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Neutracuetical Characterization, Anti Oxidant and Anti Coagulant Activities of Sea Buckthorn (*Hippophae Rahmnoides*) Leaves and Berries from Pakistan

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ABSTRACT

Sea buckthorn is a neglected multipurpose plant in Pakistan with around 300 hectares of its wild populations. The leaves and berries of two sea buckthorn genotypes (H. turkestanica and H.sinensis) were evaluated for mineral composition, phenolic contents and antioxidant and anti-coagulant activities for the 1st time from Pakistan. Anti-oxidant activities were estimated by measuring the scavenging activities of leaf extracts against 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radicals. Amongst the two genotypes of leaves, H. turkestanica showed 87% anti oxidant activity as compared to 95% in berries of H. turkestanica. The higher range of biochemical constituents. i.e. vitamin C 83.6 mg100 g^{-1}, phosphorous 41.5 mg100 g^{-1}, iron 78.1 mg100 g^{-1}, magnesium 56.8 mg100 g^{-1} and calcium 18.6 mg100 g^{-1} in leaves was observed in H. turkestanica. The total phenolic contents were 19.03 mg GAE/g in leaves of H. turkestanica thus comparatively higher than H. sinences. i.e.16.3 mg GAE/g. The in-vitro anticoagulant activity of Sea buckhorn berries acetonic extract in inhuman blood was showed very promising results and clotting time was noted upto 45 minutes which is higher than normal blood clotting time. The study revealed that Sea buckthorn leaves and berries have significant potential for utilization in food and pharmaceutical industry in Pakistan sustain the rural economy of mountain areas.

Keywords: Sea buckthorn, Pakistan, Biochemical constituents, Phenolics, Anti-oxidant, Anti-coagulant

INTRODUCTION

Plant secondary metabolites have been utilized for centuries for nutritional and medicinal purposes. There has been a growing preference amongst society for the usage of naturally derived medicines as a pose to synthetically prepared ones. This is partially due to the low purchase cost and little to no side effects. It has been evidenced from current studies that plants (either fruits, vegetables or the derived plant products) are immensely enriched with bioactive compounds. The bioactive compounds can be extracted purely and are found in flora and fauna of various geographic regions [1]. Pakistan, specifically its northern areas and state of Azad Jammu and Kashmir, has an abundance of flora and fauna with great health, food, economic and environmental benefits due to the presence of valuable bioactive compounds. Sea buckthorn, *Hippophae rhamnoides* L., is a magic multipurpose plant widely spread throughout the Northern Areas of Pakistan. The plant has gained the global attention due to its high content of vitamins, minerals, natural antioxidants, n-3 and n-6 fatty acids, and proteins. It has been extensively reported as a functional food around the globe and to have anti-proliferation properties which can stimulate the immune system. Sea buckthorn has a wide range of beneficial antioxidant, anti-inflammatory, and anti-carcinogenic effects. The plant has attracted the attention of scientists and is highly valued for its biomedicine and cosmetic industry. Its occurrence in numerous regions is due to its exceptional features that make it cultivatable in various parts of the world and can be used for multiple research areas including environmental studies.

The traditional use of sea buckthorn as a medicine for several ailments has been very common in central and Southeastern Asia. The therapeutic effect of sea buckthorn is mainly due to the bioaccumulation of a large number of bioactive compounds including carotenoids, flavonoids, lipids, tannins and amino acids. The importance of these bio-molecules is indeed irrefutable as they have been reported to play dynamic therapeutic functions including antioxidant, antitumorous, hepato-protective and immunomodulatory roles in the body [2].

Comparable to its berries, the leaves of the plant also show great diversity of bioactive compounds, thus can be regarded as largely chemo-diverse parts of the plants. The recent scientific researchers have reported several pharmacological benefits of Sea buckthorn leaf extracts such as tissue regeneration, immunomodulatory, radioprotective, adaptogenic, anti-inflammatory, anti-atherogenic and anti-stress effects. The extensive research of sea buckthorn mostly circulates around the berries. However, no adverse effects like cytotoxicity of sea buckthorn leaf extracts have been reported on the mammalian body cells, when orally administered. The leaves of the plant have been marked to have antibacterial, anti-tumor and anti-oxidative properties. No previous scientific study has been reported on the leaves of Sea buckthorn in Pakistan, therefore this research paper is mainly based on the leaves of Sea buckhorn as there is no limit for the availability of plant leaves throughout the year. They can be obtained any time of the year and are one of the most chemo-diverse sections of the plant with astounding medicinal value [3].

