Research Article

Investigation of antioxidant activity of methanolic extracts of *Hippophae rhamnoides* ssp. *turkestanica* across habitats in Cold Desert Biosphere Reserve, Trans Himalaya, India

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ABSTRACT

The present study evaluated phenolic compounds and antioxidant properties of leaf, stem and root methanolic extracts of a high altitude species, *Hippophae rhamnoides* L. ssp. *turkestanica* Rousi (Elaeagnaceae). *H. rhamnoides* ssp. *turkestanica* was collected from four distantly located populations in Cold Desert Biosphere Reserve. Total phenolic contents across populations were ranged from 62±2.81 to 78±0.61; 80±2.67 to 98±0.62 and 77±3.83 to 97±0.35 mg GAE g^{-1} of extracts in leaves stems and roots, respectively. Flavonoid contents across populations ranged from 26±0.48 to 36±0.48; 34±4.38 to 53±5.59 and 65±0.48 to 75±0.28 mg QE g^{-1} of extracts in leaves, stems and roots, respectively. Phenolic and flavonoid contents were significantly higher in the stem and root extracts as compared to leaf extracts. One way analysis of variance (ANOVA) revealed significant variation among populations. All extracts exhibit strong antioxidant activity determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and reducing power, assays. The percent inhibition was found to increase with every unit increase in concentration. The DPPH and ABTS radical scavenging reached up to 88.38 % and 99.49 % at a concentration of 400 μg mL^{-1}, respectively. Reducing power ranged from 0.203 to 0.593 at concentrations ranging from 50 to 400 µg mL^{-1}. Therefore, present study strongly supports *Hippophae rhamnoides* ssp. *turkestanica* as a potent source of antioxidant and could be utilised as natural source of antioxidants in pharmaceuticals and food industry.

Keywords: *Hippophae rhamnoides* L. ssp. *turkestanica* Rousi; Phenolic contents; flavonoid contents; antioxidant activity; DPPH; ABTS; cold desert; Trans Himalaya.

INTRODUCTION

Antioxidants, present in plants at high levels, identified as a good sources of functional ingredients[1]. An antioxidant is a compound that inhibits or delays the oxidation of substrate even if the compound is present in a significantly lower concentration than the oxidized substrate[2]. Antioxidant compounds play a vital role as reducing agents and free radical scavengers. Free radicals either formed by cellular metabolism, exogenous chemicals or stress is capable of oxidative damage to various biomolecules in cells, such as, DNA, carbohydrates and proteins which in turn lead to various chronic and degenerative diseases, including, atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, inflammation, neurodegenerative diseases and others[3,4,5]. Plants have long been a source of natural antioxidants and effective phenolic compounds, which act as scavengers of free radicals and inhibitors of lipid peroxidation[6]. The natural antioxidants are an interesting alternative to synthetic antioxidants due to safety concerns and limitation of usage[7]. The phenolic and flavonoid compounds of plants have been reported to exert multiple biological effects including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic, inhibition of hydrolytic and oxidative enzymes etc[8]. Therefore, there is an urgent need to find some external natural source of antioxidants. The high altitude medicinal plants are considered as a good source of natural antioxidants.

Hippophae rhamnoides ssp. *turkestanica* popularly known as "Seabuckthorn" is an ancient, deciduous shrub with good adaptability to various climate conditions with extensive genetic variability[9]. The genus belongs to the family Elaeagnaceae, which consists of 7 species (*H. tibetana*, *H. rhamnoides*, *H. gyantsensis*, *H. salicifolia*, *H. neurocarpa*, *H. gonoicarpa*, and *H. litangensis*) and 11 subspecies, out of which *H. rhamnoides* includes 9 subspecies (*sinensis*, *yunnanensis*, *turkestanica*, *mongolica*, *fluviatilis*, *carpatica*, *rhamnoides wolongensis* and *caucasica*), among which the most economically important one is *Hippophae rhamnoides* ssp. *turkestanica* (Figure 1)[9,10]. Seabuckthorn (SBT) is a high altitude species, widely distributed throughout the temperate zone of Asia, Europe and all over sub-tropical zone[11]. It has also been introduced in North and South America[12].

In India, SBT is widely distributed in the cold desert region of Himachal Pradesh. It is a multipurpose shrub of cold desert region, used by tribal communities for making jam, jellies, tea, etc.; considered as a good source of fuel and fodder and also used for making hedge around their agricultural fields. In addition, SBT is widely used for controlling soil erosion due to its long and extensive roots. Its roots are associated with a symbiotic mycorrhizal fungus (*Frankia*), which forms root nodules and can fix the maximum amount of atmospheric

nitrogen. The estimated capacity of SBT roots to fix nitrogen (180 kg of nitrogen/ha/year) is twice that of soybean[13]. It is also a fast growing and pioneer species, which increase soil fertility by increasing the nitrogen, phosphorus and organic matter contents in soil. It has attracted international attention as a high potential species as livelihood of the inhabitants. It is said to have "momentous economic potential" and is predicted by some as the "next major health food fad"[14]. All parts of the plant are very good source of large number of bioactive substances like carotenoids (α, β, ð-carotene and lycopene), flavonoids (isorhamnetin, quercetin, myricetin, kaempferol and their glucoside compounds), vitamins (A, C, E, riboflavin, folic acid and K), organic acids (malic acid and oxalic acid), sterols (ergosterol, stigmasterol, lanosterol and amyrins) and some essential amino acids[15]. It has been traditionally used in the treatment of various diseases, like cough, gastric ulcers, asthma, flu, cardiovascular diseases, lung disorder and skin related problems[7,16]. SBT is highly valued for its antioxidant, anti-diabetic, anti-atherogenic, cardioprotective, anti-carcinogenic, hepatoprotective, immunomodulatory, antiviral, antibacterial, anti-inflammatory and vasorelaxant effects[17]. Isorhamnetin isolated from SBT, showed significant antioxidant activity in several antioxidant assays[18].

