicatechin by comparing with authentic standards. Calibration curves using catechin and epicatechin were plotted and used for the quantification of reaction products (Figure 3.20).

Quantification of reaction products were performed using DAD responses. Epigallocatechin-phloroglucinol (21.3mM) and gallocatechin-phloroglucinol (20.3) adducts respectively had higher concentration among the reaction products. 5.6 and 2.5 mM of catechin and epicatechin phloroglucinol adducts were respectively found in hydrolyzed fractions. Gallocatechin (1.0 mM), epigallocatechin (1.8 mM), catechin (0.2 mM) and epicatechin (0.7 mM) were also detected in the fraction.

Table 3.30. Concentration of reaction products after acid catalyzed cleavage of KMWF proanthocyanidins in presence of phloroglucinol.

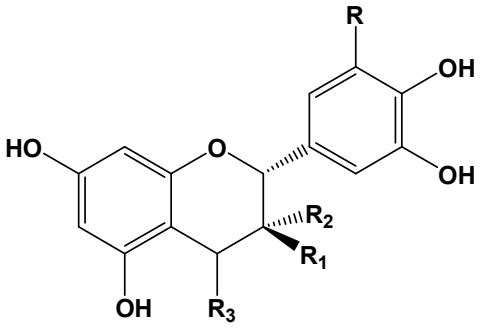
Compounds	Characteristic mass peaks (m/z)			Conc. (mM)
	$[M-H]^-$	$[M+Cl]^-$	MS^2	
gallocatechin-(4α-2)-phloroglucinol	429	465	303,261	20.3
epigallocatechin-(4β-2)-phloroglucinol	429	465	303,261	21.3
catechin-(4α-2)-phloroglucinol	413	449	287,261	5.6
epicatechin-(4β-2)-phloroglucinol	413	449	287,261	2.5
Gallocatechin	305	-	287,179	1.0
Epigallocatechin	305	-	287,179	1.8
Catechin	289	-	-	0.2
Epicatechin	289	-	-	0.7
Prodelphinidin – procyanidin ratio				4.9 : 1

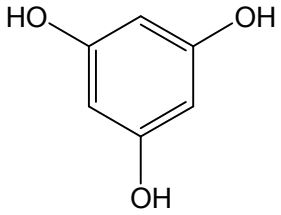
PAs can be classified into 6 sub units (procyanidins, prodelphinidins, profisetinidins, propelargonidins, prorobinetidins and proguibourtinidins) depending on the hydroxyl substitution pattern of single favan-3-ol units Procyanidins consist of catechin and epicatechin extension units and prodelphinidins of gallocatechin and epigallocatechin sub units. Acid catalysed depolymerisation of SB seeds mainly resulted (epi)catechin and (epi)gallocatechin derivatives, showing that mainly procyanidins and prodelphinidins constituted seed PAs. Therefore prodelphinidin – procyanidin ratio as ratio of (epi)gallocatechin to (epi)catechin derivatives, was estimated from the concentrations of reaction products. The purified fraction of kernel PA found to have a prodelphinidin – procyanidin ratio of 4.9:1 (Table 3.30).

Some reports on the composition of SB seed PA were published recently (165,167,168). Since these reports describe the composition of

different fraction of seed PA, no quantitative correlation could be expected between these reports. The present study dealt with the composition of purified total PA in seed kernel and results are in qualitative agreement with the available reports.

Figure 3.19. Structures of reaction products obtained by the acid catalyzed cleavage of SB PAs in presence of phloroglucinol.

