



Antioxidant, cytoprotective and antibacterial effects of Sea buckthorn (*Hippophae rhamnoides* L.) leaves

Nitin K. Upadhyay, M.S. Yogendra Kumar, Asheesh Gupta *

Defence Institute of Physiology and Allied Science (DIPAS), Timarpur, Delhi 110054, India

ARTICLE INFO

Article history:

Received 3 May 2010

Accepted 14 September 2010

Keywords:

Antibacterial activity

Antioxidant

Flavonoids

RP-HPLC

Sea buckthorn leaf

ABSTRACT

The present study was carried out to investigate the antioxidant, cytoprotective and antibacterial effects of aqueous and hydroalcoholic extracts of *Hippophae rhamnoides* L. (Sea buckthorn) (SBT) leaves by using various *in vitro* systems and analysis of marker compounds by reverse phase-high performance liquid chromatography (RP-HPLC). The chemical composition of the leaf extracts was quantified by colorimetric reaction in terms of total phenol and flavonoids contents. Further, some of its bioactive phenolic constituents, such as quercetin-3-O-galactoside, quercetin-3-O-glucoside, kaempferol and isorhamnetin were also quantified in both SBT leaf extracts by RP-HPLC. The SBT leaf extracts exhibited potent antioxidant activity determined by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. Further, both extracts were observed to have cytoprotective activity against hydrogen peroxide and hypoxanthine-xanthine oxidase induced damage to BHK-21 cell line. The SBT leaf extracts showed growth inhibiting effect against *Bacillus cereus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis*. These observations suggest that aqueous and hydroalcoholic extracts of Sea buckthorn leaves have marked antioxidant, cytoprotective and antibacterial activities.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

In healthy individuals, there is a dynamic balance between the amount of free radicals generated in the body and endogenous antioxidant defense system which scavenge them and protect the body against their deleterious effects. Oxidative stress is a process where the physiological balance between pro-oxidants and antioxidants is disrupted in favour of the former, ensuing in potential damage for the organism (Halliwell and Gutteridge, 1990). Free radicals either formed by cellular metabolism, exogenous chemicals or stress are capable of oxidising biomolecules which may cause many diseases, including cancer, diabetes, cardiovascular and neurodegenerative diseases (Bland, 1995; Thomson, 1995). Pathogenic bacteria can cause food borne illness and a wide variety

of infectious diseases (Begnami et al., 2010). Phenolic compounds such as flavonoids, phenolic acids, diterpenes and tannins have received attention for their high antioxidative and antibacterial activity (Kaur and Arora, 2009). Dietary intake of such phytochemicals may be an important strategy for inhibiting or delaying of pathological conditions and disease prevention in many paradigms.

Hippophae rhamnoides L. subspecies *turkestanica* (family Elaeagnaceae) commonly known as Sea buckthorn (SBT) is a thorny nitrogen fixing deciduous shrub, native to Europe and Asia (Rousi, 1971). In India, SBT is widely distributed in cold desert areas of the Trans-Himalayan region at an altitude of 2500–4000 m amsl. All parts of the plant are considered to be rich source of large number of bioactive substances. The plant has been used extensively in oriental traditional system of medicine for treatment of cough, skin diseases, gastric ulcers, asthma and lung disorders (Bernath and Foldesi, 1992). In the recent years, SBT leaf extracts have been scientifically investigated and various pharmacological activities such as anti-inflammatory, immunomodulatory, radioprotective, adaptogenic and tissue regeneration have been reported. Further, it has also been reported that SBT leaf has no cytotoxicity and adverse effect after oral administration (Chawla et al., 2007; Ganju et al., 2005; Geetha et al., 2005; Gupta et al., 2005; Gupta et al., 2008; Saggi et al., 2007; Upadhyay et al., 2009).