Figure 1: *Hippophae rhamnoides* ssp. *turkestanica*; (a) female plant with heavy fruiting, and (b) enlarged view

The review of literature revealed that there are lots of studies are available related to antioxidant properties of *H. rhamnoides* at international and national levels[6,7,9,19-26]. While, in Himachal Pradesh such studies on this species mainly focused on berries and seeds[7,27-29], except, a single study on the antioxidant potential of *H. rhamnoides* methanolic leaf extract[16]. Therefore, apart from well documented data on *H. rhamnoides* berries and seeds, data on antioxidant potential of whole plant is lacking in respect to *H. rhamnoides* ssp. *turkestanica* growing widely in Cold Desert Biosphere reserve, Lahaul Spiti district, Himachal Pradesh. Therefore, present study has been carried out to investigate antioxidant properties of *H. rhamnoides* ssp. *turkestanic*a and focuses on; (i) total phenolic contents of leaf, stem and root extracts as gallic acid equivalents and total flavanoid content as quercetin equivalent, and (ii) antioxidant activity profiles of these plant extracts by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, 2,2'-azinobis- (3-ethylbenzothia zoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging assay and reducing power assays.

MATERIALS AND METHODS

Collection of plant material

The leaves, stems and roots of *H. rhamnoides* ssp. *turkestanica* were collected in the month of August 2013, from four distantly located populations (i.e., Lidang-3583 m amsl, Lingti-3619 amsl, Rangrik-3613 m amsl and Rama Khas-3572 m amsl) of Cold Desert Biosphere Reserve (CDBR). These populations represented by road side, dry slope, riverine and agricultural land habitats, respectively. Specimens of the species collected and identified with the help of flora of Lahaul-Spiti: A cold desert in North West Himalaya[30]. Voucher specimen of plant has been preserved.

Preparation of extracts

The fresh plant parts cleaned thoroughly, and air dried under shade in clean environment. The dried plant parts were ground to fine powder using a mechanical grinder. The standard one "cold maceration method" was used for the extraction of plant material. Powdered plant material dipped in sufficient amount of methanol in a stoppered container and allowed to stand at cool, dry and dark place for a period of 5 days with frequent agitation. After 5 days, the mixture was filtered and filtrate was taken out. The process of filtration was repeated for two more times, and then all the filtrates were combined. After evaporation of filtrate final extracts were obtained. This extract was used for the determination of total phenolics, flavonoids and antioxidant properties.

Determination of total phenolic contents

Total phenolic contents (TPC) were determined using Folin-Ciocalteu method with slight modification[31]. Gallic acid was used as standard for the calibration curve. 1 mL of extract (1 mg methanolic extract) was diluted in 46 mL

of distilled water. Then, 1mL Folin-Ciocalteu Reagent was added and mixed thoroughly. After 3 minutes 3 mL of 2% sodium carbonate was added to the mixture and allowed to stand for 3 hours with intermittent shaking. The mixture absorbance was measured at 760 nm in a UV-visible spectrophotometer (Ultrospec 2100 Pro, Healthcare Biosciences AB, Uppsala, Sweden). Same procedure was repeated to all the standards of gallic acid solutions. The standard curve was prepared using 0, 50, 100, 150, 200 and 250 µg mL^{-1} solutions of gallic acid in methanol. TPC in extracts was quantified using standard curve and results were expressed as milligrams gallic acid equivalent (GAE) per gram of extract.

Determination of total flavonoid contents

Total Flavonoid contents (TFC) was determined by aluminium chloride colorimetric method[32]. Quercetin was used as the standard for the calibration curve. Briefly, 50 µL of crude extract (1 mg mL^{-1}) was made up to 1 mL with methanol and diluted with 4 ml distilled water. Then, 0.3 mL of 5% $NaNO_2$ solution along with 0.3 mL of 10% $AlCl_3$ solution was added. The mixture was incubated for 5 minutes and then allowed to stand for 6 minutes. After that 2 mL of 1M L^{-1} NaOH solution was added, and 10 mL double-distilled water was added to make the final volume of the mixture. After, 15 minutes the absorbance of resulting reaction mixture was measured at 510 nm. Same procedure was repeated to all the standards of quercetin solutions. The calibration curve was prepared by using quercetin solutions at concentrations 0, 50, 100, 150, 200 and 250 µg mL^{-1} in methanol. TFC of the extracts was calculated in terms of quercetin equivalent using the standard curve and expressed as milligram quercetin equivalent (QE) per gram of extract.

Measurement of antioxidant activity

Radical scavenging activity (DPPH) assay

DPPH is a stable free radical and very popular for the study of natural antioxidants. On accepting hydrogen from a corresponding donor, its solutions lose the characteristic deep purple (λmax 515–517 nm) colour[33]. Free radical scavenging activity of methanolic extracts of *H. rhamnoides* ssp. *turkestanica* was measured by using DPPH assay as described by Shimada et al[34]. 1 ml of DPPH solution (0.1 M) mixed with 3 ml of leaf, stem and root extracts in water at varying concentrations (50–400 µg mL^{-1}). The mixture was shaken vigorously and kept in dark at room temperature for 30 minutes. Reduction in the absorbance at 517 nm was recorded by spectrophotometer. Lower the absorbance higher the free radical scavenging activity of the reaction mixture. The scavenging reaction between $DPPH^{\bullet}$ and an antioxidant (H-A) can be written as

$$(DPPH^{\bullet}) + (H\text{-}A) \longrightarrow DPPH\text{-}H + (A^{\bullet})$$

(Purple) (Yellow)

The percent DPPH scavenging effect was calculated using the following equation:

DPPH scavenging effect (%) = [(Ac– At) / Ac × 100]

Where, Ac is the absorbance of the control reaction, and At is the absorbance of the extract. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration.