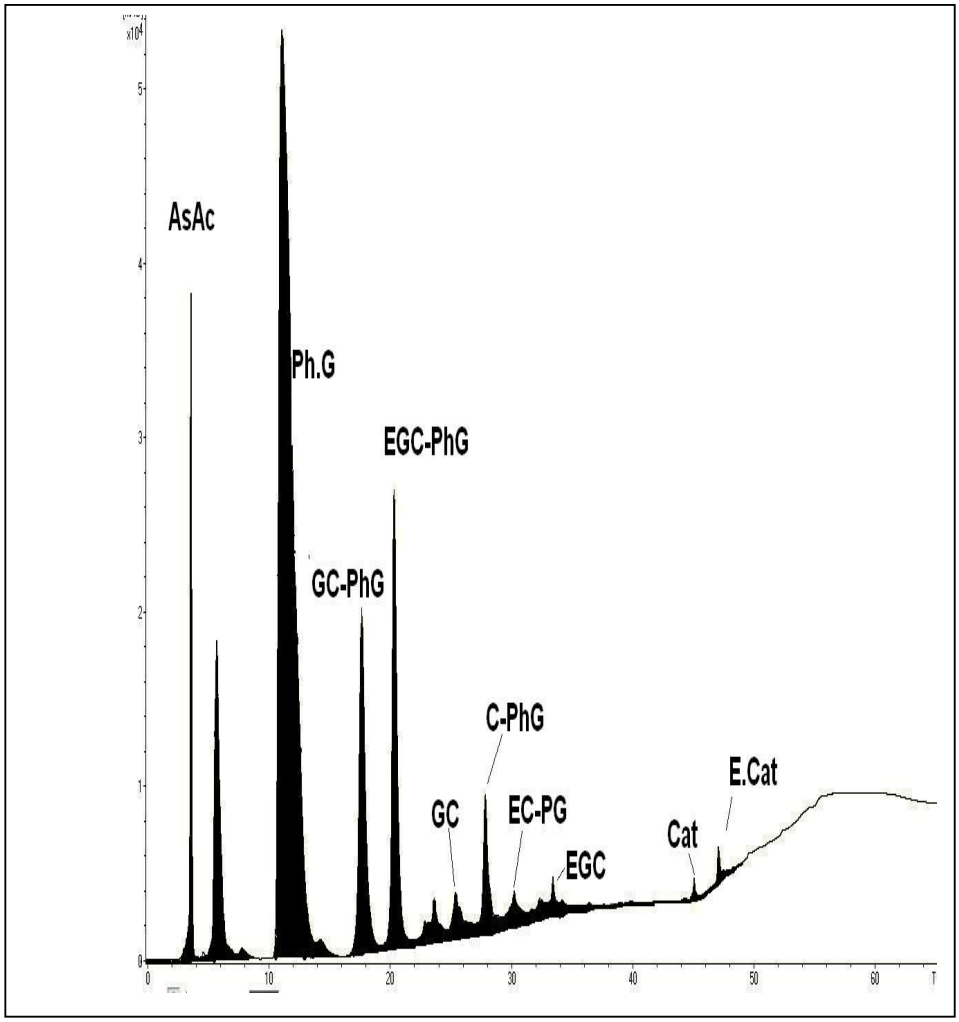




Phloroglucinol (Ph.G)

Compound	**R**	**R_1**	**R_2**	**R_3**
Catechin	H	OH	H	H
Epicatechin	H	H	OH	H
Gallocatehin	OH	OH	H	H
Epigallocatechin	OH	H	OH	H
catechin-(4α -2)-phloroglucinol	H	OH	H	Ph.G
epicatechin-(4β-2)-phloroglucinol	H	H	OH	Ph.G
gallocatechin-(4α-2)-phloroglucinol	OH	OH	H	Ph.G
epigallocatechin-(4β-2)-phloroglucinol	OH	H	OH	Ph.G

Figure 3.20. Total ion chromatogram of depolymerisation reaction mixture of purified PAs from KMWF



AsAc: ascorbic acid, PhG: phloroglucinol, GC-PhG: gallocatechin-phloroglucinol, EGC-PhG: epigallocatechin-phloroglucinol, GC; : gallocatechin, C-PhG: catechin-phloroglucinol, : EC-PhG : epicatechin-phloroglucinol, : EGC: epigallocatechin, Cat: catechin, E Cat: epicatechin

3.4.3.3c Fractionation of KMWF

Until now many chromatographic separation methods of high molecular weight polyphenols especially PA have been developed, but there is no perfect method satisfying separation efficiency, simplicity and scale of treatment. Most of these methods fail in the resolution of high molecular weight PA (280). Size exclusion chromatography is considered as a relatively simple and efficient technique to resolve moderately high molecular weight polyphenols. In this study resolution of KMWF using sephadex gel size exclusion chromatography was attempted. Most of the non phenolics in KMWF were washed out with 100% ethanol. Low molecular weight phenolics including PAs up to trimers have been reported to be eluted with ethanol (280). Therefore ethanol eluents were collected as fraction 1. Increasing concentration of water in acetone was used to elute remaining high molecular weight polyphenols mainly PA.