A healthy individual shows a dynamic balance between the free radicals produced in the body, during metabolic reactions, and the internal antioxidant defense systems that provide protection against damage and ultimately toxicity caused by these free radicals. The body suffers from oxidative stress when there is an imbalance between oxidants and antioxidants due to an increase of free radicals, resulting in higher concentration of oxidants in the body. This causes various genetic deleterious effects in the body. These effects on the cells exposed to excessive quantities of oxidants include protein oxidation, lipid peroxidation, nucleic acid instability and several mutations. The antioxidants derived from the sea buckthorn leaf extracts like vitamins, minerals, flavonoids, and polyphenolic compounds have the capability to scavenge free radicals and cause inhibition of lipid peroxidation. The sea buckthorn leaves have been found to possess pharmacological grade of anti-inflammatory and antibacterial effects in the body. Multiple problems have been generated subsequently to the excessive use of antibiotics, which produced a great interest in the use of medicinal plants as potential antibacterial medicines of human beings are dying of cardiovascular diseases every year. One of the principal causes of these disorders include thrombosis *i.e.* clot formation in blood vessels. Presently, a large number of human beings are suffering from thromboembolic disorders worldwide. Plant extracts have been used in traditional healing and display anticoagulant/antithrombotic activity. This magic shrub offers multiple benefits for the economically deprived mountain communities of the Himalayas and can improve the socio economic status of the masses residing in these rural areas. There is huge potential for developing standardized herbal products from different parts of the Sea buckthorn plant. The main objectives of the present study were:

- The phytochemical analysis by mineral estimation of sea buckthorn leaves in two genotypes *i.e. H. sinensis* and *H. turkestanica*.
- Determination of anti-oxidative potential in sea buckthorn leaves and its berries.
- Evaluation of anti-coagulant activities of sea buckthorn leaves and berries in human blood.

MATERIALS AND METHODS

Plant material

Two different genotypes of sea buckthorn were used in this study as different plant cultivars/genotypes express significant variation in secondary metabolites. The research was conducted during the period 2017-2018 and two important genotypes of sea buckthorn were used in experiments on biochemical analysis, antioxidant and anti-coagulant activities. The leaves and berries of turkestanica were collected from skardu, baltistan and leaves of sinences were collected from khaigala rawalakot, district poonch azad Jammu and Kashmir in the month of August 2017. *H. turkestanica* grows naturally in gilgit Baltistan and Sinences has been introduced from China to rawalakot, district poonch. The plant material was transported to the laboratory of department of biotechnology, university of Kotli Azad Jammu and Kashmir. The samples were separately washed, dried in shade and subjected to freezer for storage at 4°C. Later the samples were crushed to powder using pistil and mortar [4].

Biochemical analysis

The plant leaves and berries contain enormous number of antioxidants, phenols, flavonoids, vitamins and minerals. Thus, the present study significantly focused on phytochemical analysis of leaves which included examination of DPPH radical scavenging assay and basic analytical techniques, also supported. The phenolic and ascorbic acid estimation had also been examined in the current research. This analysis also explored the mineral elements in sea buckthorn leaves [5].

Mineral estimation

Leaves of sea buckthorn were subjected to digestion for the estimation of minerals from sea buckthorn leaves and berries by instrumental method.

Preparation of acid digest: The powdered leaves in were taken and transferred to digestion tubes. 5 ml of conc. HNO_3 was added and kept for 30 minutes in a treated digester at 70°C. Temperature was increased up to 140°C until the all the HNO_3 was evaporated. After cooling the tubes, 3ml of 1:1 solution (HNO_3:$HClO_4$) was added. The tube was heated to 200°C until the $HClO_4$ was evaporated. As the fumes disappeared tubes were cooled down and then the residues left behind were transferred to a volumetric flask and diluted to 50 ml with distilled water. The prepared digest was stored in the refrigerator and further used for mineral estimation [6].

Determination of iron (Fe): The iron contents were determined by thiocyanate method. On reaction iron contents in the sample developed color by reacting Iron with potassium thiocyanate. The Iron contents were estimated by spectrophotometer. Complex formation of iron developed red color and absorbance was recorded at 447 nm.

Preparation of reagents and solution

- Hydrochloric acid (6N).
- Hydrogen peroxide (30%).
- Potassium thiocyanate solution (15%).
- Standard iron solution (1 mg/100 ml): 70.2 mg $FeSO_4$ (NH_4). $6H_2O$ in 50 ml distilled water and diluted to 1000 ml with water.

Preparation of standard curve

Different concentrations were prepared from standard iron solution (5 µl, 10 µl, 15 µl, 20 µl and 25 µl). 5 ml from each dilution of standard solution were taken. 10 ml of reagents from (a), 1 ml of (b) and 5 ml of (c) were taken and diluted with water up to 100 ml. The solutions were allowed to stand for sixty minutes to produce colour. Water was used to prepare a control standard blank and the above procedure was repeated accordingly. By using spectrophotometer absorbency was monitored at 447 nm wavelength. While plotting a graph between iron concentration and absorbency a straight line was observed.

Sample assay

The amount of acid digest used was 5 ml and its dilutions were made in the same way as described above. Absorbance was also observed at 447 nm. Wavelength and results were obtained from standard curve drawn using different concentrations of standard iron against their absorbance of sample. The iron was calculated as mg/100 g of the sample.

Determination of phosphorus

Phosphorus was usually determined and expressed as phosphoric acid (P_2O_5) through the colorimetric method. Digested berries sample was then used for determination of phosphorus. The method used was colorimetric method.

Principle: Phosphorus estimation is based upon colourimetric method in which certain elements or compounds react with some reagents and develop a colour, whose intensity is measured in a spectrophotometer. The inorganic phosphorus reacts with ammonium molybdate. Ammonium phospho-molybdate was formed, which on reduction produce "molybdenum blue". The blue colour complex was measured on spectrophotometer and total amount of phosphate was determined [7].

