ANTIOXIDANT ACTIVITY AND BIOACESSIBILITY OF POLYPHENOLS FROM SEA BUCKTHORN UNDER *IN VITRO* SIMULATED GASTRO INTESTINAL DIGESTION

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CERTIFICATE

This is to certify that the work presented in this dissertation entitled **"Antioxidant activity and bioaccessibility of polyphenols from Sea buckthorn under in vitro simulated gastro intestinal digestion"** was carried out by Ms. Surabhi Chauhan and Mr. Abhay Sharma at Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat (Solan) under my supervision towards the partial fulfillment of their Bachelor of Technology in biotechnology. It is also certified that no part of this dissertation has been submitted elsewhere for award of any degree or diploma.

Signature of supervisor:

Name of Supervisor: Dr. Gunjan Goel

Date:

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DECLARATION

I do hereby declare that the dissertation entitled **"Antioxidant activity and bioaccessibility of polyphenols from Sea buckthorn under in vitro simulated gastro intestinal digestion"** submitted towards partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology of Jaypee University of Information Technology is based on the results of studies carried out under the guidance and supervision of Dr. Gunjan Goel. This dissertation or no part of this has been submitted elsewhere for award of any degree or diploma.

Signature of Supervisor:

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ABSTRACT

The present study evaluates total phenolic content (TPC) and antioxidant activity of the Sea buckthorn berries extract under in vitro gastric intestinal and colonic batch fermentation. The TPC was determined using standard Folin-ciocalteau (FC) assay whereas antioxidant activity was determined using ABTS free radical scavenging activity. In this study, the digestion of Sea buckthorn extract was also compared with commercially available Sea buckthorn juice. It was observed that pure Sea buckthorn extract which was prepared in laboratory contained more TPC content throughout undigested (2.38 folds), gastric (2.11 folds) and small intestine phase (2.14 folds) of digestion. In case of antioxidant activity, the initial antioxidant content of pure extract and commercial juice was almost same but increase in pure extract in gastric (1.53 folds) and small intestine (2.33 folds) phase was observed over commercial juice. Though, in case of both pure extract and commercial juice increase in TPC and antioxidant activity was observed after gastric and small intestine phase. For colonic batch fermentation of pure Sea buckthorn extract and small intestine digested extract were lyophilized. Lyophilized sample (0.5%) Sea buckthorn pure extract and commercial juice were added to serum bottles containing basal media and fecal slurry. In case of digested Sea buckthorn extract an increase in TPC was observed up to 72 hrs after which it gradually declined. In case of undigested samples, sudden increase in TPC content was observed after 24 hrs which also further declined indicating that during initial stages of digestion, the phenolics are extracted from the juice or extract matrix followed by their degradation under longer incubations.

LIST OF ABBREVIATIONS

SBT - Sea buckthorn

TPC - Total polyphenol content

g - Grams

µ - Micro

ºC - Degree Celsius

FC - Folin-Ciocalteu (FC) reagent

ABTS - ABTS (2, 2'-Azino-bis (3-ehtylbenzothiazoline-6-sulfonic acid))

dw - Distilled water

nm - Nanometer

hrs - Hours

GAE - Gallic acid equivalent

TEAC - Trolox equivalent antioxidant activity

L - Liters

rpm - Revolutions per minute

Mg - Milligram

ml - Milliliter

Sec - Seconds

µg - Microgram

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Nutritional composition is the main feature of food as it determines its consumption among vigilant consumers. Polyphenols are a class of mainly natural, synthetic or semisynthetic, organic chemical characterized by multiples of phenol units. They are abundant micronutrients in our diet, and evidence for their role in the prevention of degenerative diseases such as cancer and cardiovascular disease is emerging. The health benefits of polyphenol depend on amount consumed and their bioavailability and bioaccessibility.

Sea buckthorn (*Hippophae rhamnoides)* is a fascinating plant growing widely in various regions of Asia, Europe, and Northern America. Sea buckthorn is a thorny, deciduous shrub that grows widely at high altitudes of 7,000–15,000 foot of the northwest Himalayan region, like Leh, Lahaul, Pakistan and is native to Eurasia.

The fruit is round yellow orange grows in summer on female plants. It is also been domesticated and used in traditional medicine in several countries, with benefits of relieving cough, aiding digestion, invigorating blood circulation, and alleviating pain.

Oil from the seeds is also a valuable product. Sea buckthorn berries have high natural antioxidants including ascorbic acid, tocopherols, and carotenoids. The complex of polyphenols comprises flavonols, catechins, proanthocyanidins, and chlorogenic acids. While the flavonols are reported to consist of glycosides of isorhamnetin, quercetin, and kaempferol detailed structures of phenolic acids andflavan-3-ols are mostly unknown.

Sea buckthorn has diverse uses such as, controlling soil erosion, a source of horse fodder, nutritious foods, drugs, and skin-care products, also contains bioactive compounds with antioxidant properties. Oil from Sea buckthorn has shown effectiveness in skin therapy for sunburns, chemical burns, radiation burns, and eczema (Wani et al., 2016).

Products

Products from Raw Sea buckthorn such as unstrained juice, clear juice, concentrated juice, Sea buckthorn fruit oil and Sea buckthorn seed oil, Sea buckthorn fruit residual oil, raw powder, Sea

buckthorn pigment etc. have emerged in markets. Beverage with Sea buckthorn such as soft drink (including syrup), alcoholic drink (sweet wine, semi-fluid drink, wine, beer), fruit juice (clear or unstrained) aerated fruit juice, powder, nutrient solution, jam, etc. are gaining attention. This is its status in Chinese market (Mingyu et al., 1993).

Status in India:

• DRDO (DIHAR) Leh has set up a market for launch of Sea buckthorn juice, oil, tea, cream etc.

• Minchy's has launched its wine, juice and jam made from Sea buckthorn.

• Many Himachal based companies are about to launch many diverse products like Sea buckthorn cleanser, cream, body lotion, shampoo, lip guard, vinegar, capsule and anti-wrinkle cream.

Many reports have studied the effects of polyphenols and antioxidants to the body but none has studied their fate in the gut. Thus it is important to look at the metabolic profile of the antioxidants and their bioacessibility in the gut. Bioacessibility which is the Release of the compound from its matrix in the intestinal lumen and bioavailability which is the availability of these compounds for physiological functions can be estimated only after digestion of these polyphenols. Thus by many *in vivo* and *in vitro* digestion models the fate of these polyphenols in the gut can be analyzed.

In vivo digestion models are exact replicas of human conditions in which the Sea buckthorn juice samples are fed to human volunteers and their blood, urine samples are taken from time to time. Or mice are fed with the juice and the effects of polyphenols are seen on them after a certain time period. *In vivo* studies are almost accurate replicas of human conditions but owing to ethical issues concerned with the use of animals in lab it is avoided. The use of humans also causes discrepancy in reading outputs due to physiological conditions a volunteer goes through.

Therefore, more preference is given to *in vitro* studies.

In vitro digestion models should fulfill the following criteria:

a) The model has to represent the exact gut conditions as of humans.

b) Starting from the mouth the digestion proceeds till the large intestine with one of the compartment being small intestine where a majority of compounds are absorbed.

c) The experimental conditions have to be as realistic as possible.

d) The test procedure should be easily applicable, reproducible.

This model is of two main types: batch and continuous fermentation types. But the digestion of both types begins from the gastric phase and small intestine digestion in which the juice is subjected to first the gastric phase digestion with HCl, NaCl, pepsin and giving it incubation and shear by shaking at specific rpm. For small intestine phase digestion the gastric phase digesta is treated with phosphate buffer, pancreatin and bile by shaking incubation.

The continuous and the batch phase serve as the large intestine phase as it contains the microbial flora same as the gut contains, because along with the small intestine digested juice it contains faecal slurry of human volunteers.

The difference between the batch and the continuous phase is that in batch there is no timely input of feed and withdrawl of media. In batch there is only one time addition of feed.

Batch digestion models are the ones in which products of digestion are not removed during the digestion process and which do not mimic the digestion process happening *in vivo* (hydrolysis, shearing). The batch digestion models are useful for simpler digestion and not useful for complex digestion models. Many models are quite crude or simple and are involved for homogenization of food, acidification of hydrochloric acid, addition of gastric enzymes, simulating gastric residence time, addition of sodium hydroxide to balance the pH, addition of bile salts. The sample aliquots before and after digestion were tested for polyphenolic content and antioxidant activity and compared. Performing HPLC shows us the metabolic profile of polyphenols that have undergone biotransformation during digestion.

Continuous digestion models are the ones in which there is continuous ingestion of feed and the products of digestion are removed simultaneously during the digestion process. Thus it mimics to some extent the human digestion process. Here the food is subjected to high shear, and enzymatic conditions which help digest more complex food items.it has high quality mixing dynamics and hence provides a better control over digestion process. The analysis of the

digestion process is same as batch process that is polyphenol content and antioxidant activity to check the increase or decrease in polyphenols after digestion and HPLC to study if there is any reported biotransformation or release of any new polyphenol after its release from the food matrix.