3.4.3.3d. Yield and composition of KMWF fractions

Yield and composition of fractions are presented in Table 3.31. A major fraction of KMWF was eluted as F-1 (48.4%). F-4, F-5 and F-3 respectively yielded 10.9, 3.1 and 1.3% of KMWF. Other fractions had < 1% yield.

Phenolic content analysis showed that fractions except F-1 had higher concentration of phenolics than that in KMWF. High yield of F-1 with low phenolic concentration showed that most of the non-phenolics in KMWF were eluted with ethanol as F-1 and most of the phenolics (> 70%) were distributed between the remaining fractions. Fractions F-3, F-7 and F-8 together had more than 90% phenolic concentration. F-2, F-4, F-5 and F-6 found to contain 60 – 80% phenolics. F-4 accounted for the highest proportion (32.4%) of phenolics in SKWF, followed by F-1 (28.6%), F-5 (10.1%) and F-3 (6.2%).

Table 3.31 Yield and composition of KMWF fractions.

Fractions	Yield (%)	TPC (%)	PAs (%)	ADP
1	48.4	12.3 ± 0.9	1.0 ± 0.3	1.96
2	0.9	80.2 ± 1.9	37.4 ± 0.9	3.09
3	1.3	98 ± 2.2	97.8 ± 4.5	3.75
4	10.9	61.9 ± 2.9	62.9 ± 1.9	3.89
5	3.1	70.8 ± 1.4	80.5 ± 1.6	5.76
6	0.8	74.1 ± 3.2	58.5 ± 0.7	5.88
7	0.2	96.7 ± 0.9	99.1 ± 5.1	7.05
8	0.3	96.1 ± 3.7	20.0 ± 0.9	8.54

PA content in these fractions was analyzed by vanillin-HCl estimation method. Similar to phenolic concentration, all the fractions except F-1 contained higher amount of PA than that in KMWF. In contrast to phenolics F-1 accounted only for 3.8% PA in KMWF. This indicated that phenolics other than PA were preferentially eluted with ethanol. Ethanol fraction might also contain low molecular weight PA. Most of the PAs in KMWF was found as eluted with various proportions of acetone and water. F-3 and F-7 had more than 95% PA concentration. F-5, F-4 and F-6 respectively contained 80.5, 62.9 and 58.5% PA. Fractions 3 to 5 accounted for 82% of total PAs in KMWF. F-4 alone contributed 32.4% of PA in KMWF. Close association of values obtained for the phenolic and PA contents in F-3, F-4, F-5 and F-7 indicated that PA constituted most of the phenolics in these fractions. Of the 96% of phenolics in F-8, PA contributed only 20% indicating that F-8 contained polar phenolics other than PA. These results showed that size exclusion chromatographic technique used here is effective for the preparation of phenolics especially PA enriched fractions.

ADP of PA fractions. The greater parts of biological and chemical properties of PA are governed by their chemical structure and size. Therefore ADP of the PA fractions as an indication of their molecular size were estimated as the ratio of absorbance of the anthocyanins formed by the oxidative depolymerisation of PA to the absorbance of vanillin PA adduct. The fractions of water extract obtained by the sephadex gel chromatography were evaluated for their ADP and summarized in Table 3.31. The fractions 1 to 8 showed a gradual increase in their polymerization index. ADP varies from 1.96 (F-1) to 8.54 (F-8). Fractions 3 to 5 with ADP varying from 3.75 to 5.76 accounted for 82% of total PAs in KMWF. The elution of PA in the increasing order of ADP showed that the chromatographic mode is adsorption rather than size exclusion.