Inorganic phosphorus+ammonium molybdate=Ammonium phopho-molybdate

Preparation of reagents and solutions

The reagents used in phosphorus estimation are mainly sulfuric acid, ammonium vanadate, ammonium molybdate and standard potassium dihydrogen phosphate (KH_2PO_4) solution. The preparation of reagents and chemicals used for phosphorus estimation have been given in the tabular form in the following table.

Preparation of reagents for phosphorus estimation

Sulfuric acid and water was mixed with 1:6 followed by mixing 0.25 gram ammonium vandate in 50 ml water, 6 ml of sulfuric acid diluted up to 100 ml. To prepare 5% of Ammonium molybdate 5 grams of reagent was diluted in 100 ml of distilled water. Standard potassium hydrogen phosphate solution was prepared by dissolving 30 mg of reagent in 100 ml of water.

Preparation of standard curve

Different concentrations of standard KH_2PO_4 solution were prepared. After addition of 5 ml aliquot in 50 ml flask, 5 ml of each reagent (a), (b) and (c) were added successfully. The flask was shaken after the addition of each reagent. The mixture was diluted up to 50 ml and left to develop color. Repetition of steps was performed and a standard blank was prepared using 5 ml water instead of standard solution. Absorbance of standard solution at 470 nm with standard blank was recorded and standard curve was constructed by plotting absorbance verses construction (graph).

Sample assay

The sample (5 ml) was taken from the acid digest and same procedure was followed used to prepare standard solution. Absorbance was measured which determined the amount of phosphate from the standard curve. The phosphate was calculated as mg/100 g sample.

Determination of magnesium

Preparation of reagents for magnesium determination: Ethylene diamine tetra acetic acid was diluted in 1000 ml of distilled water. EDTA was heated at 800°C to remove moisture and then weighed. 0.2 g of Eriochrome Black T. Indicator (EBT) dissolved in 20 ml in absolute ethanol was used. The buffer solution (17.5 g) NH_4Cl was mixed in 142 ml concentrated ammonia solution and diluted to 250 ml with distilled water. The sample was prepared by adding berries and blended in distilled water [8].

Procedure

Sample solution (25 ml) were diluted to 100 ml with distilled water. The buffer solution (2 ml) was added, followed by 3-5 drops of EDT indicator. Prepared solution was titrated against 0.1 M EDTA until colour changed from red to blue. Titration was performed slowly until the end point since the complex does not form instantaneously. The methodology followed for magnesium estimation has already been reported by. Titration principle was the base used for determining Ca in the sample. The indicator Murexide and buffer combined produce a red coloured solution. The solution was then titrated against 0.01 M solution of EDTA until the red colour of the solution changed to bluish-violet. Tabular form of the principle is also given. A sample of 25 ml was placed in the titration flask and 25 ml of distilled water was added. 1 ml of buffer (4 M NaOH) and 1 ml of murexide indicator was also added. By using 0.01 M EDTA, solution was titrated until colour of solution changed from red to bluish-violet colour [9].

Antioxidant activity by DPPH free radical scavenging assay

The antioxidant activity of sea buckthorn berries and leave extracts was evaluated using 2,2-diphenyl-1-Picrylhydrazyl. For the DPPH radical-scavenging assay, the procedure followed the method described by Muhammad. The sample was evaluated at 100 mg/L by mixing 0.75 mL with 1.5 mL of DPPH fresh prepared solution (20 mg/L in ethanol). Each sample was mixed and incubated at room temperature for 60 min under darkness. The extract was analyzed for DPPH radical scavenging activity by measuring the absorbance at 517 nm. The blank was a solution of 1.5 mL DPPH and 0.75 mL ultrapure water. The extract blank was a solution obtained by mixing 1.5 mL extract and 0.75 mL ultrapure water. All measurements were performed in triplicate and the mean values were recorded. The antioxidant activity of the extracts was evaluated spectropho to metrically by using an Evolution 220 ultraviolet-visible spectrophotometer. The synthetic oxidant 2,2-Diphenyl-1-Picrylhydrazyl, (DPPH), was used for radical scavenging assay. The plant extract was prepared in various solvents namely acetone, ethanol and methanol [10].

Preparation of sea buckthorn extracts and DPPH free radical: One gram of dried, powdered sample of each ssp. was dissolved in 100 ml of each solvent separately. The solutions were placed in shaker for 30-40 minutes at 250 rpm and afterwards filtered using Whatman filter paper No. 41. The extract obtained in this way was subjected to refrigeration for later use. The amount of 0.004 gm of DPPH radical was taken and dissolved in 50ml of 70% methanol. For complete dissolution the solution was put in a shaker at 250 rpm for 20-25 minutes. The obtained solution was protected from light radiation by keeping it in the dark due to its photosensitivity and becoming easily oxidized [11].