Radical scavenging activity (ABTS Assay)

Traditional ABTS assay as described by Re et al[35] with slight modification was used for the determination of antioxidant activity. A concentration of 7 mM L^{-1} ABTS was prepared in water to make the stock solution. Then, an amount of 2.45 mM L^{-1} potassium persulfate was added to the ABTS stock solution and kept in the dark for 12-16 hours at room temperature for the production of ABTS cation ($ABTS^{\cdot+}$). $ABTS^{\cdot+}$ solution was diluted with 5 mM (pH 7.4) phosphate-buffered saline till an absorbance of 0.70 at 734 nm. Diluted $ABTS^{\cdot+}$ solution (1.0 mL) was added in 20 µL of methanolic leaf, stem and root extracts and the absorbance reading was taken 5 minutes after the initial mixing. The percent ABTS scavenging effect was calculated as follows:

ABTS scavenging effect = [(Ac– At) / Ac × 100]

Where, Ac is the absorbance of the control reaction, and At is the absorbance of the extract. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration.

Determination of reducing power

The reducing power of *H. rhamnoides* ssp. *turkestanica* leaf, stem and root extracts was measured according to the method prescribed by Oyaizu[36]. Different concentrations i.e., 50, 100, 200, 250 g mL^{-1} of extracts were prepared in 1 mL of distilled water. After that phosphate buffer (2.5 mL, 0.2M, pH 6.6) and potassium ferricyanide [K3Fe $(CN)_6$] (2.5 mL, 1%) was added in the prepared extracts solutions. The mixture was incubated at 50°C for 20 minutes, and then 2.5 mL of trichloroacetic acid (10%) was added to the reaction mixture. Now the prepared reaction mixture was centrifuged at 3000 rpm for 10 minutes. About 2.5 mL upper layer of centrifuged solution was mixed with 2.5 mL distilled water and 0.5 mL (0.1%) ferricchloride ($FeCl_3$). The absorbance of the reaction mixture was measured at 700 nm in a spectrophotometer. Higher absorbance of the reaction mixture indicated greater reducing power.

Statistical analysis

All the experiments were carried out in triplicate and the results were expressed as mean ± standard deviation (SD). Linear regression analysis was used to calculate the IC_{50} values, by plotting the percentage inhibition versus the concentrations. Statistical analysis was performed using SPSS 16.0, Sigma Plot 10.0 and Excel 2007. The results were also analysed statistically using One Way Analysis of Variance (ANOVA). The P value less than 0.05 (≤ 0.05) was considered to be statistically significant.

RESULTS

Total phenolic and flavonoid contents

Plant phenolic compounds such as flavonoids, phenolics, and tannins possess many potential biological activities; such activities might be related to their antioxidant activity[37]. Natural phenolic compounds are capable of decreasing oxygen concentration and contribute to the overall antioxidant activities of the plant foods[38]. Total phenolic and flavonoid contents of all the different methanolic leaf, stem and root extracts of *H. rhamnoides* ssp. *turkestanica* are presented in **Figure 2**.

Leaf extracts from different populations differed significantly (F = 41.7; P < 0.05) in their TPC, ranged between 62±2.81 (Lingti) to 78±0.61 (Rangrik) mg GAE g^{-1} of extract with an average value of 69±7.7 mg GAE g^{-1} of extract. The TPC in stem extracts ranged between 80±2.67 (Lingti) to 98±0.62 (Rangrik) mg GAE g^{-1} of extract. Average value of stem extracts is 89±7.73 mg GAE g^{-1} of extract and variation across populations were significant (F = 54.9; P < 0.05). Similarly, the amount of TPC in root extracts ranged between 77±3.83 (Lingti) to 97±0.35 (Rangrik) mg GAE g^{-1} of extract. Average value of root extracts is 84±9.27 mg GAE g^{-1} of extract and variation among population were significant (F = 52.04; P < 0.05). The above mentioned results indicated that *H. Rhamnoides* ssp. *turkestanica* growing in riverine habitat (Rangrik) contains significantly higher amount of TPC as compared to dry slope habitat (Lingti). Results also showed higher amounts of TPC in stem and root extracts as compared to leaf extracts.

Flavonoids are widespread plant secondary metabolites, shows considerable effect on human nutrition and health[39]. The TFC in leaf extracts of *H. rhamnoides* ssp. *turkestanica* varied from 26±0.48 (Lingti) to 36±0.48 (Rangrik) mg QE g^{-1} of extract, with an average value of 33±4.32 mg QE g^{-1} of extract and variation across populations were significant (F = 23.9; P < 0.05) as shown in **Figure 2**. The TFC in stem extracts ranged between 34±4.38 (Lidang) to 53±5.59 (Rangrik) mg QE g^{-1} of extract, with an average value of 42±9.23 mg QE g^{-1} of extract and variation across populations were significant (F = 20.6; P < 0.05). The TFC in the root extracts varied from 65±0.48 (Lingti) to 75±0.28 (Rangrik) mg QE g^{-1} of extract. Average value of root extracts is 71±4.2 mg QE g^{-1} of extract and variation across populations were not significant (F = 1.59; P > 0.05). Riverine habitat also shows higher TFC as compared to dry slope. Similarly, the TFC is significantly higher in stems and roots than leaves.

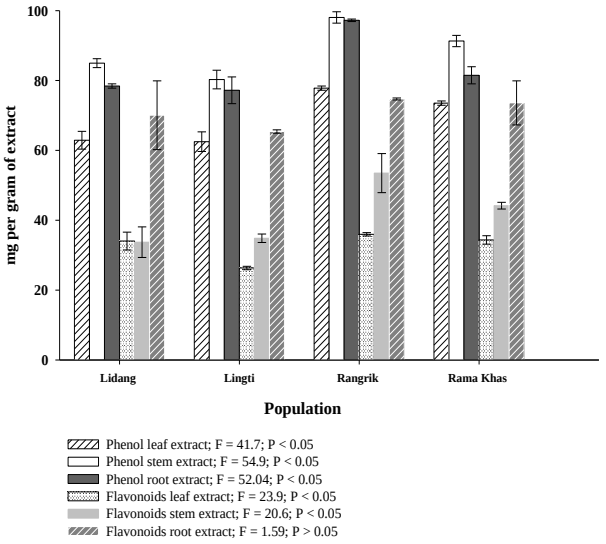

Figure 2: Total phenolic and flavonoid contents of leaf, stem and root extracts of *H. rhamnoides* ssp. *turkestanica*; All values are mean of three measurements

Antioxidant activity

DPPH radical scavenging activity

DPPH is one of the best scavenging methods because of it is easy, fast and reliable. It is a stable diamagnetic molecule and the reduction capability was determined by the decrease in absorbance induced by plant antioxidants[40]. For each sample (leaf, stem and root), four concentrations (50–400 μg mL^{-1}) of the extracts were tested. The antioxidant activity of leaf, stem and root extracts was increased with increasing concentration. IC_{50} value is negatively correlated with antioxidant activity, the lower the IC_{50} value, the higher is the antioxidant activity of tested sample.