3.4.3.3e. AO capacities of KMWF fractions

KMWF fractions obtained by the size exclusion chromatography were subjected to AO capacity studies. $DPPH^{\bullet}$ scavenging capacity evaluation showed that all the fractions except F-1 had higher radical scavenging activity ($IC_{50} \leq 3.5\mu g$) than KMWF ($IC_{50} = 4.1\ \mu g$). F-3, F-7 and F-8 showed higher activity than reference gallic acid. ($IC_{50} = 1.6\ \mu g$) (Table 3.32). $DPPH^{\bullet}$ scavenging capacity of fractions except F-8 was in correlation with their phenolics and PA content. These results showed that PA was the major phenolic AOs in fractions 1-7. F-8 might have some radical scavenging phenolics other than PA. No direct correlation was observed between the $DPPH^{\bullet}$ scavenging capacity and ADP of fractions (Table 3.31 & 3.32).

TEAC values of 1 mg of KMWF fractions are summarized in Table 3.32. F-3, F-4 and F-7 showed significantly higher TEAC values (approx. 14 nM). F-1 (4.6 nM) had the lowest TEAC value. F-2, F-5 and F-6 respectively had the TEAC values 4.7, 7.3 and 4.4 nM. In contrast to $DPPH^{\bullet}$ scavenging activity F-8 had lower TEAC value than that of 2 to 7 fractions. TEAC

values of fractions did not show any dependence on the ADP of PAs in the fractions.

Fe(III) reducing power of KMWF fractions was compared in terms of ascorbic acid equivalents (Table. 3.32). Fractions 2 to 8 had higher reducing power than KMWF. F-3 (8.74 µg) had the highest reducing power followed by F-5 (5.61 µg), F-8 (5.43 µg), F-4 (5.42 µg) and F-6 (4.81 µg). Reducing power also did not show any direct correlation with degree of polymerization of PA fractions. Activity of most of the fractions was higher than gallic acid.

Fe(II) chelation capacity of the fractions were determined as the stability of their complexes. The % of Fe(II) chelated by 1 mg of fractions is presented in Table.3.32**.** F-2 (65 %) showed the highest chelation capacity followed by F-3 (53 %), F-5 (48%) and F-6 (47 %). 1 mg of F-1 and F-4 respectively chelated 39.6 and 16.4% of Fe(II) in the system. No significant Fe(II) chelation capacity was showed by F-7 and F-8 under experimental conditions. There was no correlation between Fe(II) chelation capacity of fractions and their ADP.

Hydroxyl radical scavenging capacity of the fractions is presented in (Table.3.32**)**. Fractions 2 to 6 had higher scavenging capacity than KMWF. F-3 (17 µg) showed the highest activity and the activity of other fractions decreased in the order of F-4, F-2, F-6 and F-8.

SOS capacity of the fractions was evaluated and given in Table.3.32**.** All the fractions except F-1 showed higher SOS activity than KMWF. Among the KMWF fractions F-3 (IC_{50} = 11.8 µg) had the highest activity followed by F-2 (IC_{50} = 12.2 µg), F-4 (IC_{50} = 14.2 µg) and F-7 (IC_{50} = 19.8 µg). Fractions F-5 (IC_{50} = 38.2 µg), F-6 (IC_{50} = 27.8 µg) and F-7 (IC_{50} = 25.4 µg) also showed high SOS efficacy.

All the fractions found to inhibit the XO inhibitory activity in a dose dependent manner. F-2 (IC_{50} = 3.9 μg) had higher XO inhibition capacity than allopurinol. All the fractions showed higher inhibition activity than KMWF. The IC_{50} values of remaining fractions varied between 17.0 to 71.9 μg. Inhibition activity of the fractions did not show any dependence on the degree of polymerization.

Sephadex gel size exclusion chromatography was effective in resolving PAs in increasing order of molecular size. Fractions F-3 to F-5 found to contribute larger portion of PA in the kernel. These fractions with ADP 3.7 to 5.8 had significantly high AO capacity among the fractions. No direct correlation was observed between the ADP and in-vitro AO efficacy of fractions. Number of phenolic groups has a positive correlation with radical scavenging efficacies of phenolic compounds. In this case molecular size and number of phenolic groups per molecules increased from fractions 2 to 7. Molecular size and stereochemistry also contribute to the activity of molecules. After certain limits solubility of high molecular weight compounds decreases with molecular size. Steric hindrance to active sites of molecules increases with molecular size. These opposing factors might contribute to the poor correlation between ADP and in vitro AO capacities of PA fractions.