Abbreviations: ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; amsl, above mean sea level; ATCC, American Type Cell Culture; BHK, Baby Hamster Kidney; DPPH, 2,2-diphenyl-1-picryl-hydrazyl; FRAP, ferric reducing antioxidant power; HX-OX, hypoxanthine-xanthine oxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; RP-HPLC, reverse phase-high performance liquid chromatography; SBT, Sea buckthorn; TPTZ, 2,4,6-tripryridyl-s-triazine; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

* Corresponding author. Tel.: +91 11 23883312; fax: +91 11 23914790.

E-mail address: asheeshgupta2001@gmail.com (A. Gupta).

The objective of the present study was to investigate the antioxidant, cytoprotective and antibacterial effects of aqueous and hydroalcoholic leaf extracts of SBT by various *in vitro* assays and further quantitative analysis of marker compounds using reverse phase-high performance liquid chromatography (RP-HPLC).

2. Materials and methods

2.1. Collection of plant material

The SBT leaves were collected from the hilly regions of the North-West Himalayas (the region lies between latitude 32–36° North and longitude 76–79°) in the month of September 2006, where the plant grows widely under natural conditions. Plant material (Voucher specimen SBTL-2006) was characterized by Dr. O.P. Chaurasia, an ethnobotanist at the Defence Institute of High Altitude Research (DIHAR), Leh, India.

2.2. Preparation of extracts

Fresh leaves were cleaned thoroughly with nanopure water, dried under shade in a clean and dust-free environment. Aqueous and hydroalcoholic extracts of SBT leaves were prepared by soaking the powdered dry leaves separately with nanopure water and 70% ethanol (1:5 w/v) at room temperature (25 ± 1 °C). After 24 h, supernatants were decanted and residues were re-soaked in respective fresh solvent. The process was repeated three times for complete extraction. The respective supernatant was pooled, filtered through muslin cloth and centrifuged at 5000g for 10 min at 4 °C. Ethanol content of the hydroalcoholic extract was evaporated using Buchi Rotavapor R-124 (Buchi Labortechnik AG, Postfach, CH-9230, Flawil, Switzerland) at 40 °C. Finally, both the supernatant solutions were lyophilized in a Heto lyophilizer (HITOSICC, Heto-Holten A/S, Denmark) and the dried extracts were stored in airtight dark bottles at 4 °C.

2.3. Determination of total phenolics and flavonoids content

2.3.1. Total phenolics content

Total phenolics content was determined with the Folin–Ciocalteu reagent according to a method described by Singleton and Rossi (1965). A mixture of 150 µl of the extract (0.2 mg/ml), 2400 µl of nanopure water and 150 µl of 0.25 N Folin–Ciocalteu reagent was prepared and allowed to react for 3 min. Then 300 µl of 1.0 N Na₂CO₃ solution was added into the reaction mixture. After incubation for 2 h at room temperature, the absorbance relative to that of prepared blank was measured at 725 nm using a spectrophotometer (SmartSpec 3000, BIO-RAD, CA, USA). Gallic acid was used as a reference standard and the results were expressed as mg gallic acid equivalents/g dry leaf.

2.3.2. Total flavonoids content

Total flavonoids content was determined as described previously by Zou et al. (2004). A mixture of 1.0 ml of the extract (1 mg/ml), 2.0 ml of nanopure water and 0.15 ml of 5% NaNO₂ was prepared and allowed to react for 6 min. Then 0.15 ml of 10% AlCl₃ solution was added and mixed thoroughly. After 6 min, 2.0 ml of 4% NaOH solution was added and allowed to stand for another 15 min. Absorbance of the mixture was measured at 510 nm versus prepared blank. Rutin was used as standard compound for the quantification of flavonoids content. Results were expressed in mg of rutin equivalents/g of dry leaf.