Determination of phenolic contents

Sample was prepared by dissolving 0.1 gm of sample in 10ml of solvent. The total phenolic content of plant sample was determined using the folin ciocalteu reagent. In a test tube, 0.5ml of the extract was added to 2 ml of 7.5% aqueous sodium carbonate solution and mixed well. Then 2.5 ml of 10% folin ciocalteau reagent was added to the mixture it will give greenish blue colour. After shaking, it was incubated at 45°C for 40 min and absorbance was measured at 750 nm in a spectrophotometer against a blank control. The total phenolic contents were calculated based on a calibration curve of Gallic acid and results were expressed as milligram gallic acid equivalents per gram (mg/g) of dry weight of the extract. Following formula was used for calculations:

Total phenolic contents=Abs × Gallic Acid Equivalent, GAE (1.15) × 100

Ascorbic acid assessment

Sample was prepared by dissolving 0.1 gm of sample in 10 ml of solvent. The total phenolic content of plant sample was determined using the folin ciocalteu reagent. In a test tube, 0.5 ml of the extract was added to 2 ml of 7.5% aqueous sodium carbonate solution and mixed. Then 2.5 ml of 10% folin-ciocalteau reagent was added to the mixture. It will give a greenish blue colour. After shaking, it was incubated at 4°C for 40 min and absorbance was measured at 750 nm in a spectrophotometer against a blank control. The total phenolic contents were calculated based on a calibration curve of gallic acid and results were expressed as milligram gallic acid equivalents per gram (mg/g) of dry weight of the extract. Following formula was used for calculations:

Total phenolic contents = Abs × Gallic Acid Equivalent, GAE (1.15) × 100

Ascorbic acid assessment: As ascorbic acid is a reducing agent, the estimation was done by titration method using an oxidizing agent 6-dichlorophenol indophenol. It is a blue coloured dye which upon addition of one drop of pure glacial acetic acid, discharges its blue colour changing into pink and becomes colourless upon titration with the plant sample. Oxalic acid solution (0.4%) was dissolved in 100 ml of distilled water and stored in a refrigerator. In 100ml of distilled water, 40 mg of 2,6-dichlorophenol-indophenol was added to make the solution. After filtration the solution should be kept in the refrigerator and should be use within three days. One drop of glacial acetic acid was added in 0.5 ml of dye in a test tube that changed the colour of dye from blue to pink. This solution was titrated against the sample until the pink colour disappeared. The volume to be used for the sample was calculating using the actual amount of vitamin C in mg/100 gm. The calculations were made using the following method: As 0.5 ml of dye reacts with 0.1 ml of the standard ascorbic acid solution.

Oil estimation from berries

Sea buckthorn berries (5 g) was taken and then kept in oven for 24 hours at 70°C to remove moisture content. After drying, the sample was taken out from the oven, cooled and 5 gm dried sample of the berries was ground then weighed accurately for the extraction of oil and wrapped in pre-weighed filter papers. Then filter paper was again weighed with the sample. The sample was put in the thimble and then it was placed in the extraction tube of soxhlet apparatus at the temperature of 70°C to 80°C for about five hours using approximately 150 ml ether in the flask. The whole apparatus was assembled. The water inlet and hot plate both were turned on to start the process of oil extraction. The sample was then refluxed for 4 hours. The ether that remained in the

extraction tube was recovered for further use [12]. The extract was transferred into a pre-weighed clean flask with ether washing and remaining ether was evaporated by placing flask in an oven at 105°C for an hour. It was then cooled in a desiccator and weighed.

Empty thimble=w_1, Thimble with sample=w_2, Weight of sample=p

Crude fat percentage=$(w_2-w_1)/p \times 100$

Anticoagulant activity

The berries of the plant were weighed to 1 g, washed, dried, powdered and finally mixed with 10 ml of the solvent.

Blood collection: Blood samples were drawn *via* venipuncture from healthy volunteers (n=3) of ages ranging between 21-26 years old. The blood placed separately in containers containing sodium citrate to prevent the clotting process.

Anticoagulation assay

Clotting time was determined from blood sample of each individual. The plant extracts (25, 50 and 75 µL) were added separately to the blood samples in a water bath at 37°C with gentle shaking. Subsequently the stopwatch was used to measure the time period for the clot formation. This time is called the prothrombin time.

Statistical analysis

The IC_{50} values were calculated for radical scavenging assay. Correlation was measured for mineral and ascorbic acid concentrations. For all other activities, mean values have been expressed with the standard deviation [13].

RESULTS AND DISCUSSION

The Figure 1 illustrates vitamin C and minerals estimated in sea buckthorn leaves and berries. It is evident that significant amount of vitamin C in sea buckthorn has been revealed not only in berries but also in leaves. Among both genotypes, the leaves of turkestanica contained higher value of vitamin C *i.e.* 83.33 mg/100g. The comparison of both genotype revealed that minerals composition including iron, calcium, phosphorus, potassium in *H. turkestanica* leaves were greater as compared to *H. sinensis*. The value of vitamin C is highest in the berries as well as leaves of both subspecies. The mineral elements occur largely in the berries and leaves of turkestanica. Calcium specifically has lower amount in the leaves.