The DPPH radical scavenging activity of leaf, stem and root extracts is shown in **Figure 3a**, **b** and **c**. The highest DPPH radical scavenging effect was obtained in leaves collected from Lidang village with the lowest IC_{50} of 38.78±6.47 μg mL^{-1} (**Table 1**) with significant variation among populations (F= 920, P < 0.05). While in case of stem and root extracts, the highest antioxidant activity was observed in plant parts collected from Rangrik village (riverine habitat) with lowest IC_{50} values 37.51±3.45 μg mL^{-1} (F= 129, P < 0.05) and 46.69±3.23 μg mL^{-1} (F= 1.67, P < 0.05), respectively, with significant variation among populations as shown in **Table 1**. While considering antioxidant activity riverine habitat (Rangrik) population also showed highest scavenging with lowest IC_{50} value (37.51±3.41). The average IC_{50} values of leaf (90.78 μg mL^{-1}), stem (78.06 μg mL^{-1}) and root (90.16 μg mL^{-1}) extracts showed better scavenging ability of stem and roots.

Table 1: DPPH radical scavenging of methanolic leaf, stem and root extracts of *H. rhamnoides* ssp. *turkestanica*

Conc. (µg/ml ± S.D.)	DPPH radical scavenging ability of leaf, stem and root extracts of all populations (% inhibition)											
	Lidang			Lingti			Rangrik			Rama Khas		
	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root
50	49.24 ±0.25	55.72 ±0.39	46.21 ±0.25	48.82 ±0.53	56.06 ±0.25	45.79 ±0.39	28.79 ±1.26	52.1± 0.64	50±0. 25	21.38 ±0.81	53.7± 0.64	26.42 ±3.18
100	53.62 ±1.05	73.65 ±0.53	52.86 ±0.39	52.53 ±0.25	71.21 ±0.51	53.54 ±0.76	64.06 ±0.39	64.98 ±0.15	68.43 ±0.25	42.43 ±0.43	64.06 ±0.53	36.4± 0.2
200	68.69 ±0.51	78.28 ±0.51	73.23 ±0.25	71.72 ±0.51	76.26 ±0.67	71.63 ±0.39	81.84 ±1.02	77.1± 0.39	76.43 ±0.53	50.42 ±0.64	81.4± 0.89	50.08 ±0.29
400	78.11 ±0.29	88.38 ±0.51	84.34 ±0.25	80.39 ±1.25	88.38 ±0.67	84.01 ±0.73	83.42 ±1.25	86.11 ±0.25	84.85 ±0.25	76.01 ±1.34	85.61 ±0.67	82.16 ±1.36
IC_{50}	**38.78 ±6.47**	118.7 7±5.2	58.34 ±1.17	43.73 ±4.38	98.4± 7.86	60.56 ±3.34	75.34 ±2.5	**37.51 ±3.45**	**46.69 ±3.23**	205.2 6±3.5	57.55 ±5.34	195.0 3±3.6

**Values calculated from three replicates (n=3) were expressed as mean±standard deviation.*

ABTS radical scavenging activity

The ABTS free radical scavenging activities of leaf, stem and root extracts of *H. rhamnoides* ssp. *turkestanica* are shown in **Figure 3d**, **e** and **f**. The ABTS radical scavenging ability of all extracts was increased with increasing concentration. While considering IC_{50} value the highest radical scavenging effect was obtained in roots (229.76±3.4) followed by leaves and stems collected from Rama Khas population as shown in **Table 2**. Significant variations were found among leaf (F= 6.19, P < 0.05), stem (F= 4.19, P <0.05) and root (F=3.81, P <0.05) populations. This radical scavenging method also indicated roots as better antioxidant organ.

Table 2: ABTS radical scavenging of methanolic leaf extracts of *H. rhamnoides* ssp. *turkestanica*

Conc. (µg/ml ± S.D.)	ABTS radical scavenging ability of leaf, stem and root extracts of all populations (% inhibition)											
	Lidang			Lingti			Rangrik			Rama Khas		
	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root
50	86.22 ±0.5	76.33 ±0.12	80.16 ±0.12	77.42 ±0.45	71.12 ±0.01	68.26 ±0.35	75.26 ±0.01	66.24 ±0.01	72.10 ±0.04	66.02 ±0.22	68.37 ±0.4	65.15 ±0.22
100	88.31 ±0.43	78.72 ±0.12	85.43 ±0.12	80.16 ±0.12	78.13 ±0.02	76.23 ±0.2	80.19 ±0.10	77.95 ±0.02	75.34 ±0.04	77.13 ±0.12	74.15 ±0.13	79.08 ±0.12
200	97.62 ±0.22	81.89 ±0.62	95.31 ±0.12	92.78 ±0.45	82.35 ±0.02	79.25 ±0.26	87.85 ±0.06	78.58 ±0.01	77.06 ±0.01	91.63 ±0.12	83.26 ±0.24	88.38 ±0.45
400	99.49 ±0.12	89.03 ±0.12	97.55 ±0.25	95.09 ±0.45	88.67 ±0.02	85.28 ±0.39	95.43 ±0.03	80.95 ±0.02	86.76 ±0.07	93.94 ±0.22	89.20 ±0.18	95.53 ±0.12
IC_{50}	938.1 6±4.7	705.1 4±4.5	647.1 1±8.4	523.3 8±9.3	497.2 3±0.4	455.2 9±3.5	437.5 6±5.5	646.4 5±0.7	506.5 8±0.9	**252±5 .2**	**318.2 3±8.2**	**229.7 6±3.4**