Table 3.32: In vitro AO capacities of KMWF fractions (mean±SD N=5).

Fractions	DPPH Radical scavenging capacity IC_{50} (μg)/mL	TEAC (nM)	% of Fe(II) Chelation/1 mg	Fe(III) reducing power/100 μg [AAE (μg)]	Hydroxyl radical scavenging capacity IC_{50} (μg)	SOS IC_{50} (μg)	XO Inhibition IC_{50} (μg)
1	10.9 ± 0.5	4.6 ± 0.4	39.6 ± 1.1	83 ± 0.04	131 ± 12	63.5	30.3
2	3.5 ± 0.4	4.7 ± 0.2	65.0 ± 3.4	831 ± 0.86	22 ± 5	12.2	3.9
3	1.5 ± 0.2	14.2 ± 0.7	53.0 ± 7.1	874 ± 0.65	17 ± 4	11.8	17.03
4	2.9 ± 0.2	14.0 ±1.1	16.4 ± 2.5	542 ± 0.36	21 ± 2	14.2	57.6
5	3.9 ± 0.5	7.3 ± 0.9	48.0 ± 1.9	561 ± 0.94	37 ± 4	38.2	44.0
6	2.8 ± 0.2	4.4 ± 0.1	47.0 ± 3.4	481 ± 0.69	23 ± 3	27.8	60.0
7	0.9 ± 0.9	14.5 ± 0.3	NS	612 ± 1.02	61 ± 4	19.8	48.0
8	1.5 ± 0.7	3.0 ± 0.7	NS	543 ± 0.86	78 ± 5	25.4	71.9

Tannins are wide spread in plant derived foods and thus may be important dietary AOs. Polymeric polyphenols are reported as more potent AOs than simple phenolics. Tannins are reported to have little or no pro-oxidant effects, whereas many small phenolics are pro-oxidants (281). Several biological effects are attributed to PAs. Most of these activities are related to their AO activities. Faria et al; demonstrated a positive correlation between AO activity of grape PA fractions and their capacity to inhibit cell proliferation (282). Tannins form strong complexes with protein and are resistant to degradation. Tannins are important AOs in digestive tract by sparing with other AOs and thus indirectly increasing the AO levels in other tissues. Tannins may also protect proteins, carbohydrates and lipids in digestive tract from oxidative damage during digestion (283).

This is the first comprehensive study on the in vitro AO capacities of SB seeds and their correlation with the chemical composition of active extracts. Chemical finger printing of AO active fractions of SB seeds using modern analytical techniques such as LC-MS/MS was attempted here for the first time. Polyphenols particularly flavonols, phenolic acid derivatives and PAs were found as the major AO compounds in SB seeds. Phenolics are generally nontoxic and manifest a diverse range of beneficial biological activities. The role of phenolics in cancer prevention, cardioprotection and prevention of neurodegenerative diseases are widely discussed. Several studies have shown that phenolics are extensively metabolized in vivo resulting a significant alteration in their AO capacities (283). The physiological significance of dietary AOs depends on their mechanism of absorption and biotransformation, thus warranting further investigations on the bioavailability of SB phenolics. Isolation of major active compounds and subsequent activity evaluation is required in order to gather knowledge on their structure activity relationships.

Chapter 4

SUMMARY AND CONCLUSION

Let food be thy medicine, thy medicine shall be thy food

.....Hippocrates

4.1. The inverse association between intake of fruits and vegetables and delayed onset of chronic diseases are well documented by epidemiological studies. Recently detailed studies on the chemical composition of fruits and vegetables and its relation with their physiological activities attained much interest among researchers. The knowledge on interactions between phytochemicals and physiological systems are particularly important in assessing their biological benefits as well as their possible toxic effects. In the present study SB *(H. rhamnoides)* berries were investigated from this perspective. SB, particularly in India, is a underutilized medicinal plant. Detailed chemical profiling of H. rhamnoides berries, quality evaluation of berries belonging to major SB species grown in India, development of a green integrated process for processing of fresh berries for nutraceutical and cosmaceutical applications, evaluation of AO activity of berry extracts and chemical profiling of active fractions were therefore set as the main objectives of the present study. The results from this investigation thus could facilitate commercial utilization of Indian SB berries.