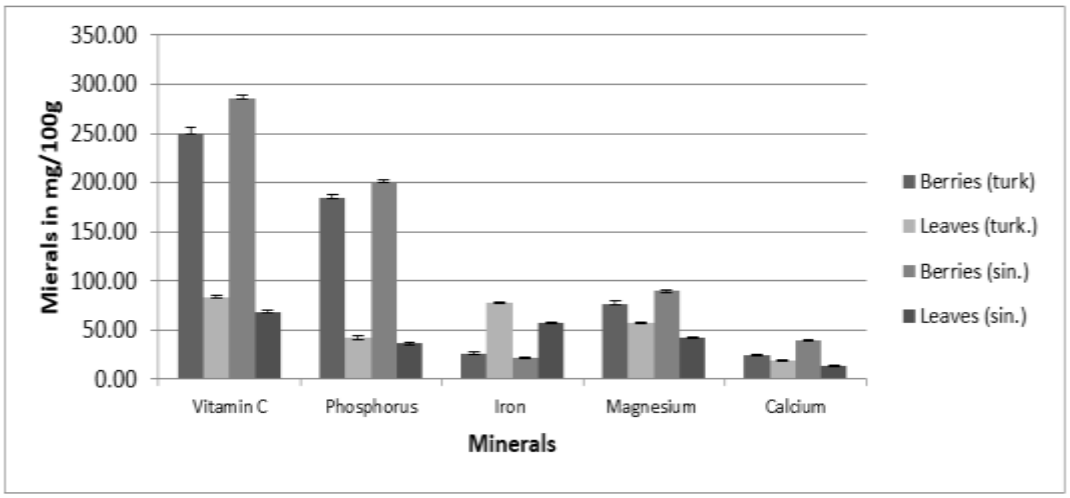

Figure 1. Mineral contents in sea buckthorn (tukestanica and sinences) berries and leaves

The Figure 2 depicts that the total phenolic contents in two genotypes of sea buckthorn leaves and berries. The range has been found between 16.33 to 61.33 mg GAE/g. The highest amount of phenolic contents has been observed in *H. turkestanica* berries and the lowest in *H. sinensis* leaves. Among the leaves of two genotypes *H. turkestanica* has comparatively higher phenolic content *i.e.* 19.0 mg GAE/g. The higher antioxidant activity of genotype *H. turkestanica* leaves and berries has been observed which is due to larger phenolic contents. Similar findings have been reported by and quoted that phenolic components play an important role in the antioxidant capacity of the sea buckthorn berries *H. sinensis* leaves as well as berries have shown lower antioxidant potential due to lesser contents of phenols. It has been illustrated that sea buckthorn oil contains large amount of phenols particularly polyphenols about 120-550 mg GAE/g that are reported to have antioxidant potential. In another study illustrated the antioxidant capability of sea buckthorn is due to the presence of phenolic compounds; this effect is considered meager to that of ascorbic acid. The oil contents calculated in dried berries was found to be 91.96% as maximum from the 5 gm of sample used. Our study verifies the findings of who has reported that sea buckthorn leaves offer the variety of biochemical constituents and should be utilized for human food and health. The scavenging activity shown in sea buckthorn leaf extracts against DPPH+ was clearly concentration dependent. There are significant differences in the scavenging percentage between extracts of different solvents. The results clearly designate that all extracts exhibited highly significant antioxidant activity [14].

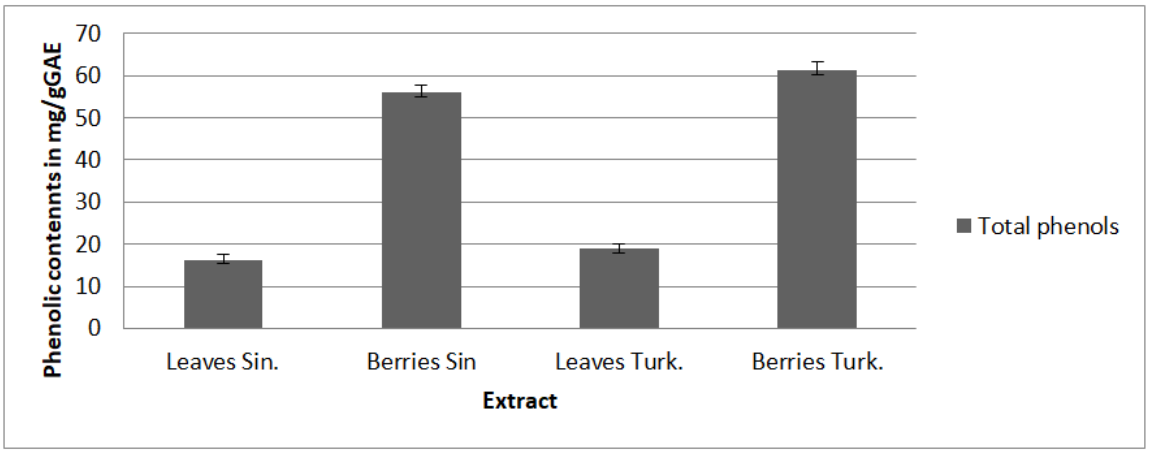

Figure 2. Total phenolic contents in sea buckthorn (tukestanica and sinences) berries and leaves

It is clearly evident from the (Figures 3-6) that sea buckthorn leaf extracts in all the solvents show considerable antioxidant activity. The highest value of RSA has been achieved from acetonic extract i.e. 87%. On the other hand, the lowest value has been shown by methanolic extract *i.e.* 51.1%. The leaves of ssp. *H. sinensis* also show significant radical scavenging activity. The highest RSA value has been attained from the acetonic extract *i.e.* 77.6%. The lowest RSA value has been observed in methanolic extract which is 53.1%. Our findings are in agreement with the findings of who reported that sea buckthorn leaves can offer a good source of antioxidants which can potentially counteract the oxidative stress-induced mitochondrial dysfunction and apoptosis of neuronal cells.