**Values calculated from three replicates (n=3) were expressed as mean±standard deviation*

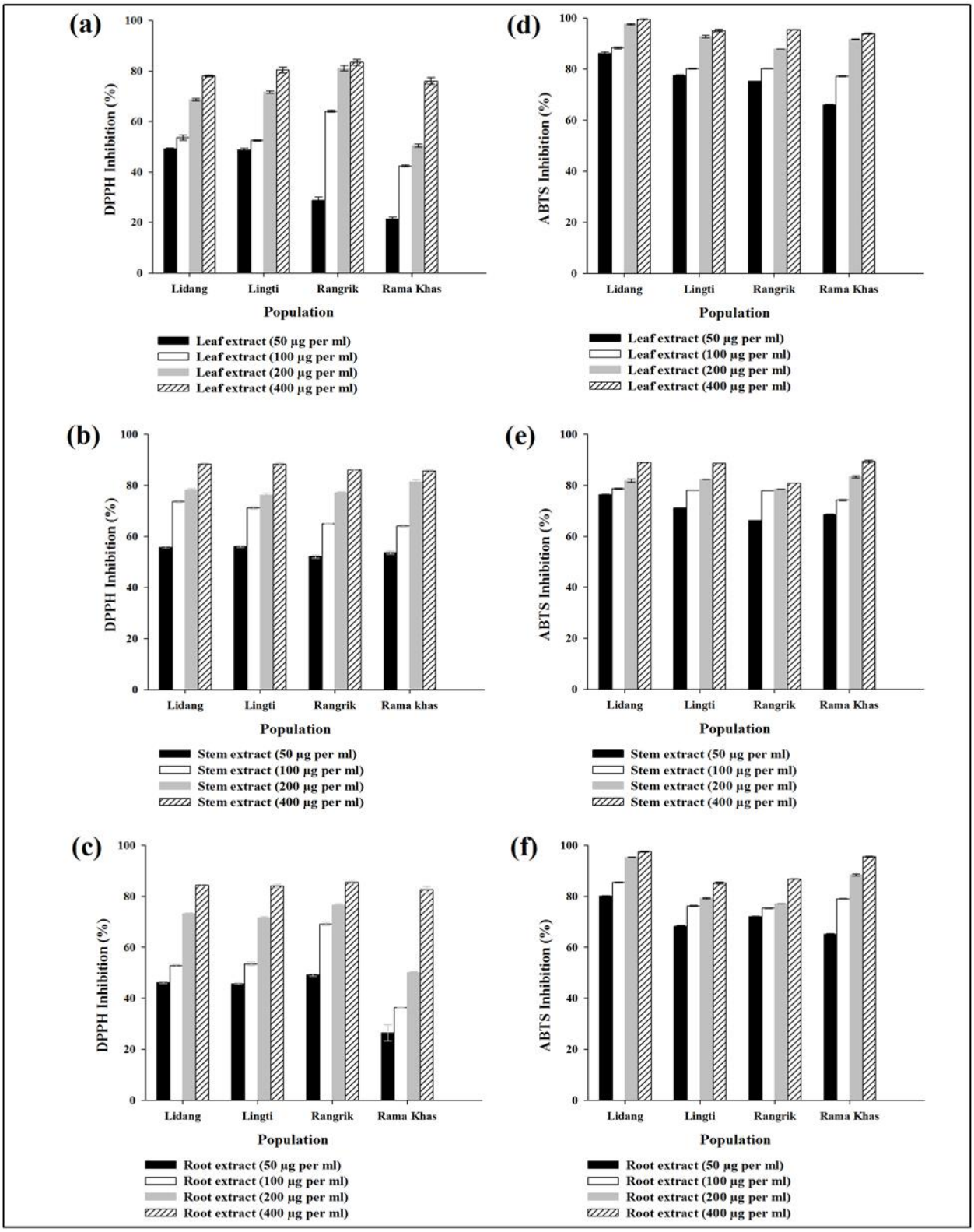

Figure 3: DPPH radical scavenging of the methanolic **(a)** leaf, **(b)** stem and **(c)** root extracts; and ABTS radical scavenging of **(d)** leaf, **(e)** stem **(f)** root extracts of *H. rhamnoides* ssp. *turkestanica*; all values are mean of three measurements

Reducing power

The reductive ability measurement at low pH, reduction of ferric (Fe^{3+}) complex to ferrous form (Fe^{2+}) which has an intense blue colour can be monitored by measuring the change in absorption. A good correlation between antioxidant activity and reducing power in plant extracts has been established and used as an indicator of potent antioxidant activity. **Figure 4a**, **b** and **c** shows the dose response curves for the reducing power of leaf, stem and root extracts of *H. rhamnoides* ssp. *turkestanica*, respectively.