The objective of the study is thus:

1) Determination of antioxidant potential and total phenolic content of Sea buckthorn juice.
2) Effect of *in vitro* simulation on antioxidant activity and polyphenol content on Sea buckthorn.

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Sea buckthorn

Sea buckthorn (*Hippophae rhamnoides L.*) is a unique plant currently being domesticated in several parts of the world. Approximately >250000 mature trees have been planted mainly for shelterbelts, enhancement of wildlife habitat, and land reclamation .it is a thorny, deciduous shrub that grows widely at high altitudes of 7,000–15,000 foot of the northwest Himalayan region, mainly the Hindukush foothills of Pakistan, Ladakh (India) (Wani et al.,2016). The plant is reputed to have considerable medicinal value being useful for the treatment of skin disorders resulting from bed confinement, stomach and duodenal ulcers, cardiovascular diseases, and perhaps growth of some tumors. These beneficial effects have made Sea buckthorn products, especially its oils, desirable for medicinal and cosmetic purposes. Products on the market from Sea buckthorn range from oil, juice, and food additives to candies, jellies, cosmetics, and shampoos (Beveridge et al., 1999).

Figure 1 : Sea buckthorn berries

2.1.1. Products of Sea buckthorn

2.1.1.1 Pigment a by-product from Sea buckthorn berries

“Sea buckthorn yellow”, is a pigment that may be extracted from the berries, pressed juice, or pulp and is made in two ways. First the pigment is extracted by a low percentage alcohol, probably after the juice or residue was adjusted or concentrated to be 11-13 °Brix. In the second method, the juice may be concentrated to 11-13 °Brix at 48-52 °C. Solution in both ways is concentrated by spray-drying to yield a yellow powder. It contains carotene and vitamin E. In alcohol solution it absorbs light maximally at 213, 315, and 445 nm or at 450 nm in aqueous solution. The pigment may be useful when it comes to coloring pharmaceutical or cosmetic creams or for addition to foods where yellow-orange colors may be desirable such as ice cream or water ices.

2.1.1.2. Sea buckthorn Oil.

The oils from Sea buckthorn vary in their vitamin E content depending on whether they are derived from seed oil (64.4-92.7 mg/100 g of seed), juice oil (216 mg/100 g of berries), or the pulp after juice and seed removal (481 mg/100 g of berries) . Carotenoids also vary depending upon the source of the oil. Fatty Acid Composition of Oil Triacylglycerol of Sea buckthorn (H. rhamnoides) seed oil pulp oil UV-B range and may therefore be used as a natural sunscreen absorber.

2.1.1.3. Sea buckthorn seed oil.

Sea buckthorn seed oil has high levels of unsaturated linoleic and linoleic acids .These essential fatty acids are claimed to relieve chronic eczema, cure dermatitis, and maintain healthy skin. Furthermore, they are claimed to cure clinical neurological symptoms as an anticancer agent.. The fatty acid profile along with the high content of carotenoids and tocopherols may be responsible for reported anti mutagenic properties, therapeutic action on eye burns, stimulating effects on skin wound healing, and prevention and treatment of peptic ulcer .

2.1.1.4. Sea buckthorn Leaves.

Sea buckthorn leaves contain many nutrients and bioactive substances. It was reported that the flavonoid content in leaves ranges from 319 to 2100 mg/100 g of air-dried leaves. Numerous products are made from Sea buckthorn leaves such as leaf extract, tea, tea powder, and animal feed (Beveridge et al., 1999).

2.1.2. Bioactive compounds and antioxidant properties

Bioactive substances like vitamins (A, C, E, riboflavin, folic acid, and K), carotenoids (α, β, ð-carotene, and lycopene), flavonoids, organic acids (malic acid and oxalic acid), sterols (ergosterol, stigmasterol, lanosterol, and amyrins) and some essential amino acids have been found in all parts of the plant. The ripe fruit is a source of exceptionally high contents of vitamins (A, C, E, and K), carotenoids, flavonoids, and organic acids.

Oil from Sea buckthorn contains several bioactive components such as vitamin E, vitamin K, carotenoids, and β-70 sitosterol. Oil extracts obtained from the berries of Sea buckthorn are rich in monounsaturated fatty acids (MUFA), carotenoids, and other bioactive compounds. The leaves of Sea buckthorn are rich in kaempferol-3-O- β-D-(6″-O-coumaryl) glycoside, 1-feruloyl-β-D-glucopyranoside, isorhamnetin-3-O-glucoside, quercetin-3-O-β glucopyranoside, quercetin-3-O-β-D-glucopyranosyl-7-O-α-L-rhamnopyranoside, and isorhamnetin-3-O-rutinoside.

The assays like 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP), which are often used to test the antioxidant activity, have revealed that the antioxidant activity of seed and root extracts is better than that of leaf and stem extracts .(Wani et al.,2016).

2.1.3 Composition of polyphenols in Sea buckthorn

Identification of Flavonols

Besides small amounts of the aglycone, isorhamnetin 7, we identified the 3-O-rutinoside 4 and the 3-O-glucoside 5 of isorhamnetin by comparison with authentic reference substances. The 3-O-rutinoside 1 of quercetin is known to occur in fruits of Sea buckthorn. The presence of quercetin 3-O-glucoside 2 has only been reported for the leaves of this plant. The presence of

isorhamnetin 3-O-glucoside-7-O-rhamnoside 3 and isorhamnetin 7-O-rhamnoside 6 by NMR and LC-ESI-MS investigations has been confirmed.

2.1.4 Identification of Phenolic Acids.

The use of electrochemical detection allowed the determination of very low amounts of phenolic compounds with a catechol or pyrogallol structure. Gallic acid (3, 4, 5-trihydroxybenzoic acid) 9 and protocatechuic acid (3,4-dihydroxybenzoic acid) 10 in Sea buckthorn juice by comparison with the retention times of standards could be identified. Identification of Flavan-3-ols. The HPLC-ECD chromatogram of Sea buckthorn juice showed also the occurrence of (+) - catechin 11 and (-)-epicatechin 12. Sea buckthorn fruits are reported to contain also flavan-3- ols of higher molecular weight (Rosch et al., 2003).

Using high-performance liquid chromatography (HPLC Thirty-four samples were analyzed including 15 RS (*Hippophae rhamnoides ssp. sinensis*) samples, 7 RY (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. In the HPLC chromatograms, 12 compounds were identified as flavonoids, including quercetin 3-O-sophoroside-7-rhamnoside, kaempferol 3-O-sophoroside-7-O-rhamnoside, isorhamnetin 3-O-sophoroside-7-O-rhamnoside, isorhamnetin 3-O-glucoside-7-O-rhamnoside, quercetin 3-O-rutinoside, quercetin 3-O-glucoside, isorhamnetin 3-O-rutinoside, isorhamnetin 3-O-glucoside, quercetin, kaempferol 7-O-rhamnoside, kaempferol and isorhamnetin. (Chen et al., 2007)

Glucuronidation and excretion of Sea buckthorn and lingo berry flavonols were investigated analyzing the intact forms of flavonol glycosides as well as glucuronides in plasma, urine, and feces. Four study subjects consumed Sea buckthorn (study day 1) and lingonberry (study day 2) breakfasts, and blood, urine, and fecal samples were collected for 8, 24, and 48 h, respectively. Glycosides and glucuronides of the flavonol quercetin as well as kaempferol glucuronides were detected in urine and plasma samples after the consumption of lingonberries; solely glucuronides of flavonols isorhamnetin and quercetin were found in plasma after the consumption of Sea buckthorn berries. Only glycosides were detected in the feces after each berry trial. The berries seemed to serve as a good flavonol supply, providing steady flavonol input for the body for a relatively long time. (Maria et al., 2010)

2.2 Polyphenols

Polyphenols are the most abundant micronutrients in our diet and are found in abundance in fruits, vegetables, cereals, olive, dry legumes, chocolate and beverages, such as tea, coffee and wine providing antioxidant activity. Polyphenols offer protection to cell and organnales against oxidative stress and prevent degeneration. The polyphenols cause inhibition or reduction of different enzymes, among which telomerase, cycloxygenase, lipoxygenase, and the interaction with signal transduction pathways and cell receptors. The study of bioavailability and bioaccessibility will not only tell us the amount of polyphenols utilized but also their health benefits (Archivio et al., 2007).

2.2.1 Classification of Polyphenols

One of the most widespread groups in plant kingdom is that of the dietary phenolic. Flavonoids being the most abundant form of dietary polyphenols which constitute approximately 4000 of the current 8000 known polyphenols. Polyphenols are chemically characterized as compounds with phenolic structural; this group has a huge diversity with many sub groups. Fruits, vegetables, whole grains and other types of foods and beverages such as tea, chocolate and wine are rich sources of polyphenols. Due to the huge variance there has been a need to classify polyphenols, which have been classified by their source of origin, biological function, and chemical structure. Also, the majority of polyphenols in plants exist as glycosides with different sugar units and acylated sugars at different positions of the polyphenol skeletons.