2.4. Identification and quantification of marker compounds by RP-HPLC

The HPLC system consisted of a Waters HPLC equipped with 515 HPLC pump, 717 plus auto-sampler and 2487 UV detector (Waters Corporation, Milford, MA). The separation was performed on a Symmetry C18 250 × 4.6 mm ID; 5 µm column (Waters, USA) by maintaining the gradient flow rate 0.75 ml/min of the mobile phase (Solution A; water: o-phosphoric acid 99.7:0.3 and Solution B; acetonitrile: methanol 75:25) and peaks were detected at 370 nm. Identification and quantification of marker compounds was performed on the basis of the coinjections and retention time matching with standards. For the preparation of the calibration curve, standard stock solution of quercetin-3-O-galactoside, quercetin-3-O-glucoside, kaempferol and isorhamnetin (0.5 mg/ml) were prepared in methanol, filtered through 0.22 µm filters (Millipore), and appropriately diluted (0.01–100.0 µg/ml) to obtain the desired concentrations for identification and quantification.

2.5. Antioxidant activity determination

Antioxidant activity of plant extract cannot be evaluated by only a single method due to the complex nature of phytochemicals. Therefore, in the present study antioxidant activity was evaluated by three different assays viz. 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and Ferric Reducing Antioxidant Power (FRAP).

2.5.1. DPPH assay

The free radical scavenging activity of SBT leaf aqueous and hydroalcoholic extracts on DPPH radical was determined by the method as described previously by Blois (1958) with some modifications. Stock solution of DPPH was prepared by dissolving 24 mg DPPH with 100 ml methanol and then stored at –20 °C until use. The working solution was prepared by mixing 10 ml stock solution with 45 ml methanol to obtain an absorbance of 1.10 ± 0.02 units at 515 nm using the spectrophotometer. Then 150 µl of the extract (0.2 mg/ml) was added to 2850 µl of DPPH working solution. The reaction mixture was vortexed thoroughly and allowed to stand for 2 h in dark. Absorbance was measured at 515 nm and samples were analyzed in triplicate. The standard curve was linear between 25 and 200 ppm Trolox. Results were expressed in mg of Trolox equivalents/g of dry leaf.

2.5.2. ABTS assay

ABTS assay was conducted by the method as described by Re et al. (1999) with some modifications. Firstly, to produce the radical cation ABTS⁺, 7 mmol/L ABTS diammonium salt and 2.4 mmol/L potassium persulfate were mixed in a volume ratio of 1:1, the reaction mixture was allowed to stand in the dark for 12 h at room temperature. The solution was then diluted by mixing 1 ml ABTS⁺ solution with 60 ml methanol to obtain an absorbance of 1.10 ± 0.02 units at 734 nm using the spectrophotometer. ABTS⁺ solution was freshly prepared for each assay. Then 150 µl of the extract (0.1 mg/ml) was allowed to react with 2850 µl of the ABTS⁺ solution and the absorbance was measured at 734 nm after 2 h using the spectrophotometer. The standard curve was linear between 25 and 150 ppm Trolox. Results were expressed in mg of Trolox equivalents/g of dry leaf.

2.5.3. FRAP assay

The FRAP assay was carried out according to the method as described previously by Benzie and Strain (1996). The stock solutions included 300 mM acetate buffer (3.1 g C₂H₃NaO₂·3H₂O and 16 ml C₂H₄O₂), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine) solution in 40 mM HCl and 20 mM FeCl₃·6H₂O solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml FeCl₃·6H₂O. The temperature of the solution was raised to 37 °C before use. Then 150 µl of the extract (0.1 mg/ml) was allowed to react with 2850 µl of the FRAP reaction solution for 30 min in dark. Readings of the colored product (ferrous tripyridyltriazine complex) were measured at 593 nm using the spectrophotometer. The standard curve was linear between 25 and 150 ppm Trolox. Results were expressed in mg of Trolox equivalents/g of dry leaf.

2.6. Determination of reducing potential

The reducing potential was determined as described previously by Negi et al. (2005) with some modifications. Briefly, 1.0 ml of the extract (0.2–1.0 mg/ml) was mixed with 0.2 M phosphate buffer, pH 6.6 (2.5 ml) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated in water bath at 50 °C for 20 min. Then 10% trichloroacetic acid (2.5 ml) was added and centrifuged at 3000 rpm for 10 min. The upper layer of supernatant (2.5 ml) was mixed with nanopure water (2.5 ml) and 0.1% ferric chloride solution (0.5 ml). Absorbance was measured spectrophotometrically at 700 nm.