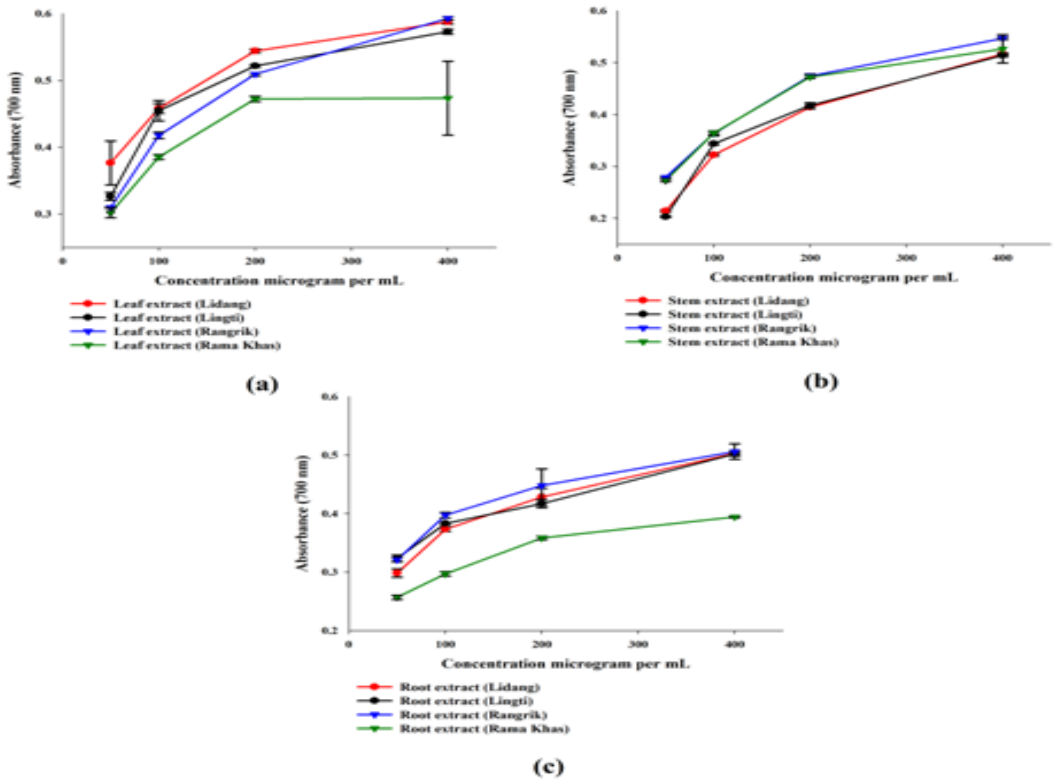

Figure 4: Reducing ability measurement of the methanolic **(a)** leaf, **(b)** stem and **(c)** root extracts of *H. rhamnoides* ssp. *turkestanica*; all values are mean of three measurements

Reducing ability increased with increasing concentration of leaf, stem and root extracts. Significantly higher reducing power was observed in leaf (0.593±0.003), stems (0.547±0.002) and roots (0.506±0.013) collected from Rangrik at 400 μg mL-1. This also supports higher antioxidant potential in riverine habitat (Rangrik) population.

DISCUSSION

Antioxidants play an important role in prevention, interception and repairing of the body by stopping the formation of free radicals and repairing the enzymes involved in the process of cellular development[41]. Phenolic compounds are known to exert preventive activity against infectious and degenerative diseases, inflammation and allergies via antioxidant, antimicrobial and proteins/enzymes neutralization/modulation mechanisms[42]. Several investigators recognised *H. rhamnoides* as a good source of high phenolic compounds with excellent antioxidant activity[6,7,9,12,19-29] and also its important therapeutic uses[43]. In the present study total phenolics, flavonoids and antioxidant activity were determined to analyse the chemical composition of *H. rhamnoides* ssp. *turkestanica* leaf, stem and root methanolic extracts. Results indicate that considerable phenolics, flavonoids and antioxidant compounds were present in leaf, stem and root extracts, which can be used to improve human health by reducing the oxidative stress in cells.

The mean value of phenolic contents of *H. rhamnoides* ssp. *turkestanica* across five distantly located populations in our studies were 69±7.7 mg GAE g^{-1} of extract (leaf), 89±7.73 mg GAE g^{-1} of extract (stem) and 84±9.27 mg GAE g^{-1} of extract (root) was in accordance to previous studies (28.35-402 mg/g in leaf, 25-95 mg/g in stem and 42-139 mg/g in root) reported by many workers in *H. rhamnoides* at national and international levels[6,9,21,23,24]. While, it is found that in our study the TPC of leaf extracts is slightly higher than the other studies carried out on *H. rhamnoides* growing in North West Himalaya[16,19]. Upadhyay et al[19] evaluated TPC of SBT leaves in aqueous and hydroalcoholic extracts as 40.49 and 56.28 mg GAE g^{-1}, respectively, and Gill et al[16] reported the TPC as 34.6 mg GAE $100g^{-1}$ in SBT leaves.

Flavonoids are one of the major secondary metabolite synthesized by *Hippophae* species in response to a wide range of microbial infection. High levels of phenolic compounds in the plant extracts are indicators of the better radical scavenging ability. The average value of flavonoid contents across population is 33±4.32 in leaves, 42±9.23 in stems and 71±4.2 mg QE g^{-1} of extract, in roots. It is similar to earlier reported flavonoid contents in *H. rhamnoides* leaves (14.14-74 mg/g) and branches (30-64 mg/g)[6,9]. But, TFC in leaf extracts is also slightly higher than the study carried out in *H. rhamnoides* growing in North West Himalaya by Upadhyay et al[19], they evaluated 14.90 mg RE g^{-1} and 20.76 mg RE g^{-1} TFC in aqueous and

hydroalcoholic leaf extracts, respectively. While, Gill et al[16] reported 18.1 mg QE 100g^{-1} TFC in SBT leaves.

In detail, a study performed by Perk et al[9] on ethanolic and aqueous extract of SBT leaves and branches (processed and unprocessed) was evaluated for their TPC and TFC. The TPC was ranged between 25.8-75.9 mg GAE g^{-1} and TFC was 21.2-74 mg RE g^{-1}. In another study, Michel et al[23] investigated antioxidant and phytochemicals in SBT leaf, stem and root. The TPC in different ethanolic extracts ranged from 53-92 in leaf, 52-95 in stem and 36-139 mg GAE g^{-1} in root. Similarly, a study on bioactivity guided extraction carried out by Kumar et al[24] showed TPC in various SBT leaf extracts from 35.62-402.19 mg GAE g^{-1}. Likewise, Kim et al[21] studied phenolic and antioxidant activity of SBT leaves and found the TPC ranged between 48-477 mg GAE g^{-1} in different extracts. A mini review on phytochemicals of SBT carried out by Anbarasu et al[26] showed TPC of leaves across population ranged from 54.4 to 86.4 mg GAE 0.25g^{-1}.