2.2.1.1 Phenolic Acid

Based on C1–C6 and C3–C6 backbone are the Non-flavonoid polyphenolic compounds further divided into benzoic acid and cinnamic acid derivatives. Fruits and vegetables contain many free phenolic acids, in grains and Seeds, phenolic acids are often in the bound form. Acid or alkaline hydrolysis, they can be broken.

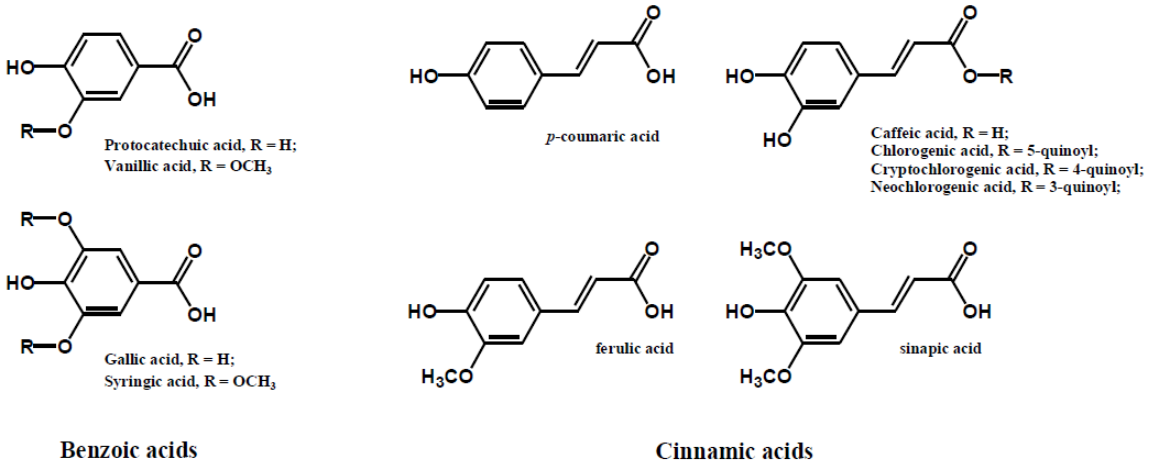


Figure 2: phenolic acids in food left benzoic right cinnamic acids . adapted from (Tsao,2010)

2.2.1.2. Flavonoids

Flavonoids have the C6–C3–C6 general structural backbone in which the two C6 units (Ring 1and Ring 2) are of phenolic nature. Flavonoids can be further divided into different sub-groups such as anthocyanin, flavan-3-ols, flavones, flavonones and flavonols due to the hydroxylation pattern and variations in the chromane ring (Ring 3).

Chalcones, that lack the heterocyclic Ring 3, are still categorized as members of the flavonoid family. These basic structures of flavonoids are aglycones; however, in plants, most of these compounds exist as glycosides. Biological activities of these compounds, including antioxidant activity, depend on both the structural difference and the glycosylation patterns.

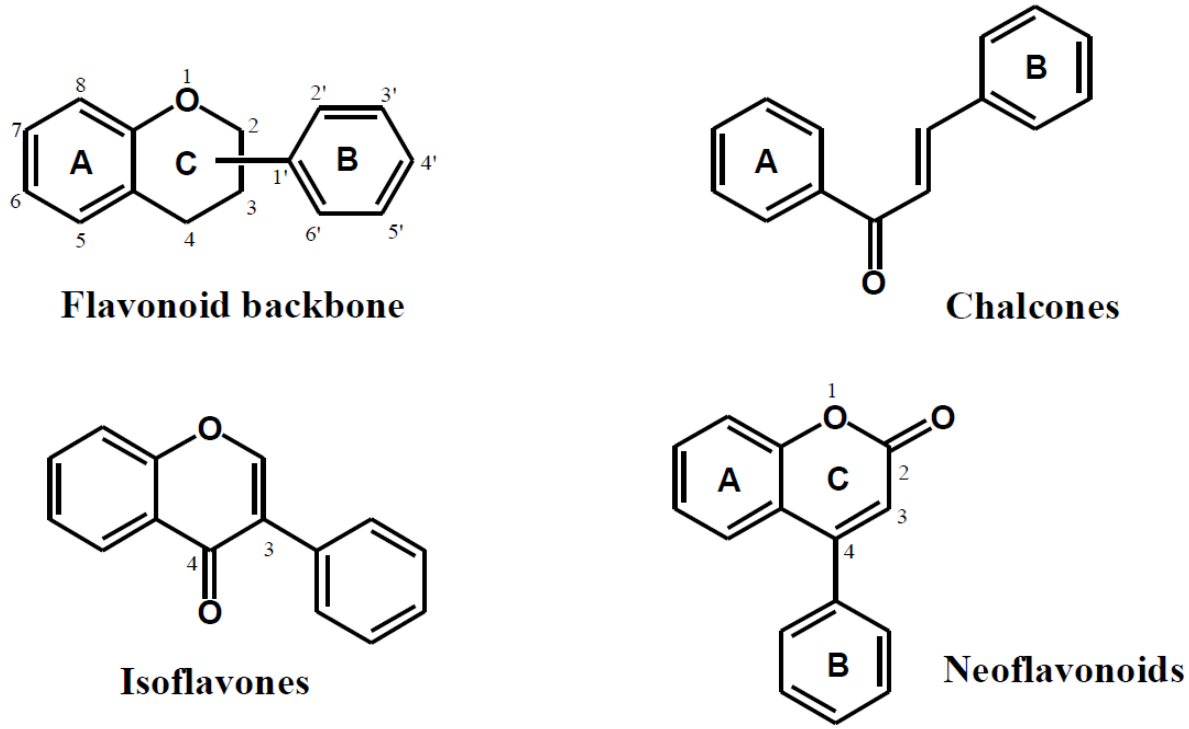


Figure 3: Basic flavonoids structure adapted from (Tsao, 2010)

2.2.1.3 Isoflavones, Neoflavonoids and Chalcones

With their Ring 2 attached to the C3 position of Ring 3 they are known as isoflavones. They are leguminous plant parts with soybean, as a major part of the diet in many cultures

Genistein and daidzein are the two main isoflavones found in soy along with glycetein, biochanin A and formononetin .They are also found in red clovers. All these isoflavone aglycones are mostly found as 7-O-glucosides and 6"-O-malonyl-7-O-glucosides. Neoflavonoids are not often found in food plants, with dalbergin as the most common and relatively widely distributed neoflavone in the plant kingdom. The open-ring chalcones are found in fruits such as apple and hops or beers.

2.2.1.4. Flavones, Flavonols, Flavanones and Flavanonols

Most common, throughout the plant kingdom. They are the largest group because the Flavones have 3-hydroxy derivatives flavonols, including their glycosides, methoxides and other acylated products on all three rings. The most common flavonol aglycones, quercetin and kaempferol, alone have at least 279 and 347 different glycosidic combinations, respectively. .

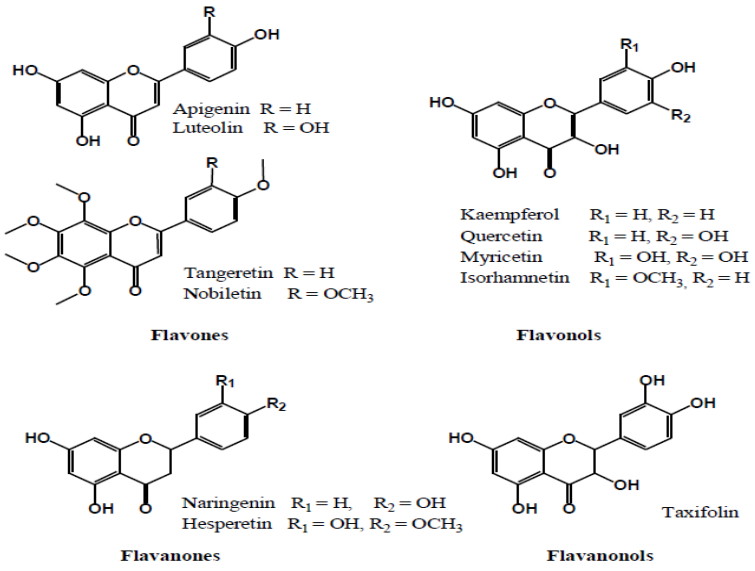


Figure 4: Structure of flavones, flavonols, flavonones, and flavononols adapted from (Tsao.2010)

2.2.1.5. Flavanols and Proanthocyanidins

Flavanols or flavan-3-ols are often commonly called catechins. With no double bond between no double bond between C2 and C3, and no C4 carbonyl in Ring C of flavanols they are quite different from the others. This and the hydroxylation at C3 allows flavanols to have two chiral centers on the molecule (on C2 and C3), thus four possible diastereoisomers.