2.7. *In vitro* cytoprotective activity

The cytoprotective effect of aqueous and hydroalcoholic extracts of SBT leaves on the viability of the BHK-21 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenases in viable cells (Phan et al., 2001). The hydrogen peroxide (H₂O₂) or hypoxanthine-xanthine oxidase (HX-OX) was used to induce oxidative damage.

2.7.1. Cell culture

BHK-21 cell-line was purchased from the National Centre for Cell Science (NCCS), Pune, India. The cells were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO₂ and cultured in Glasgow's Minimum Essential Medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 µg/ml), and neomycin sulphate (50 µg/ml) (Invitrogen Co., Carlsbad, CA).

2.7.2. Cell viability assay

The BHK-21 cells were seeded in 24-well plates (Iwaki Glass Co., Ltd., Tokyo, Japan) at densities of 1 × 10⁵ cells/ml. Sixteen hours after plating, the cells were treated with various concentration of the SBT leaf extracts (125, 250 and 500 µg/ml) and simultaneously H₂O₂ (2 × 10⁻⁴ mol/l) or HX-OX (2 × 10⁻² units/ml) in Hank's balanced salt solution were added for 3 h. Hundred microlitres of MTT stock solution (5 mg/ml) was then added and incubated for 4 h. The formazan crystals generated in each well were dissolved in 1.0 ml of acidified isopropanol and absorbance was measured by using spectrophotometer at 570 nm. The percents of cell viability were calculated with the following equation:

$$\text{Cell viability (\%)} = (A_1/A_0) \times 100\%$$

where A_0 was the absorbance of the control cells and A_1 was the absorbance of the treated cells.

2.8. Antibacterial activity

Antibacterial activity of SBT leaf extracts using ampicillin (10 µg/disc) (Hi Media Laboratory Pvt. Ltd., Mumbai, India) as positive control was evaluated against *Bacillus cereus* (ATCC 13601), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212) and *Escherichia coli* (ATCC 9837). Sensitivity of different bacterial strains to various extracts was measured in terms of zone of inhibition using agar-diffusion assay (Bauer et al., 1966). The plates containing Mueller–Hinton agar were spread with 0.2 ml of the inoculum. Wells (8 mm diameter) were cut out from agar plates using a sterilized stainless steel borer. Stock solutions (5 mg/ml) of both aqueous and hydroalcoholic extracts of SBT leaves were prepared and each well was filled with 0.1 ml of solution at final amount of 125, 250, 375, and 500 µg extract. The plates inoculated with different bacteria were incubated at 37 °C for 24 h and diameter of resultant zone of inhibition was measured. The experiment was repeated thrice.

Table 1
Determination of total phenols and flavonoids contents of Sea buckthorn leaf aqueous and hydroalcoholic extracts.

Sample	Total phenols (mg gallic acid equivalents/g dry leaf)	Total flavonoids (mg rutin equivalents/g dry leaf)
Aqueous leaf extract	40.49 ± 2.10	14.90 ± 1.10
Hydroalcoholic leaf extract	56.28 ± 2.30	20.76 ± 1.35

Data are presented as the mean ± standard deviation of three determinations.

2.9. Statistical analysis

Data are expressed as mean ± standard deviations of three parallel measurements. Data for MTT assay was analyzed by one-way ANOVA followed by Dunnett's test using Graph Pad Prism 2.01 (Graph Pad Software Inc., La Jolla, CA). A p -value < 0.05 was considered statistically significant.

3. Results

3.1. Total phenolics and flavonoids contents

Aqueous and hydroalcoholic extracts of SBT leaves were found to be rich in total phenolics and flavonoids contents (Table 1).