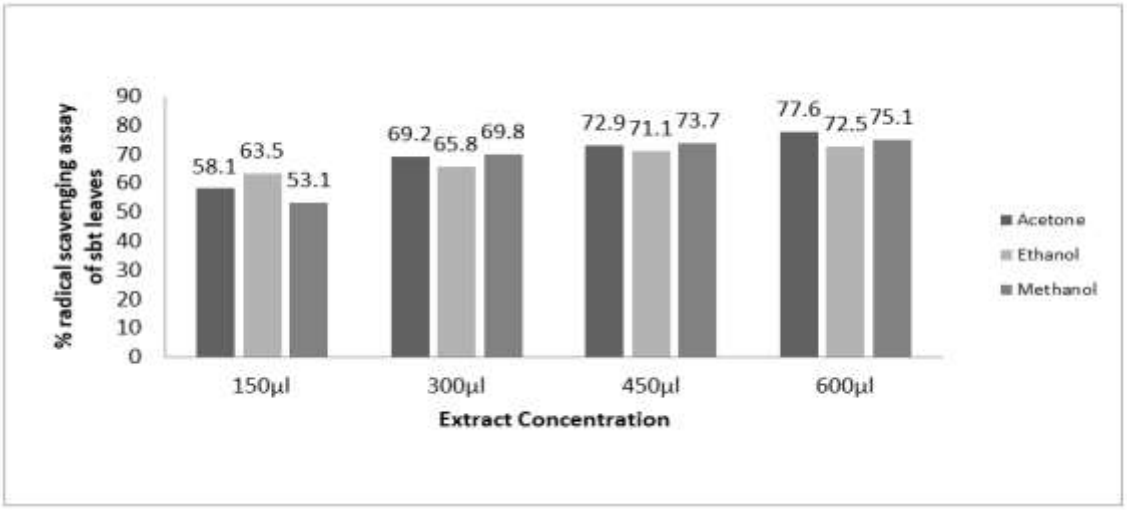

Figure 3. Free radical scavenging %age of sea buckthorn leaves (Sinences)

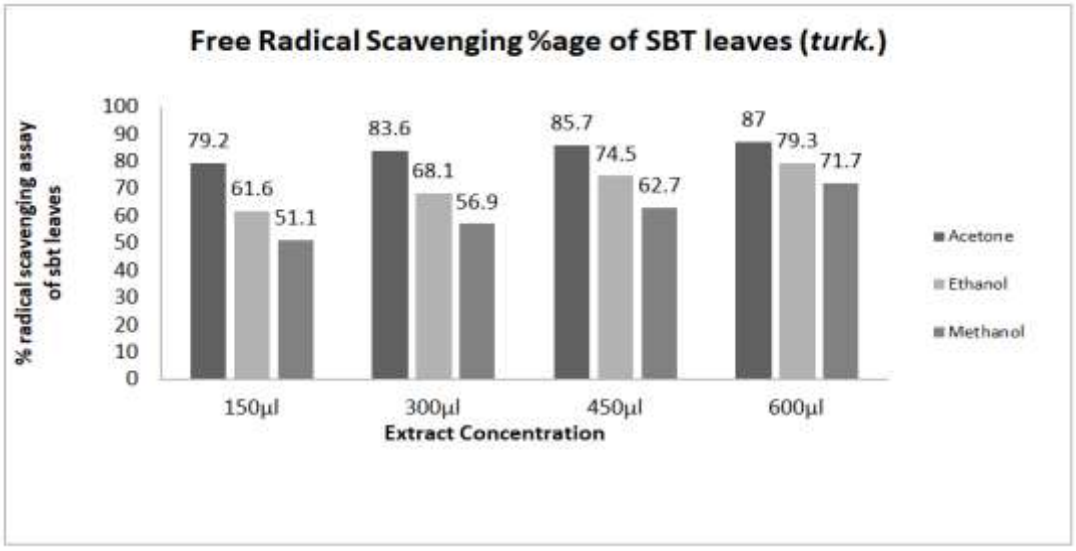

Figure 4. Free radical scanvenging % age of sea buckthorn leaves ssp *H. turkestanica*

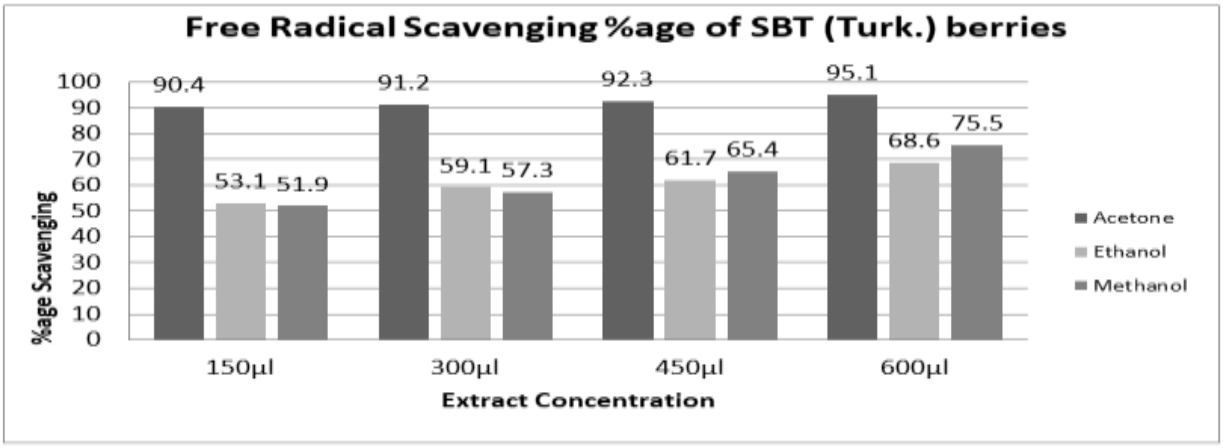

Figure 5. Free radical scavenging %age of sea buckthorn berries ssp. *H. turkestanica*