Catechin is the isomer with trans configuration and epicatechin is the one with cis configuration. Each of these two configurations has two steroisomers, i.e., (+)-catechin, (−)-catechin, (+)-epicatechin and (−)-epicatechin. (+)-Catechin and (−)-epicatechin are the two isomers often found in food plants.

Flavanols occur in skins of grapes, apple and blueberries. Monomeric flavanols (catechin and epicatechin), their derivatives (e.g., gallocatechins) are the major flavonoids in tea leaves and cacao bean (chocolate).

Catechin and epicatechin can form polymers, which are often referred to as proanthocyanidins because an acid-catalyzed cleavage of the polymeric chains produces anthocyanidins.

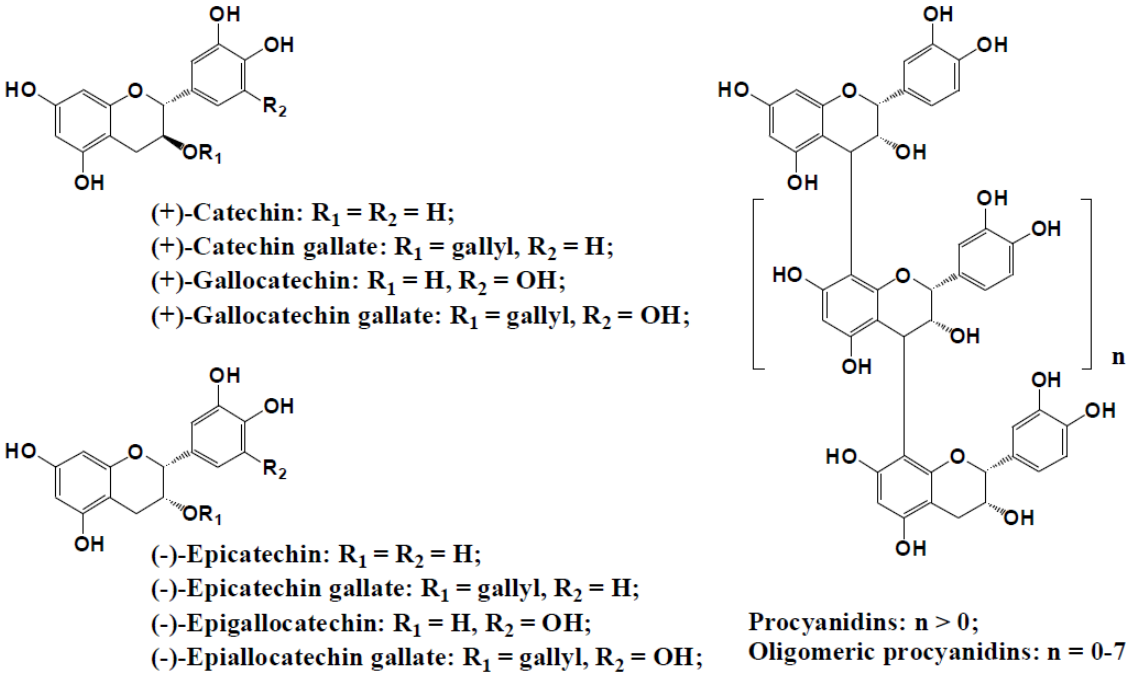


Figure 5: structure of flavolnol and procyanadins adapted from (Tsao, 2012)

2.2.1.6. Anthocyanidins

The principal components of the red, blue and purple pigments of the majority of flower petals, fruits and vegetables, and certain special varieties of grains, e.g., black rice are the anthocynadins.Anthocyanidins mainly exists in glycosidic forms which are commonly referred to as anthocyanins.

Cyanidin, delphinidin and pelargonidin are the most widely found anthocyanidins, along with more than two dozen other monomeric anthocyanidins. A total of more than 500 anthocyanins are known depending on the hydroxylation, methoxylation patterns on the B ring, and glycosylation with different sugar units.

The color of anthocyanin is pH-dependent, i.e., red in acidic and blue in basic conditions. However, other factors such as degree of hydroxylation, or methylation pattern of the aromatic rings, and the glycosylation pattern, i.e., sugar vs. acylated sugar can also affect the color of anthocyanin compounds. Anthocyanin is chemically stable in acidic solutions.

2.2.1.7. Polyphenolic Amides

N-containing functional substituents may be present in some polyphenols. Two such groups of polyphenolic amides are of significance for being the major components of common foods: capsaicinoids responsible for hotness in chili peppers and avenanthramides in oats. Capsaicinoids have also been found to have strong antioxidant and anti-inflammatory properties.

2.2.1.8. Other Polyphenols

In addition to the phenolic acids, flavonoids and phenolic amides, there are several non-flavonoid polyphenols found in foods. Eg. Resveratrol is unique to the grapes and red wine; ellagic acid and its derivatives are found in berry fruits, e.g., strawberries and raspberries, and in the skins of different tree nuts. Lignans exist in the bound forms in flax, sesame and many grains; structures shown below are hydrolysis products. Curcumin is a strong antioxidant from turmeric. Rosmarinic acid is a dimer of caffeic acid, and ellagic acid is a dimer of gallic acid. (Tsao. 2010).

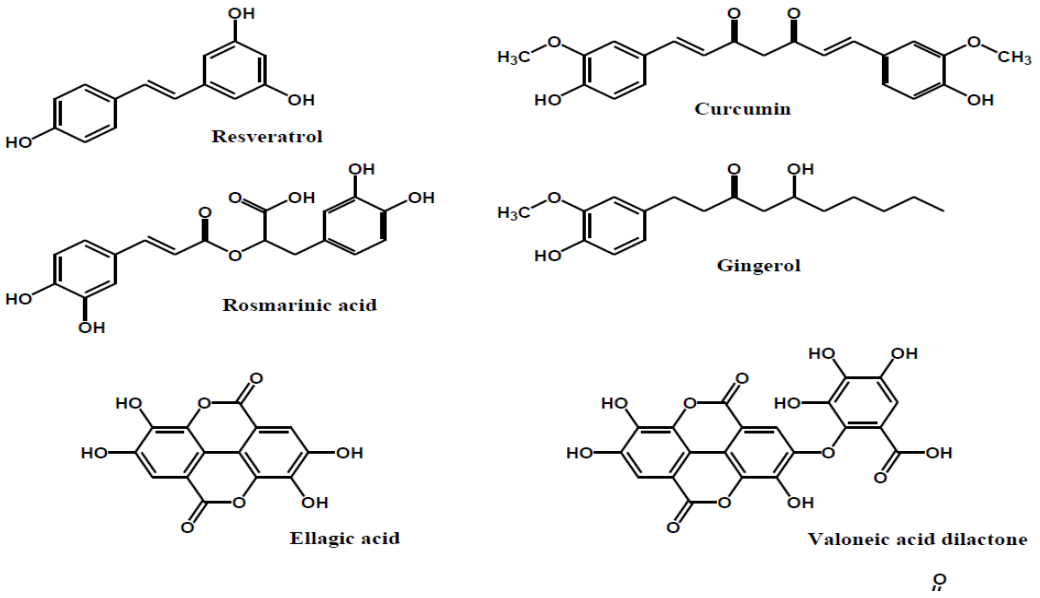


Figure 6: other polyphenols adapted from (Tsao, 2010)

2.3. Antioxidant Activity

Plants produce many secondary metabolites to protect themselves from other organisms eg polyphenols. High intake of polyphenols in the form of fruits, vegetables and whole grains, which are rich in polyphenols, loweres the risk of many chronic diseases including cancer, cardiovascular disease, chronic inflammation and many degenerative diseases . Phytochemicals, especially polyphenols, are the predominant contributor to the total antioxidant activities of fruits, rather than vitamin C. Polyphenols are strong antioxidants that can neutralize free radicals by donating an electron or hydrogen atom. Polyphenols suppress the generation of free radicals, thus reducing the rate of oxidation by inhibiting the formation of or deactivating the active species and precursors of free radicals acting as chain terminators. Polyphenols are also known as metal chelators, Chelation of transition metals such as Fe2+ can directly reduce the rate of Fenton reaction, thus preventing oxidation caused by highly reactive hydroxyl radicals

Several *in vitro* antioxidant model systems have been developed to evaluate the total antioxidant activities. they may portray well how polyphenols function as antioxidants, and thus shed light on the actual role of polyphenols in human health. (Tsao et al., 2010).

2.4 .Medicinal properties of Sea buckthorn

2.4.1 Cardiovascular system

The total flavonoids of Hippophae (seabuckthorn) (TFH) extracted from the leaves and fruit is a group of compounds containing seven kinds of flavonoids. Of these, the main components are isorhamnetin and quercetin. Wang Bingwen and others of Xi'an Medical University investigated the effects of TFH extracted from the leaves of seabuckthorn on white rats' cardiac function. The internal pressure peak of the left ventriculus and its maximum rate of change (dp/dtmax) increased distinctly.The mechanism might be related to its effect on both the inward flow of extra-cellular Ca2+ of the cardiac muscles and the Ca2+ release from intracellular reservoirs.. Seabuckthorn oil could decrease cholesterol, triglyceride and ß-lipoprotein (LP).