3.2. Identification and quantification of marker compounds by RP-HPLC

Identification and quantification of marker compounds was performed on the basis of the coinjections and retention time matching with standards. The HPLC fingerprint of aqueous and hydroalcoholic extracts of SBT leaves revealed peaks at the retention time 12.9, 13.5, 30.3 and 30.5 min at 370 nm and analysis confirmed the presence of quercetin–3-galactoside, quercetin–3-glucoside, kaempferol and isorhamnetin respectively (Fig. 1). Further, quantitative determination of these compounds indicated the presence of higher quercetin–3-O-galactoside and quercetin–3-O-glucoside content in the aqueous extract whereas higher

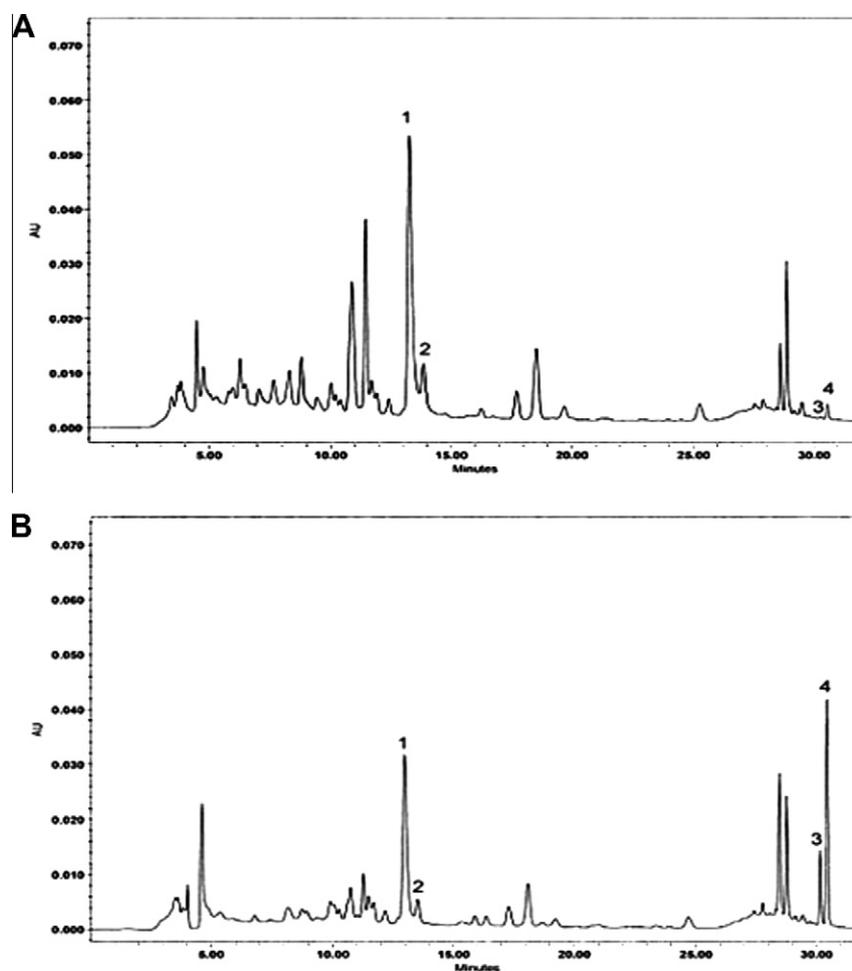


Fig. 1. HPLC chromatogram of Sea buckthorn leaf (A) aqueous and (B) hydroalcoholic extracts. Column - symmetry C18, 5 µm particle size, 4.6 × 250 mm; gradient flow rate (0.75 ml/min); mobile phase - (Solution A; water: O-phosphoric acid 99.7:0.3 and Solution B; acetonitrile: methanol 75:25); detection 370 nm; injected sample volume 10 µl; Peaks identified were: (1) quercetin-3-O-galactoside, (2) quercetin-3-O-glucoside, (3) kaempferol, (4) isorhamnetin.

Table 2

Quantitative determination ($\mu\text{g/g}$ of dry leaf) of the marker compounds by RP-HPLC analysis in Sea buckthorn leaf aqueous and hydroalcoholic extracts.