Our study also indicates that *H. rhamnoides* ssp. *turkestanica* extracts are effective in scavenging ABTS and DPPH radicals. This indicates the methanolic extracts have strong proton-donating scavenging ability, which could be utilized as free radical inhibitors, possibly acting as primary antioxidants[16]. Highest DPPH radical scavenging effect observed in leaves collected from Lidang village with the lowest IC_{50} of 38.78±6.47 μg mL$^{-1.}$ Similarly, Kumar et al[24] observed DPPH IC_{50} ranged between 5.99 to 56.07 μg mL^{-1} in different extracts.

However, this comparison is difficult because recent study shows that storage time, genetic, extrinsic and physiological factors have a strong influence on phenol contents[40]. In addition, the chemical and phytochemical composition of SBT varies with origin, climate and method of extraction[43]. The variation in TPC and TFC contents across populations is due to the variations in altitude, slope and habitat. The population growing in riverine habitat shows significantly higher concentration of phenolic compounds and radical scavenging activity. Present study also concluded that stem and roots are the best antioxidant organ. Similar results were also observed previously by Michel et al[23], they recorded roots as the antioxidant rich organ. Therefore, the overall study shows higher phenolic compounds and free radical scavenging capacity of *H. rhamnoides* ssp. *turkestanica* and considered it as a potential source of antioxidants.

CONCLUSION

Habitat-wise investigation of antioxidant potential of *H. rhamnoides* ssp. *turkestanica* leaves, stems and roots of cold desert biosphere reserve, Trans Himalaya have been done for the first time. Present study highlights the pharmaceutical importance of *H. rhamnoides* ssp. *turkestanica*. Being a good source of plant phenolic compounds and antioxidant activity, it can be taken as dietary supplement as well as can be used to prepare antioxidant rich herbal tea. Various ecological factors combined with processing factors are responsible for wide variation in TPC, TFC and antioxidant activities across populations. Present study strongly supports its ethenobotanical use as traditional medicinal herb and can be suggested it as a significant source of natural antioxidant and might replace synthetic antioxidants in pharmaceutical industries. However, further research is required for isolation, identification and investigation of other active compounds.

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REFERENCES

1. Gursoy N, Sarikurkcu C, Cengiz M, Solak MH, Antioxidant activities, metal contents, total phenolics and flavonoids of seven *Morchella* species, Food and Chemical Toxicology, 47, 2009, 2381-2388.
2. Hallywell B, Gutteridge J, Free radicals in biology and medicine, Oxford University Press, New York, USA, 2007.
3. Sun Y, Free radicals, antioxidant enzymes, and carcinogenesis, Free Radical Biology and Medicine, 8, 1990, 583-599.
4. Stadtman ER, Protein oxidation and aging, Science, 257, 1992, 1220-1224.
5. Veeru P, Kishor MP, Meenakshi M, Screening of medicinal plant extracts for antioxidant activity, Journal of Medicinal Plants Research, 3, 2009, 608-612.
6. Kumar MY, Dutta R, Prasad D, Misra K, Subcritical water extraction of antioxidant compounds from Seabuckthorn (*Hippophae rhamnoides*) leaves for the comparative evaluation of antioxidant activity, Food chemestry, 127, 2011, 1309-1316.
7. Dhar P, Bajpai PK, Tayade AB, Chaurasia, OP, Srivastava RB, Singh SB, Chemical composition and antioxidant capacities of phytococktail extracts from Trans-Himalayan Cold Desert. BMC Complementary and Alternative Medicine, 13, 2013, 2-15.
8. Patel RD, Mahobia NK, Singh MP, Singh A, Sheikh NW, Alam G, Singh SK, Antioxidant potential of leaves of *Plectranthus amboinicus* (lour) spreng, Der. Pharmacia Lettre, 2, 2010, 240-245.
9. Perk AA, Ceylan FD, Yanar O, Boztas K, Capanoglu E, Investigating the antioxidant properties and rutin content of sea buckthorn (*Hippophae rhamnoides* L.) leaves and branches, African Journal of Biotechnology, 15, 2016, 118-124.
10. Swenson U, Bartish IV, Taxonomic synopsis of *Hippophae* (Elaeagnaceae), Nordic Journal of Botany 22, 2002, 369-374.

11. Germplasm Resources Information Network (GRIN), Species Records of *Hippophae*. Germplasm Resource Information Network. United States Department of Agriculture, Agriculture Research Service Beltsville Area, Maryland, US, 2007. http://www.ars-grin.gov/cgi-bin/npgs/html/splist.pl?5698

12. Papuc C, Diaconescu C, Nicorescu V, Antioxidant activity of Sea Buckthorn (*Hippophae rhamnoides*) extracts compared with common food additives, Romanian Biotechnological Letters, 13, 2008, 4049-4053.

13. Christaki E, *Hippophae rhamnoides* L. (Sea Buckthorn): A potential source of nutraceuticals, Journal of Food and Public Health. 2, 2012, 69-72.

14. Lu R, Seabuckthorn–a multipurpose plant for fragile mountains, International Centre for Integrated Mountain Development (ICIMOD) occasional paper no. 20. ICIMOD, Kathmandu, Nepal, 1992.

http://lib.icimod.org/record/24762/files/c_attachment_364_4397

15. Small E, Catling P, Blossoming treasures of biodiversity: 21. Sugarcane: An old star with a new act, Biodiversity Journal, 7, 2006, 37-46.

16. Ahmed Wani T, Wani S, Shah A, Masoodi F, Optimizing conditions for antioxidant extraction from Sea Buckthorn leaf (*Hippophae rhamnoides* L.) as herbal tea using response surface methodology (RSM), International Food Research Journal, 20, 2013, 1677-1681.

17. Gill N, Sharma R, Arora R, Bali M, Antioxidant and antibacterial activity of *Hippophae rhamnoides* methanolic leaf extracts from dry temperate agro-climatic region of Himachal Pradesh, Journal of Plant Sciences, 7, 2012, 194-200.

18. Krejcarova J, Strakova E, Suchy P, Herzig I, Karaskova K, Sea Buckthorn (*Hippophae rhamnoides* L.) as a potential source of nutraceutics and its therapeutic possibilities-a review, Acta Veterinaria Brunensis, 84, 2015, 257-268.