2.4.2 Immune System

Nanjing Medical College of the Railway Ministry experimented on mice and guinea pigs with the compound extracts of Hippophae (CEH) (Sea buckthorn). CEH can strengthen non-specific immunity functions. In mice, the serolysin level and the serum-accelerator level were distinctly increased.

Shanxi Cancer Institute experimented on mice with the bone marrow micronucleus technique. It was shown that Sea buckthorn seed oil had the capacity to restore, under inhibited states of immune function, the natural killer cell level.

2.4. 3. Anti-inflammation and anti-radiation

Xu Mingyu et al. Of Xiyaun Hospital of the Academy of Traditional Chinese Medicine of China showed that Sea buckthorn oil had obvious effects on eliminating inflammation and slough, easing pain, promoting immune function and strengthening body resistance. All this provided a scientific basis for clinical treatments of the chronic bed sore (pressure sore). L. D. Lebedeva et al. Of the Chemical Institute of the Tadzhikistan Academy of Sciences injected experimental animals with seabuckthorn oil. During 20 to 30 days, the development of the artificially induced inflammation of mouse subcutaneous tissue was inhibited, and the antiinflammation effects was strengthened

2.4.4. Development of medicines and health products from seabuckthorn.

- Raw Seabuckthorn such as unstrained juice, clear juice, concentrated juice, seabuckthorn fruit oil and seabuckthorn seed oil, seabuckthorn fruit residual oil, raw powder, seabuckthorn pigment, TFH, etc.
- Beverage with seabuckthorn such as soft drink (including syrup), alcoholic drink (sweet wine, semi-fluid drink, wine, and beer), and fruit juice (clear or unstrained) aerated fruit juice, powder, nutrient solution, jam, etc.
- Cosmetics with Sea buckthorn such as hair shampoo, skin care cream, beauty cream, body lotion.
- Medicines such as cough remedy (relieving cough, dissolving phlegm, and treating chronic tracheitis), seabuckthorn (Acetylsalicylic) Flavonoid Tablets (treating the ischemic cardiopathy and remitting the angina cordis), compound oil-embolus extractum, and capsule (for inflammation and ulcer), Healthcare Medicine, including seabuckthorn oil, instant powder or granule preparation of seabuckthorn, and seabuckthorn dried cream.(Mingyu et al.,1997)

2.5. Digestion

Plant based food and food products are the main sources of nutrients such as carbohydrates, proteins, lipids and dietary fiber along with essential elements for human beings particularly for vegetarians.

Digestion is very essential for health where the food we take is broken down to minute nutrients that are assimilated for further growth of the organisms.when food is getting digested two main processes are taking place one is the mechanical where food is broken into smaller particles and the other is enzymatic where macromolecules are hydrolyzed to simpler elements to be readily absorbed.

The materials not absorbed till the small intestine travel to the large intestine for absorption of water certain salts etc. gut micro biota are helpful for human health like in absorption of nutrients. Microbial infections and imbalance in gut cause gut diseases (Moon et al., 2016).

All mineral content present in diet may not be available for absorption and/or utilization for normal health and physiological functions of humans. Bioavailability is the efficiency with which the nutrients are absorbed in the body and may be available for further use.

Bioavailability of various elements is determined by either *in vivo* administration to similar species to humans, eg, rats or *in vitro* methods by simulating digestive system conditions in the laboratory.

In *in vivo* techniques, amount of an element of interest is estimated as the difference in the concentration of the element in ingest and excreta, using radiotracers eg, Fe and Zn .Basic disadvantage of the method is the exposure of ionizing radiations to the sensitive human groups such as pregnant women, infants and young children certain limitations . Also there is massive variation due to temperature and varying body conditions. Also sample withdrawl adds to further discrepancy.

In vitro methods were developed which are rapid and inexpensive. *In vitro* procedures involve the simulation of the gastric and intestinal digestive conditions in the laboratory. As the experiments are carried out under ‘simulated’ digestive conditions, the results may not be as accurate as those obtained by *in vivo* studies (Kulkarni et al., 2007).

Human large intestine:

About 1-2 kg of human body weight is due to the colonic bacteria in the gut basically most of them resided in the gut because of the conditions being so supportive eg. pH, nutrient availability etc. the specialized enzymes that are needed for digestion are provided by specific members of the microbial community.

2.6. Bioaccessibility and Bioavailability

2.6.1 Bioaccessibility

Bioaccessibility has been defined as the fraction of a compound which is released from the food matrix and is available for intestinal absorption. The process starts by mastication in the presence of saliva from mouth and ends with the action of digestive enzymes in gastrointestinal lumen. bioaccessibility is influenced by the composition of the digested food matrix, the synergisms and antagonisms of the different components and a variety of gut microflora but also by physicochemical properties, such as pH, temperature and texture of the matrix .The digested food is predominantly broken down in the small intestine by bile, pancreatic and other enzymes secreted from the intestinal mucosa.

2.6.2 Bioavailability

Bioactive food compounds can be absorbed in the gastrointestinal tract and used for physiological actions. The absorption of these compounds can be influenced by solubility, interaction with other dietary ingredients, molecular transformations, different cellular transporters, metabolism and the interaction with the gut microbiota, resulting in changes to their bioavailability .The different solubility of lipophilic and hydrophilic compounds results in different absorption mechanisms (Rein et al., 2013).

CHAPTER 3

MATERIALS AND METHOD

3. MATERIALS AND METHODS

3.1. Materials

3.1.1 Chemicals required

ABTS (2,2'-Azino-bis(3-ehtylbenzothiazoline-6-sulfonic acid)), bile, bile salt, calcium chloride hexahydrate, dipotassium phosphate, Folin-Ciocalteu (FC) reagent (2N, Merck), gallic acid, hemin, hydrochloric acid, L Cysteine, HCl, magnesium sulfate heptahydrate, pancreatin, phosphorus buffer, pepsin, peptone, potassium dihydrogen phosphate, potassium per sulphate, sodium bicarbonate, sodium carbonate (Fischer scientific), sodium chloride, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), vitamin K1, yeast extract. Sea buckthorn juice extract, Sea buckthorn juice.

3.1.2 Physical requirements

Gassing manifold, autoclave, micropipettes, eppendorf, test tube, test tube stand, serum bottles, syringes, gloves, reagent bottles, pH meter, weighing balance, conical flasks, shaking incubator, centrifuge, lypholIizer, and refrigerator.

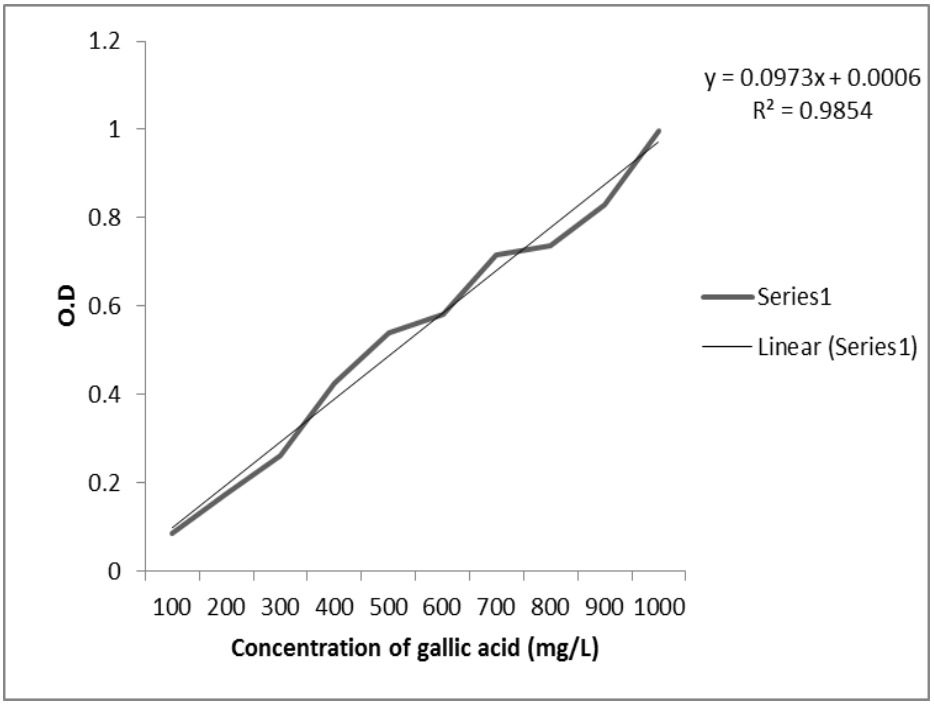


Figure 7 : Gallic acid standard

3.2 Analysis of Juice

3.2.1 Total phenolic content

Total phenolic content: using Folin-ciocalteau (FC) reagent and gallic acid as standard.