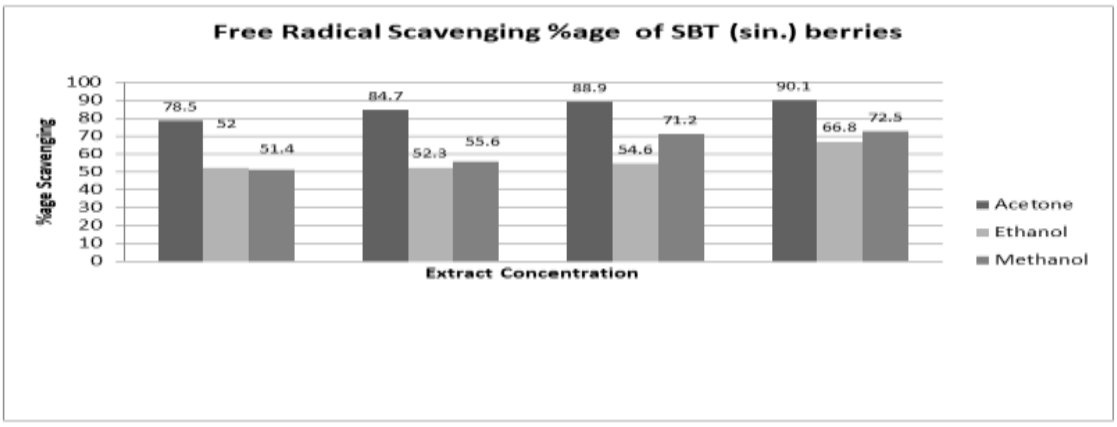

Figure 6. Free radical scavenging %age of sea buckthorn berries spp. *H.Sinences.*

On comparison of the two ssp. *H. turkestanica* and *H. sinensis*, it has been noted that sub specie. *Turkestanica* has a comparatively higher RSA value *i.e.* 87% which depicts that it is more potent sub species for antioxidant activity with greater potential of scavenging free radicals. The acetonic extract of berries shows the highest %RSA *i.e.* 95.1%. The %RSA of berries is used as a positive control for its comparison with %RSA of leaves. Upon this comparison, it can easily be deduced that sea buckthorn leaves having 87% RSA with antioxidant potential near to that of its berries *i.e.* 95.1%. The IC_{50} value (extract concentration that causes 50 per cent scavenging) was also determined from the graph of scavenging effect percentage against the extract concentration. From the IC 50 values of different extracts, it was observed that acetonic extract was better scavenger than ethanolic and methanolic extracts for the DPPH free radical. Our findings differ with the results reported by who reported that ethanolic extracts from *Zanthozyulum armatum* seeds expressed maximum anti oxidant activity. The efficiency of solvent varies with the type of species [15].

The potential derivatives of oxygen known as reactive oxygen species, ROS such as superoxide anion (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) are produced in the human body as a result of exogenous exposure of toxic substances or endogenous metabolic processes involving redox enzymes or bioenergetic electron transport. The sea buckthorn leaves and berries offer a potential source to neutralize the impact of free radicals produced in human body. Many plants have been investigated to seek the anticoagulant or antithrombotic activity of their extracts. Among these grape seed oil, garlic and red onion; *Allium cepa*, are found to have antithrombotic effects through prevention of coagulation and clot formation.