4.2 Detailed chemical profiling of SB berries belonging to the most common species in India i.e. *H. rhamnoides* was conducted for the first time. The berries were separated into pulp, seed coat and kernel and analyzed in detail. They were characterized in terms of their proximate composition, lipid profile, phenolic and organic acid profiles. Pulp and seed showed considerable variations in terms of their proximate composition. GC analysis of pulp and seed oil FAs indicated unique FA profiles for both oils. The occurrence of large proportion (48.2%) of palmitoleic acid in pulp oil is unique among the oils of plant origin. Seed oil was rich in the two essential fatty acids, linoleic (29.5%) and α-linolenic acids (26.2%). Pulp oil was found to have significantly higher amount of carotenoids (2548 mg/Kg) whereas seed oil contained moderate amount of carotenoids (389 mg/Kg). HPLC analysis showed that both pulp and seed oils were rich in tocols. Pulp oil had

1394 mg/kg of tocols with α-tocopherol accounting for 70% along with β-tocopherol, δ-tocopherol, γ-tocopherol, α-tocotrienol, γ-tocotrienol and δ-tocotrienol in minor amounts. Seed oil with 1193 mg/kg of tocols also had α-tocopherol as the major tocol. In contrast to the pulp oil, seed oil had high amount of γ-tocopherol. Presence of α-tocotrienol, β-tocopherol and δ-tocotrienol were also detected in seed oil. HPLC analysis of sterols shown that β-sitosterol and stigma sterol were the major sterols in pulp and seed oils. Seed oil (1.62%) had significantly higher amount of phytosterols than that of pulp oil (0.41%). Fresh SB Berries were highly acidic in nature with 3.6% (malic acid equi.) titrable acids. Quinic acid isolated from the berry pulp along with other organic acids was used to optimize RP-HPLC-DAD analytical conditions for the profiling of organic acids. HPLC analysis showed that quinic acid (2.80%) as the major organic acid in SB berries, along with malic acid (1.60%), citric acid (0.16%) and traces of fumaric acid and maleic acids. HPLC analysis also revealed that Indian berries *(H. rhamnoides)* also contained high levels of vitamin C (2232 mg/kg of fresh wt.).

4.3 Detailed profiling of polyphenols in *H. rhamnoides* berries were also attempted. Pulp, Seed coat and kernel respectively contained 2.73, 3.95 and 8.58% of phenolics on dry weight. A flavonol named isorhamnetin was isolated from the hydrosylate of kernel methanol extract. RP-HPLC-DAD conditions for the quantitative profiling of SB flavonols were optimized. Pulp, seed coat and kernel respectively had 288, 18 and 553 mg/100g of flavonols. Quercetin, kaempherol and isorhamnetin were identified as the major flavonols in the SB berries. PAs were found as a major class of phenolics in SB berries. PAs constituted 46, 22 and 51% of total phenolics in pulp, seed coat and kernel respectively. Extraction conditions for the preparation of PA rich extracts were optimized. ADP of purified PA factions of pulp, seed coat

and kernel were respectively 7.4, 8.2 and 5.6. RP-HPLC-DAD conditions for the qualitative and quantitative profiling of phenolic acids in SB berries were optimized and validated. Phenolic acids in the berries were separated into free, glycosides and esters and analyzed. Gallic, protocatechuic, p-hydroxybenzoic, vanillic, salicylic, cinnamic, p-coumaric, ferulic and caffeic acids were identified and quantified in the extracts.