- Sample = 125µl
- Water = 7.5ml
- FC reagent = 0.625ml
- Na_2CO_3 = 1.88ml
- dH_20 = final 2.4 ml

1. Total polyphenol content was determined by Folin-Ciocalteu method (Singleton and Rossi, 1965).
2. Briefly, a 125µl of sample of seabuckthorn juice were diluted with 7.5 ml distilled water.
3. To this mixture0.625ml of FC reagent (2N, Merck) and 1.88 ml sodium carbonate (20%w/v, Fischer scientific) were added and final volume were made up to 12.5 ml. after incubation at R.t. in dark for 2 hours , the absorbance at 765 nm was measured.
4. Total phenolic content was expressed as milligram of gallic acid equivalent per litre (mg GAE/L)

3.2.2 Antioxidant activity

Antioxidant activity: using ABTS.

Set absorbance of ABTS 0.750-1 by dilution with 80% ethanol

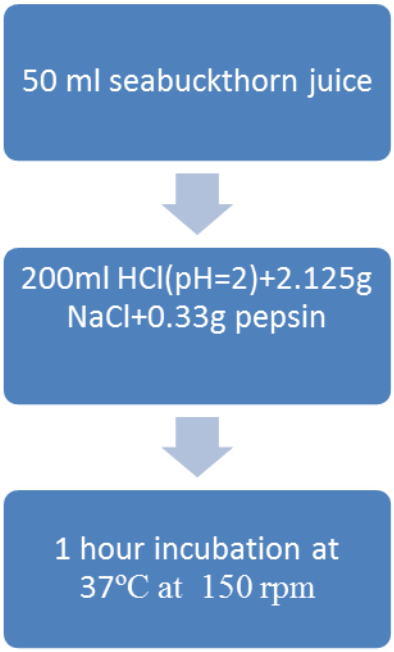
- ABTS =2.9ml
- Sample =100µl

1. The antioxidant activity was determined by ABTS method as described by (Pellergini et al).
2. A 7mM solution of ABTS was prepared by mixing a stock solution with potassium per sulphate (2.45 mam) in an equal quantities and left to stand for 12-16 h at R.T. in the dark until reachimg a stable oxidative state.

3. The ABTS solution was diluted with 80% ethanol to an absorbance of 0.80 +0.005 at 734 nm.
4. For determination of antioxidant activity in different samples, 100µl samples of Sea buckthorn juice were added to 2.9 ml ABTS solution and were incubated at R.T.for 30 min at dark. The absorbance was measured at 734nm against 80%ethanol as blank.
5. The calibration curve for ABTS was obtained using6-hydroxy-2,5,7,8-tetyramethylchroman-2-carboxylic acid(Trolox) ,a water soluble analog of α-tocopherol as standard and antioxidant activity was expressed as Trolox equivalent antioxidant activity micro molar per litre (TEAC µM/L).

3.3. *In vitro* simulated gastro intestinal digestion

Gastric phase digestion



Small intestine phase digestion

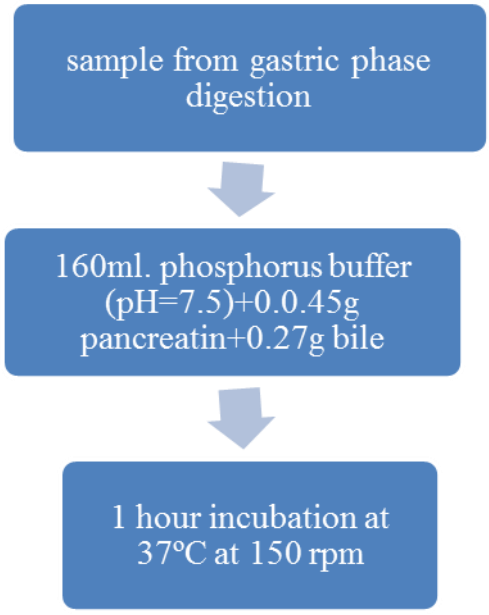


Figure 6: Flowchart gastro-intestinal digestion

1. Seabuckthorn juice was subjected to successive gastric and small intestine digestion.
2. The fate of polyphenols from the mouth to the stomach remains unchanged , it remains embedded in matrix which may be carbohydrates, etc. to proceed with gastric phase digestion *in vivo*,
3. We treated 50ml of the Sea buckthorn juice with 200ml HCl (pH=2) to reciprocate the acidic condition of the stomach, also 2.125g NaCl and 0.33g Pepsin was added.
4. And 1 hr incubation at 37ºC was given at 135 rpm to reciprocate *in vivo* condition and copy the churning motion.
5. After the gastric phase digestion ends, the fate of small intestine digestion starts in which the juice from the digested gastric phase was taken and subjected to the conditions it would face in the small intestine during digestion,
6. To that juice 160ml of phosphate buffer was added to create a basic condition of pH around 7.5 also 0.045g of pancreatin was added and 0.27 of bile all this was kept for 1 hour of incubation at the same condition as the gastric phase digestion (Gross G et al.2010).

3.4. Batch fermentation by gut microbiota

Table 1: Media composition for batch fermentation

Components	g/l
Peptone	2
Yeast extract	2
Hemin	0.02
Bile salt	0.5
NaCl	0.1
NaHCO3	2
K2HPO4	0.08
KH2PO4	0.08g
MgSO4.7H2O	0.01g
CaCl2.6H2O	0.01g
Vitamin K1	10mg
L-Cysteine HCl	0.5g

1. Three independent fermentation experiments were carried out using faeces from different volunteers, who had not ingested antibiotics for at least 3 months.

2. Briefly, 150ml glass fermentation vessels were filled with 45ml of sterile medium. The medium was adjusted to pH=7.0 and continuously sparged with O_2-free N_2 overnight.
3. The pH was maintained at 6.8 and the temperature at 37˚C in order to mimic conditions located in the distal region of the human large intestine.
4. Vessels were inoculated with 5ml faecal slurry (10% (w/v)). Then, Seabuckthorn extract and small intestine digested extract were added to separate stirring batch-culture vessel containing faecal slurry at final concentration of 0.5% (w/v).
5. Batch cultures were run under anaerobic conditions for a period of 144 hrs during which samples were collected at 0th hr to every 24th hr till 120th hr (0, 24, 48, 72, 96, 120 hr) for total phenolic content and antioxidant activity analysis. For this latter analysis, samples were stored at -20˚C until required.

3.5. Total phenolic content analysis

1. Total phenolic content was determined by Folin-Ciocalteu method (Singleton and Rossi, 1965).
2. Briefly, a 125µl of sample of Seabuckthorn juice were diluted with 7.5ml distilled water. To this mixture 0.625ml of FC reagent and 1.88 ml sodium carbonate (20%w/v) were added and final volume was made up to 12.5ml.
3. After incubation at room temperature in dark for 2 hrs, the absorbance at 765 nm was measured. Total phenolic content was expressed as milligram of gallic acid equivalent per litre (mg GAE/L).

3.6. Antioxidant activity analysis

1. The antioxidant activity was determined by ABTS method as described by (Pellergini et al).
2. A 7mM solution of ABTS was prepared by mixing a stock solution with potassium per sulphate (2.45mM) in an equal quantities and left to stand for 12-16 hrs at room temperature in the dark until it reaches a stable oxidative state.
3. The ABTS solution was diluted with 80% ethanol to an absorbance of 0.80 ± 0.005 at 734 nm.

4. For determination of antioxidant activity in different samples, 100µl samples of Seabuckthorn juice were added to 2.9ml ABTS solution and were incubated at room temperature for 30 min. in dark.
5. The absorbance was measured at 734nm against 80% ethanol as blank. The calibration curve for ABTS was obtained using trolox, a water soluble analog of α-tocopherol as standard and antioxidant activity was expressed as trolox equivalent antioxidant activity micro molar perlitre (TEACµM/L)