Sample	Quercetin-3-O-galactoside	Quercetin-3-O-glucoside	Kaempferol	Isorhamnetin
Aqueous leaf extract	1447.66 \pm 13.35	105.12 \pm 3.10	2.73 \pm 0.63	13.53 \pm 1.01
Hydroalcoholic leaf extract	1029.66 \pm 12.10	54.30 \pm 2.30	93.36 \pm 2.10	238.13 \pm 6.18

Data are presented as the mean \pm standard deviation of three determinations.

kaempferol and isorhamnetin content was observed in hydroalcoholic extract (Table 2).

Table 3

Determination of antioxidant activity of Sea buckthorn leaf aqueous and hydroalcoholic extracts.

Sample	DPPH (mg trolox equivalents/g dry leaf)	ABTS (mg trolox equivalents/g dry leaf)	FRAP (mg trolox equivalents/g dry leaf)
Aqueous leaf extract	109.03 \pm 2.10	119.86 \pm 3.18	114.68 \pm 2.70
Hydroalcoholic leaf extract	143.21 \pm 3.60	166.67 \pm 4.15	153.42 \pm 2.80

Data are presented as the mean \pm standard deviation of three determinations.

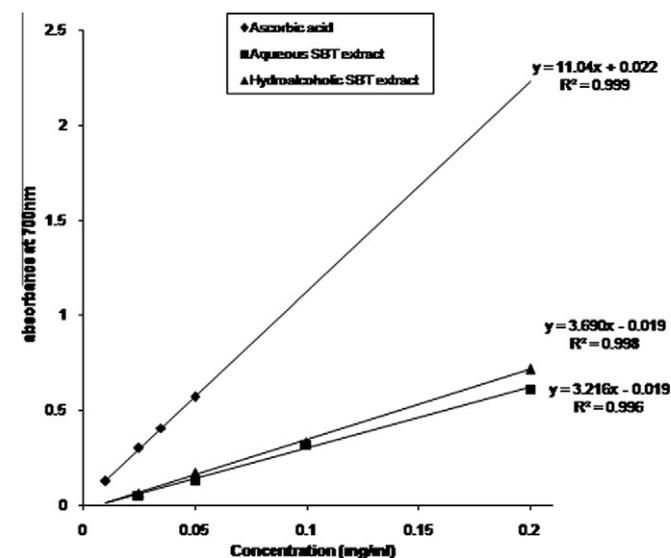


Fig. 2. Reducing potential of ascorbic acid, aqueous and hydroalcoholic Sea buckthorn leaf extracts. Values are means \pm SD of three parallel determinations.

3.3. Antioxidant activity

Both aqueous and hydroalcoholic extracts of SBT leaves exhibited potent antioxidant activity when analyzed by DPPH, ABTS and FRAP assays (Table 3).

3.4. Reducing potential

It was observed that reducing potential of SBT leaf extracts increased with increase in amount of extract. The equation of reducing potential (y) and amount of extract (x) was $Y = 3.6901X - 0.00197$ ($r^2 = 0.9988$) for the hydroalcoholic extract and $Y = 3.2163X - 0.00198$ ($r^2 = 0.9965$) for the aqueous extract which indicated that reducing ability correlated well with amount of extracts. The reducing potential decreased in the order of ascorbic acid > hydroalcoholic leaf extract > aqueous leaf extract (Fig. 2).

3.5. Cytoprotective activity

The cytoprotective effect of extracts was assessed on BHK-21 cells subjected to exposure with H_2O_2 (2×10^{-4} mol/l) or HX-OX (2×10^{-2} units/ml) as a cytotoxic agent and oxidative stress inducer. The results demonstrated that this concentration of H_2O_2 or HX-OX had significant cell toxicity and their cytotoxic effect was reduced in the presence of both aqueous and hydroalcoholic SBT leaf extracts. However, maximum cytoprotective activity was observed with 250 $\mu\text{g/ml}$ of each extract and results are depicted in Fig. 3.