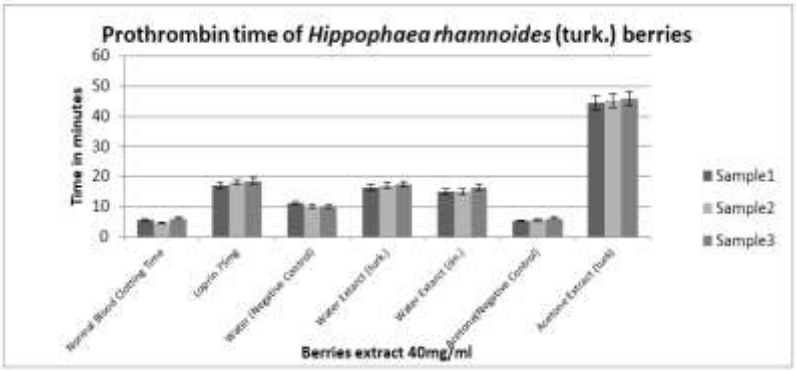

Figure 7. Prothrombin time of Hippophae rhamnoides spp turkestanica berries

The anticoagulant activity presented in Figure 7 was performed to investigate the cardio protective potential of sea buckthorn. For this clotting time of human blood *in vitro* was measured by using plant berry extracts. Normally human blood clots within 5-7 minutes. The clotting time was found to be 45 minutes 10 seconds of sea buckthorn berry extract in acetone, while acetone when added in blood as a negative control, and immediately coagulates the blood within minutes and 20 seconds. The clotting time of loprin (anteric coated asperine) taken as a positive control was about 17 minutes. Thus, upon comparison with acetonic extract of sea buckthorn berries, it is observed that it may contain phytochemical constituents that are capable of anticoagulation of human blood. This characterises sea buckthorn as a potential of causing anticoagulation and can be used as an anticoagulative agent. Sea buckthorn leaves are a particular potent source of phytochemicals, specifically phenols and flavonoids including isorhamnetin and quercetin, carotenoids, sterols, triterpenols and isoprenols, etc. These compounds show therapeutic effects including anti-cancerous, anti-bronchitis, anti-inflammatory, wound and burns healing, stimulant, memory revitalizer including palliative antiviral and antimicrobial properties. All these effects have been observed and studied in the sea buckthorn leaves. On the basis of phytochemical constituents in the berries and leaves of *H. rhamanoides*, three main components were elucidated; it is clear from the biplot diagram (Figure 8) that PC1 imparts more variance *i.e.* 92.31 with eigen value 5.54, while all other components carried very small variations. Total phenolics, vitamin C and minerals including showed significant positive correlation with each other while the iron showed negative correlation with all other phytochemical constituents [16].

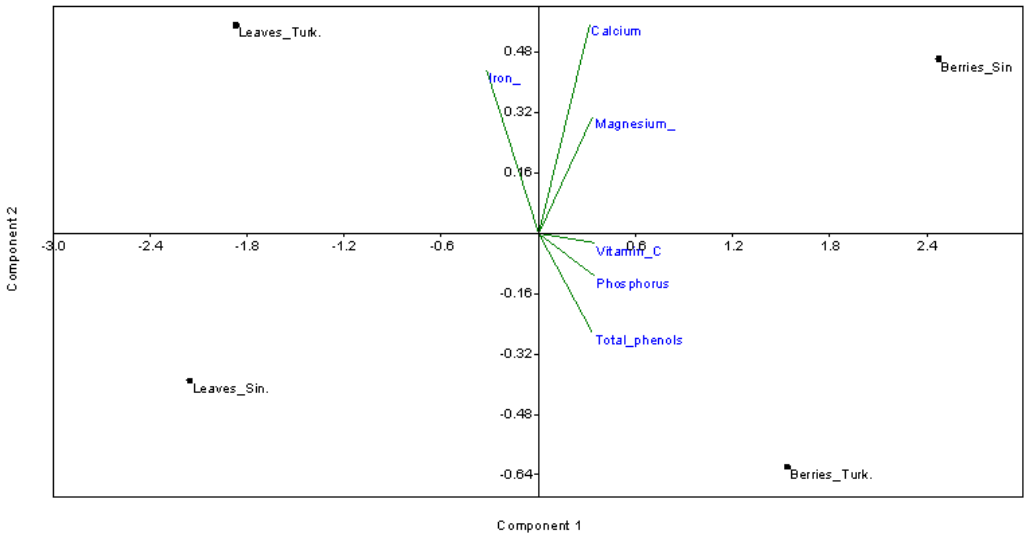

Figure 8. Correlation among the biochemical constituents in sea buckthorn and leaves

CONCLUSION

Sea buckthorn is a popular multipurpose plant known for its potential uses in nutrition, health and environment benefits throughout the world. There has been research reported in Pakistan about the biochemical and antioxidant properties of sea buckthorn but there hasn't been any report thus far about the properties of the sea buckthorn leaves of ssp *H. turkestanica*. This study has been reported for the first time in Pakistan using both sea buckthorn leaves and berries. The results obtained through various investigations confirmed the biochemical properties, antioxidant and anticoagulant activities in the berries and leaves of sea buckthorn located in Pakistan. The phytochemical analysis indicated that antioxidant potential, phenolic contents, mineral elements and ascorbic acid were found to be in greater quantity in *H. turkestanica*. Upon investigation, antioxidant and anticoagulant activities of the sea buckthorn were found to be an effective agent against oxidative stress and helps in scavenging free radicals. This antioxidants potential of Sea buckthorn leaves and berries can help in reducing the risk of terminal diseases like cancer. The blood clotting time has been prolonged by sea buckthorn leaves and berries thus reducing the risks of coronary heart diseases due to the rich contents of phenolic compounds. Based on these potential health benefits, the leaves of Sea buckthorn *H. turkestanica* along with the berries are recommended to be used to develop products with immense value to society riddled with diseases caused by oxidative stresses and exposure to excessive free radical activity in the human body. It is concluded that Sea buckthorn *Hipphae rhamnoides* ssp turketanica has an enriched source of medicinal and nutritional ingredients compared to ssp sinences. Henceforth it should be propagated and genetically improved to develop pharmaceutical and nutritional products to aid the eradication of malnourishment, strengthen the carrying capacity of ecosystem and sustain the rural economies on sustainable basis.

DISCLOSURE STATEMENT

The authors declare and confirm that there is no conflict of interest in submitting and publishing this research in the journal of horticultural science and biotechnology.

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