19. Bhartee M, Basistha B, Pradhan DS, Seabuckthorn-a secret wonder species: Review, SMU Medicinal Journal, 1, 2014, 102-115.

20. Upadhyay NK, Kumar MY, Gupta A, Antioxidant, cytoprotective and antibacterial effects of sea buckthorn (*Hippophae rhamnoides* L.) leaves, Food and Chemical Toxicology, 48, 2010, 3443-3448.

21. Goyal AK, Basistha BC, Sen A, Middha SK, Antioxidant profiling of *Hippophae salicifolia* growing in sacred forests of Sikkim, India, Functional Plant Biology, 38, 2011, 697-701.

22. Kim JS, Kwon YS, Sa YJ, Kim MJ, Isolation and identification of Sea Buckthorn (*Hippophae rhamnoides*) phenolics with antioxidant activity and α-glucosidase inhibitory effect, Journal of Agriculture and food chemistry, 59, 2010, 138-144.

23. Chaman S, Syed NI, Danish Z, Khan FZ, Phytochemical analysis, antioxidant and antibacterial effects of Sea Buckthorn berries, Pakistan Journal of Pharmacautical Science, 24, 2011, 345-351.

24. Michel T, Destandau E, Le Floch G, Lucchesi ME, Elfakir C, Antimicrobial, antioxidant and phytochemical investigations of Sea Buckthorn (*Hippophaë rhamnoides* L.) leaf, stem, root and seed, Food chemistry, 131, 2012, 754-760.

25. Kumar SA, Paul AD, Tanveer N, Bioactivity guided extraction of Seabuckthorn (*Hippophae rhamnoides* L. ssp. *turkestanica*) leaves, Journal of Scientific and Industrial Research, 72, 2013, 307-311.

26. Rop O, Ercisli S, Mlcek J, Jurikova T, Hoza I, Antioxidant and radical scavenging activities in fruits of 6 Sea Buckthorn (*Hippophae rhamnoides* L.) cultivars, Turkish Journal of Agriculture and Forestry, 38, 2014, 224-232.

27. Anbarasu S, Radhakrishnan M, Suresh A, Joseph J, Phytochemical, ethnomedicinal and pharmacological potentials of Seabuckthorn-a mini review, International Journal of Pharma and Bio Sciences, 38, 2015, 224-232.

28. Kant V, Mehta M, Varshneya C, Antioxidant potential and total phenolic contents of Seabuckthorn (*Hippophae rhamnoides*) pomace, Free Radical Antioxidants, 2, 2012, 79-86.

29. Varshneya C, Kant V, Mehta M, Total phenolic contents and free radical scavenging activities of different extracts of Seabuckthorn (*Hippophae rhamnoides*) pomace without seeds, Inernational Journal of Food Science and Nutrition, 63, 2012, 153-159.

30. Mehta M, Kant V, Varshneya C, Screening of *in vitro* antioxidant potential of Seabuckthorn seedcake extracts, Journal of Interculture Ethnopharmacology, 2, 2013, 99-104.

31. Aswal B, Mehrotra BN, Flora of Lahaul-Spiti: A cold desert in North West Himalaya, Bishen Singh Mahendra Pal Singh, Dehradun, 1994.

32. Slinkard K, Singleton VL, Total phenol analysis: Automation and comparison with manual methods, American Journal of Enology and Viticulture, 28, 1997, 49-55.

33. Chang CC, Yang MH, Wen HM, Chern JC, Estimation of total flavonoid content in propolis by two complementary colorimetric methods, ournal of Food and Drug Analysis 10, 2002, 178-182.

34. Tirzitis G, Bartosz G, Determination of antiradical and antioxidant activity: Basic principles and new insights, Acta biochimica Polonica, 57, 2010, 139-142.

35. Shimada K, Fujikawa K, Yahara K, Nakamura T, Antioxidative properties of Xanthan on the autoxidation of Soybean oil in cyclodextrin emulsion, Journal of Agriculture and Food Chemistry, 40, 1992, 945-948.

36. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C, Antioxidant activity applying an improved abts radical cation decolorization assay, Free Radical Biology and Medicine, 26, 1999, 1231-1237.

37. Oyaizu M, Studies on products of browning reaction--antioxidative activities of products of browning reaction prepared from glucosamine, Japanese Journal of Nutrition, 44, 1986, 307-315.

38. Sowndhararajan K, Kang SC, Free radical scavenging activity from different extracts of leaves of *Bauhinia vahlii* Wight and Arn, Saudi Journal of Biological Sciences, 20, 2013, 319-325.

39. Enujiugha VN, Talabi JY, Malomo SA, Olagunju AI, DPPH radical scavenging capacity of phenolic extracts from African

Yam Bean (*Sphenostylis stenocarpa*), Food and Nutritional Sciences, 3, 2012, 7-13.

40. Pourmorad F, Hosseinimehr S, Shahabimajd N, Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants, African Journal of Biotechnology, 5, 2006, 1142-1145.
41. Chaouche TM, Haddouchi F, Ksouri R, Atik-Bekkara F, Evaluation of antioxidant activity of hydromethanolic extracts of some medicinal species from South Algeria, Journal of Chinese Medical Association, 77, 2014, 302-307.
42. Rawat S, Jugran A, Giri L, Bhatt ID, Rawal RS, Assessment of antioxidant properties in fruits of *Myrica esculenta*: A popular wild edible species in Indian Himalayan Region, Evidence Based Complementary and Alternative Medicine, 2011. http://dx.doi.org/10.1093/ecam/neq055
43. Ozcan T, Akpinar-Bayizit A, Yilmaz-Ersan L, Delikanli B, Phenolics in human health. International Journal of Chemical Engineering and Applications, 5, 2014, 393-396.
44. Zeb A, Important therapeutic uses of Sea Buckthorn (*Hippophae*): a review, Journal of Biological Sciences, 4, 2004, 687-693.

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