4.4 The quality of SB berries belonging to major species and different geo-climatic regions in Indian Himalayas were compared in terms of their composition of bioactive components. SB berries belonging to *H. rhamnoides, H. tibetana* and *H. salcifolia* species were collected from the cold deserts of Himalayas (Lahaul, Ladakh and Spiti) and characterized in terms of their chemical profile of pulp oil and vitamin C and phenolic content. Composition of SB berries varied significantly from species to species. Total carotenoids varied from 692 to 3420 mg/kg in pulp oils of fresh berries. The amount of total tocols in the pulp oils ranged from 666 to 1788 mg/kg. *H. salicifolia* berries showed small amount of pulp oil with lower levels of carotenoids and tocopherols. No much difference in the proportion of individual tocols in pulp was noticed among the varieties. α-tocopherol alone constituted 40-60% of total pulp tocols in berries. Pulp oils had palmitoleic (32 – 53%) as the most abundant fatty acid followed by palmitic (25 – 35%), oleic (8 – 26%), linoleic (5 – 16%) and linolenic (0.6 – 2.6%), with highest deviation observed in the proportion of palmitoleic acid in these berries. *H. rhamnoides and H. tibetana* species contained the highest amount of lipophillic constituents like carotenoids and tocols. *H. salicifolia* berries had significantly higher amount of lipophobic constituents like vitamin C and flavonols. This is the first comprehensive report on the systematic chemical profiling of SB berries of Indian origin. For the first time the distinct nature of *H. salcifolia* berries, which is found only in Himalayan region, with high content of hydrophilic components like flavonols and vitamin C and very low

content of oil and lipophilc components like carotenoids and tocopherols was revealed. Nutritional quality of berries of *H. tibetana*, with its limited distribution in India, is reported here for the first time.

4.5. An efficient process for the production of nutraceutical and cosmaceutical products from fresh SB berries was developed at pilot scale and process streams and products were chemically evaluated. In this process fresh berries were subjected to high-pressure continuous dewatering using screw press. The separated liquid phase containing 80–90% of pulp oil was clarified at 80^{o} C and centrifuged to obtain pulp oil, clear juice and sludge. The pulp oil yield was 2.7–2.8% of fresh berry weight with 66–70% extraction efficiency. The pulp oil was remarkably rich in carotenoids, tocopherols and sterols, with a characteristic fresh berry flavor with palmitoleic acid as the major fatty acid. The clear juice obtained was free from oil and contained high amounts of vitamin C and other phytochemicals such as polyphenols and flavonoids. Isorhamnetin along with quercetin and kaempherol were the major flavonoid in the juice. The juice was very acidic (pH 3), with high concentrations of organic acids. 50 to 80% of the bioactive phytochemicals in the pulp was found to be retained with juice. Supercritical CO_2 conditions for the extraction of oil from the seeds were optimized. The seeds separated from the pressed cake were subjected to supercritical CO_2 extraction. Chemical composition of seed oil thus obtained was compared with that of oil extracted with hexane. Such a product development approach with out using chemicals and organic solvents with high retention of the bioactive phytochemicals in the end products is reported for the first time. The process was developed at pilot scale and techno-economic feasibility was established.

4.6 In the present study it was attempted to fill the gap in the knowledge on AO capacity of the berries and their chemical composition. AO activity guided fractionation of berries and chemical profiling of active fractions were attempted. The berry extracts particularly kernel extracts showed high AO activities and high yield. Activity guided fractionation showed that major AO active phytochemicals in kernel were concentrated in its ethyl acetate and water fractions of methanol extract. Therefore, these fractions were further subjected to detailed chemical profiling.

4.7. Phenolics especially flavonoids were found as the major active components in the ethyl acetate fraction. HPLC-DAD-MS/MS conditions for the analysis of flavonoid glycosides were therefore optimized and ethyl acetate fraction was subjected to HPLC-MS/MS analysis. Nine flavonoid glycosides and 3 flavonols were identified as the major components in the ethyl acetate extract. The identified flavonoids could account for the major fraction (approx. 88%) of phenolics in the ethyl acetate fraction. This is the first report on the LC-MS profiling of SB seed flavonoids.

4.8. Water fraction was analyzed for its tannin composition as tannins are the major high polar phenolics in the plants. Water fraction was hydrolyzed and subjected to HPLC analysis for phenolic acid composition. Gallic acid constituted 62.7% of total phenolic acids in the fraction indicating that gallotannins could be the major hydrolysable tannins in kernel. Total PA content in water fraction constituted 60% of total phenolics in the extract. Therefore characterization of PA the extract was also conducted. PAs in the water fraction was purified and subjected to depolymerisation in presence of a nucleophile (phloroglucinol). The reaction products were analyzed in LC-MS/MS. Epigallocatechin-phloroglucinol and gallocatechin-phloroglucinol adducts had higher concentration among the reaction products. Catechin and epicatechin phloroglucinol adducts and free gallocatechin, epigallocatechin,