Figure 8: Digestion protocol

CHAPTER 4

RESULT AND DISCUSSIONS

4. RESULTS AND DISCUSSION

4.1 Analysis of juice

Table 2: TPC Analysis of juice

Samples	TPC mgGAE/L
SBT extract	**628.39**
SBT juice	**263.23**

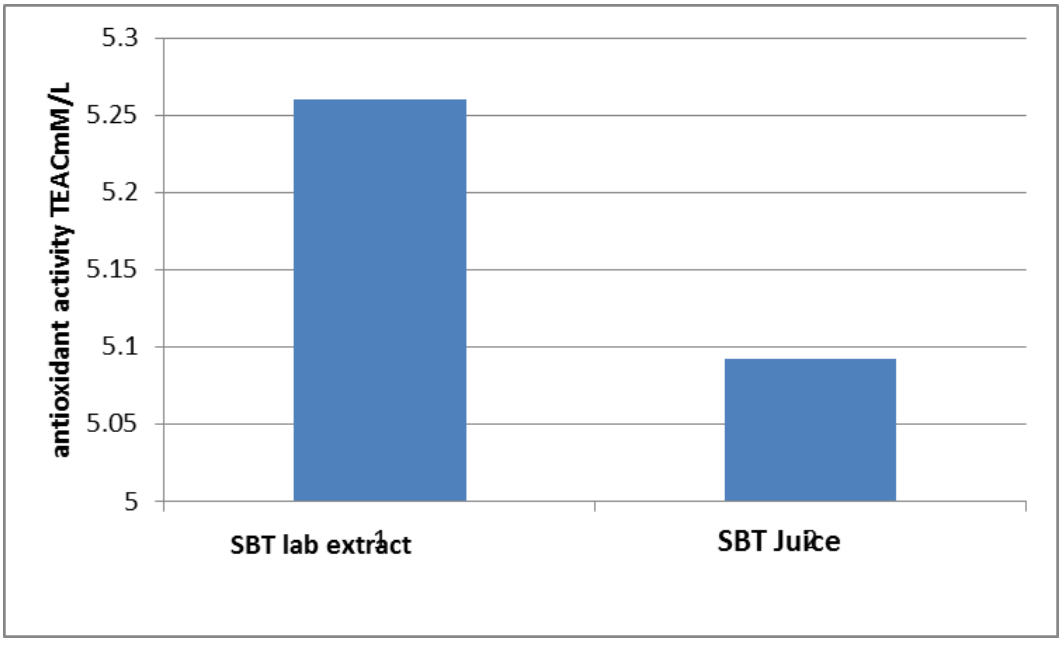


Figure 10: antioxidant activity SBT lab extract vs. SBT juice

It was seen that both the antioxidant activity and TPC content of the extract was more than the juice because extracts are more concentrated than juices.

4.2 Gastro intestinal digestion

This was the first phase of digestion and in this the juice was subjected to gastric and small intestine digestion. After the digestion all the samples were analyzed for antioxidant activity and the polyphenolic content by the ABTS method and TPC using FC reagent. Here we had taken the Sea buckthorn extract which was already available in the lab and the Sea buckthorn juice from DIHAR labs.

Table 3: TPC content of the Sea buckthorn extract and juice

Sample	Lab extract (mg GAE/L)	Juice (mg GAE/L)
Pure (undigested)	**628.39**	**263.23**
Gastric phase (digested)	**675.59**	**319.65**
Small intestine (digested)	**1042.9**	**486.5**

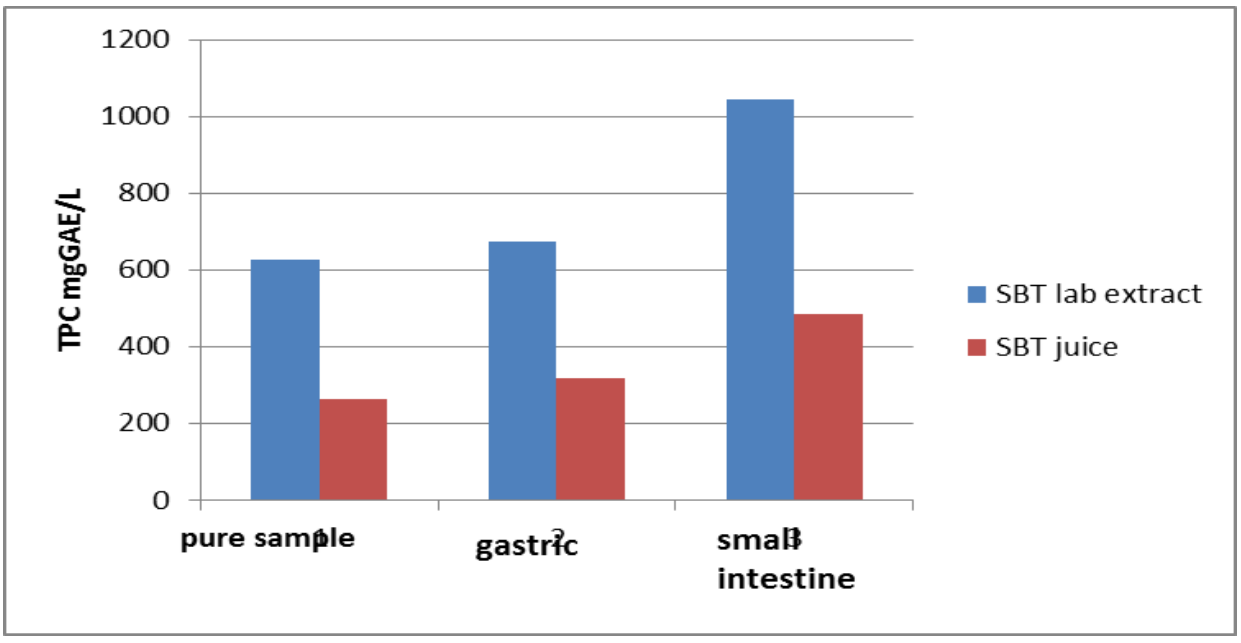


Figure 11: TPC content of Sea buckthorn extract vs Sea buckthorn juice after gastric phase

Table 4: Antioxidant activity of SBT lab extract and juice

Sample	Lab extract(TEAC µM/L)	Juice (TEAC µM/L)
Pure (undigested)	**5261.5**	**5092.3**
SBT gastric phase(digested)	**26865.3**	**17461.45**
Small intestine(digested)	**45023.0**	**19280.75**

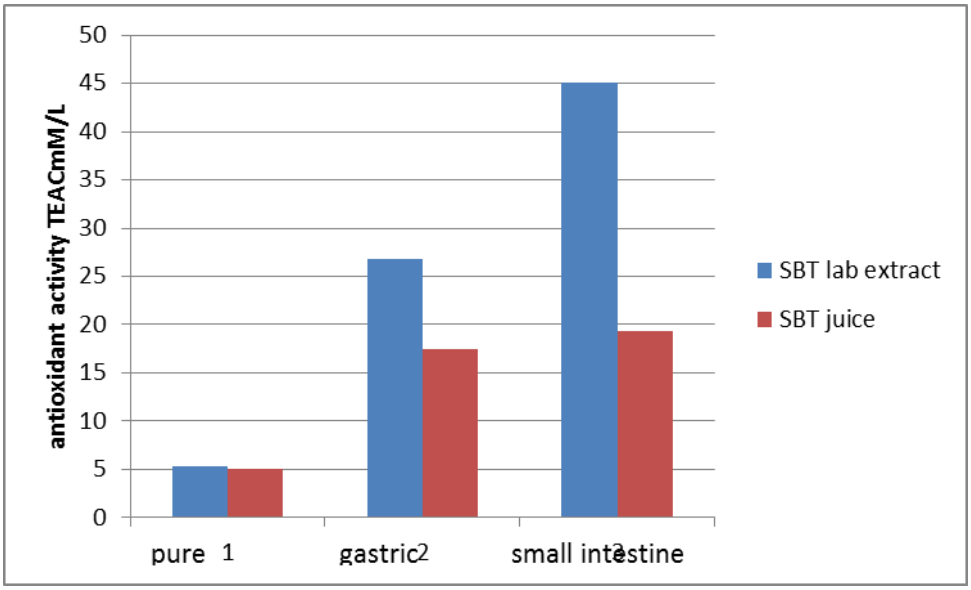


Figure 12: Antioxidant activity Sea buckthorn lab extract vs Sea buckthorn juice

From the above observations it was quite visible that the polyphenolic content and antioxidant activity of the lab extract was on a higher side .Therefore, the lab extract was taken into account for conducting rest of the study.

4.3 TPC for lyophilized sample

After the small intestine and gastric phase digestion it is time to subject the polyphenols to the gut microbiota and observe the action of these on the polyphenols. Before the large intestine phase the digested samples were lyophilized so that they could be easily inoculated in the media.

Table 5: TPC of lyophilized samples

Samples	**TPC(mg GAE/L)**
SBT pure	**34.63**
SI	**21.04**

The readings showed a vast difference therefore the TPC had to be equalized.

4.4 Batch phase digestion

To find the fate of food in the large intestine digestion, faecal samples were pooled from three different individuals and a media was prepared.to this equal amounts of gastric and small intestine digested media was inoculated.to check the difference various categories of control, gastric phase and small intestine phase were kept. Since this was a batch mode of fermentation no feed was given after the initial one and the digesta was taken out only after the period got over. The fermentation continued for 5 days after the inoculation with samples being withdrawn after every 24 hrs.