catechin and epicatechin were also detected in the hydrolysed fraction. PAs in the kernel fraction was found to have a prodelphinidin – procyanidin ratio of 4.9. Resolution of kernel PAs according to their molecular size was also attempted with the help of size exclusion chromatography. The fractions thus obtained were subjected to AO capacity assays and characterized in terms of their degree of polymerization. PAs were found to fractionate in their increasing order of molecular size. Most of the PA fractions had significantly high AO activity. No direct correlation was observed between AO capacity of these fractions and degree of polymerization.

Quest for health friendly phytochemicals as nutraceuticals, food additives, chemopreventive and therapeutic agents is far stronger than ever before. On the commercial point of view not many natural sources have been investigated for their therapeutic and chemopreventive efficacy and chemical composition. Availability and renewability of plant sources over petrochemical based synthetic molecules enhance their importance. SB grown in the cold deserts of Indian Himalayas with its high nutritional quality and therapeutic efficacy, in India is an under utilized natural resource. In vitro AO efficacy of SB berries in relation to its chemical composition was investigated in this study. Evidence for their therapeutic and chemopreventive effects of SB berries and other plant parts warrants further studies using in vivo models. Detailed investigations on chemical composition particularly tannin and protein composition are also suggested for the future work. Systematic approach for the collection, storage, processing and value addition of SB plant products would be helpful in the economic development of tribal areas in the Himalayan foot hills where SB is mostly grown.

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List of Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ACN	Acyl carbon number
ADP	Average degree of polymerization
ALA	α-linolenic acid
AO	Antioxidant
APCI	Atmospheric pressure chemical ionization
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CVD	Cardio vascular disease
DAD	Diode array detector
DPPH	2,2-Diphenyl-1- picrylhydrazyl
ECD	Electrochemical detector
EGCG	Epigallocatechin gallate
ESI	Electrostatic ionization
ESR	Electron spin resonance
ET	Ellagitannin
FA	Fatty acid
F-C	Folin-Ciocalteau's
FID	Flame ionization detector
GAE	Gallic acid equivalent
G-I	Gastro-intestinal
GPL	Glycerophospholipid
HAT	Hydrogen atom transfer
HPTLC	High Performance Thin Layer Chromatography
KME	Kernel methanol extract
KMEF	Ethyl acetate fraction of kernel methanol extract
KMWF	Water fraction of kernel methanol extract
KWE	Kernel water extract
LDL	Low density lipoprotein
MDA	Malondialdehyde

PA	Proanthocyanidin
PDA	Photo diode array
PDT	Photo dynamic therapy
PG	Propyl gallate
PUFA	Poly unsaturated fatty acids
PME	Pulp methanol extract
PWE	Pulp water extract
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP	Reverse phase
SB	Sea buckthorn
SC-CO_2	Super critical carbondioxide
SCME	Seed coat methanol extract
SCWE	Seed coat water extract
SD	Standard deviation
SET	Single electron transfer
SOD	Superoxide dismutase
SOS	Superoxide radical scavenging
TAG	Triacylglyceride
TBHQ	t-butyl hydroxy quinone
TEAC	Trolox equivalent antioxidant capacity
T_R	Retention time
XO	Xanthine oxidase

PUBLICATIONS

Papers

1. **A. Ranjith**, K. Sarin Kumar, V.V. Venugopalan, C. Arumughan, R. C. Sawhney, and Virendra Singh.
   Fatty Acids, tocols and carotenoids in pulp oil of three sea buckthorn species (*H. rhamnoides, H. salcifolia and H.tibetana*) grown in Indian Himalayas.
   *Journal of American Oil Chemists Society,*Vol 83 (2006) 359-364.
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   C. Arumughan, V. V. Venugopalan, **A. Ranjith**, K. Sarin Kumar, R. C. Sawhney, O. P. Chaurasia and D. P. Attrey.
3. HPLC-DAD-APCI-ESI-MS analysis of flavonoid glycosides in antioxidant active extracts of sea buckthorn (*H. rhamnoides*) seeds.
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.....miles to go before I sleep and I have promises to keep.