Table 6: TPC of batch phase digestion

Time (hrs)	TPC (SBT) mgGAE/L	TPC (SI) mg GAE/L
0 hr	21.40[a]	30[bc]
24 hr	47.81[e]	30.86[bc]
48 hr	41.63[d]	34.04[cd]
72 hr	33[c]	37.13[d]
96 hr	31.18[bc]	31.54[c]
120 hr	24.63[bc]	29[ab]
144 hr	28.04[b]	24.84[a]

*a-e=means in the column with same superscript letter are not significantly different($p<0.05$)as measured by 2 sided turkey's-post-hoc range test between replications

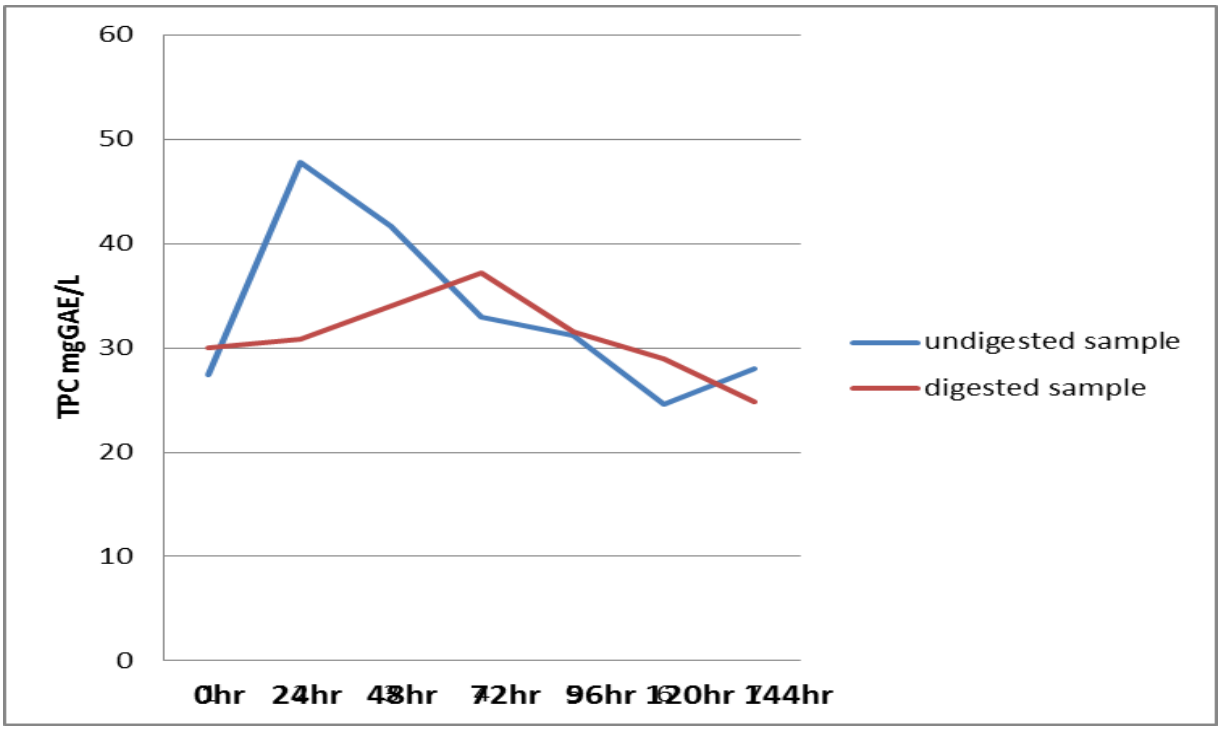


Figure 13: TPC content during 7 days of batch fermentation

In case of small intestine digested Sea buckthorn extract an increase was observed upto 72hrs.this increase may be due to transformation of polyphenol into another form or multiple smaller units resulting higher TPC content. After 72hrs. decrease in polyphenol content was noticed. Sudden increase in TPC content was observed after 24 hrs. which may be release of the polyphenols from the food matrix due to enzymatic action of gut microbes after 24hrs.there was decline in TPC content. This may be due to degradation of polyphenols, which may result in lower polyphenolic content.

Table 7: Antioxidant activity of batch phase digestion

Time (hrs)	**Antioxidant activity (SBT) TEAC µM/L**	**Antioxidant activity (SI) TEAC µM/L**
0 hr	**8192.3**	**8069.7**
24 hr	**8284.6**	**8292.7**
48 hr	**8169.23**	**8246.14**
72 hr	**7953.8**	**8400**
96 hr	**8230.7**	**8286.14**
120 hr	**8161.5**	**8146.46**
144 hr	**8207.6**	**8023.0**

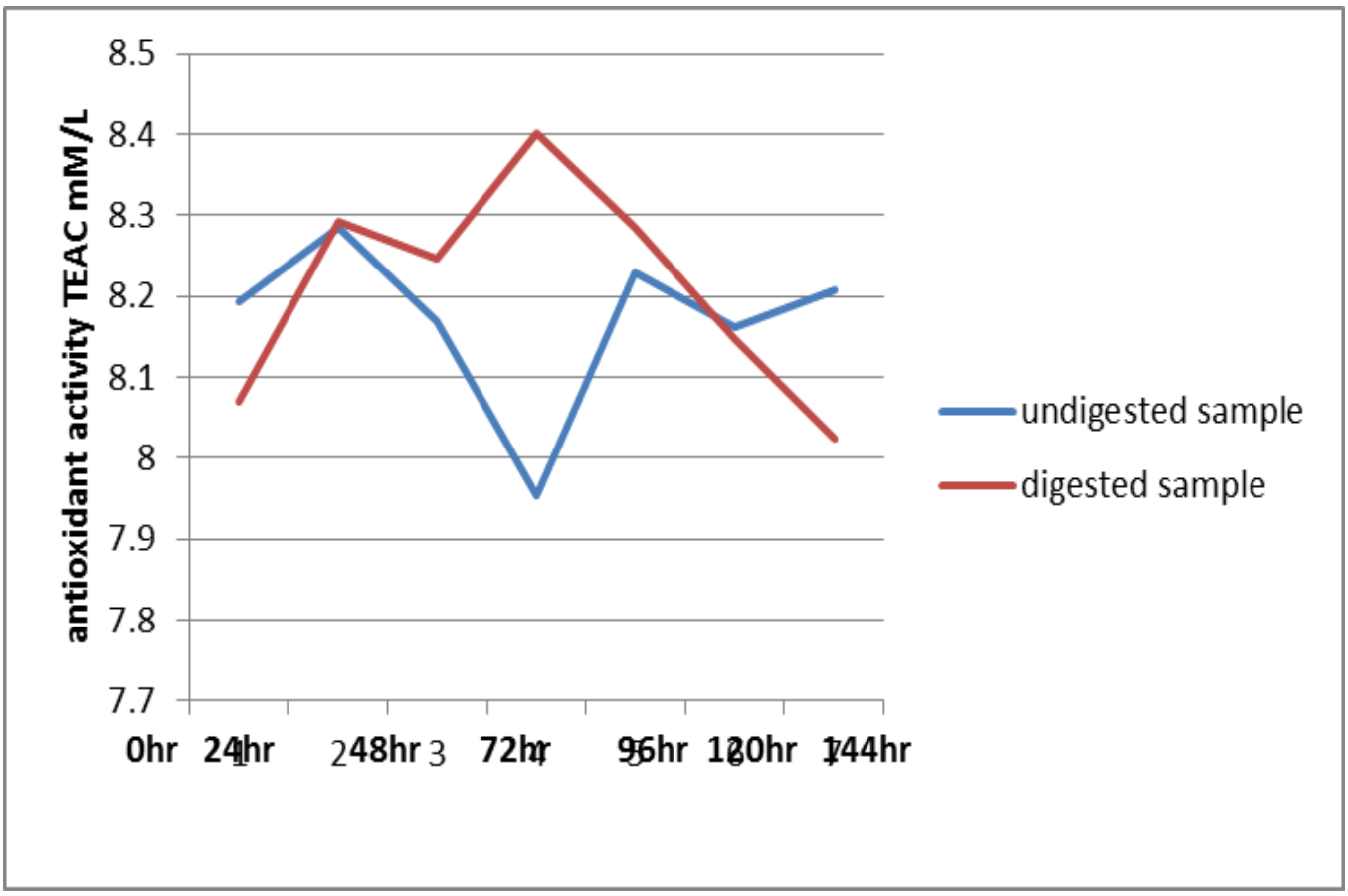


Figure 14: antioxidant activity after 7 days of batch fermentation

In case of digested Sea buckthorn TPC content was higher up to 72 hrs. overall antioxidant content of the Sea buckthorn was almost stable throughout six day study.though,antioxidant content of digested Sea buckthorn remained higher than undigested SBT.

CHAPTER 5

SUMMARY AND CONCLUSIONS

5. SUMMARY AND CONCLUSIONS

The present study reports that Sea buckthorn pure extract and commercially available juice are a valuable source of antioxidants and polyphenols under in vitro gastro intestinal digestion. Dietary polyphenols are mostly considered as xenobiotic by the human body and their bioavailability is therefore relatively low in comparison to other nutrients. However, their role in providing antioxidants first have to be released from the food matrix and solubilized therefore; the present study investigates the impact of gastric and pancreatic digestion on the bioaccesibility of phenolics from sea buckthorn berries.

Gut microbiota also play important role in transformation and breakdown of polyphenols into smaller units. These smaller units have the tendency of getting absorbed in large intestine in colon and have positive effects on human body.

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