3.6. Antibacterial activity

The results of antibacterial effect of aqueous and hydroalcoholic extracts of SBT leaves in terms of zone of inhibition (mm) were presented in Table 4. The aqueous leaf extract had maximum zone of inhibition (18 mm) against *P. aeruginosa* and minimum zone of inhibition (9 mm) for *E. coli*. Similarly, hydroalcoholic leaf extract had maximum zone of inhibition (19 mm) for *B. cereus* and minimum zone of inhibition (9 mm) for *E. Coli*.

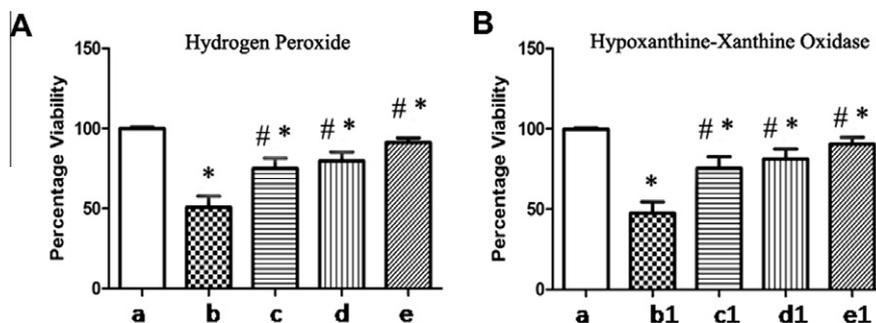


Fig. 3. Cytoprotective effect of Sea buckthorn (SBT) leaf aqueous and hydroalcoholic extracts (250 $\mu\text{g/ml}$) after (A) hydrogen peroxide (H_2O_2) and (B) hypoxanthine-xanthine oxidase (HX-OX) induced damage to BHK cell line. Values are means \pm SD of three parallel determinations. $p < 0.05$ when compared with normal cell culture, # $p < 0.05$ when compared with H_2O_2 /HX-OX treated group. (a) Normal cell culture, (b) Hydrogen peroxide exposed, (c) Hydrogen peroxide + SBT aqueous leaves extract, (d) Hydrogen peroxide + SBT hydroalcoholic leaves extract, (e) Hydrogen peroxide + ascorbic acid, (b1) Hypoxanthine-xanthine oxidase exposed, (c1) Hypoxanthine-xanthine oxidase + SBT aqueous leaves extract, (d1) Hydrogen peroxide + SBT hydroalcoholic leaves extract, (e1) Hypoxanthine-xanthine oxidase + ascorbic acid.

Table 4
Antibacterial activity determination of Sea buckthorn leaf aqueous and hydroalcoholic extracts using agar-diffusion assay.

Sample	Zone of Inhibition (mm)				
	<i>B. cereus</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>E. faecalis</i>	<i>E. coli</i>
Aqueous (125 µg)	–	–	–	–	–
Hydroalcoholic (125 µg)	11 ± 0.75	12 ± 1.79	–	–	–
Aqueous (250 µg)	12 ± 0.75	12 ± 1.67	10 ± 0.55	11 ± 0.75	–
Hydroalcoholic (250 µg)	14 ± 1.17	14 ± 1.60	11 ± 0.75	9 ± 1.55	–
Aqueous (375 µg)	13 ± 0.89	15 ± 2.19	12 ± 1.67	13 ± 1.03	–
Hydroalcoholic (375 µg)	19 ± 1.41	15 ± 1.67	12 ± 0.75	10 ± 0.55	–
Aqueous (500 µg)	14 ± 0.98	18 ± 0.77	14 ± 0.60	15 ± 0.32	9 ± 0.17
Hydroalcoholic (500 µg)	19 ± 2.07	16 ± 0.82	14 ± 0.30	13 ± 0.52	9 ± 0.55
Ampicillin (10 µg)	28 ± 2.59	12 ± 0.32	18 ± 0.79	20 ± 0.57	15 ± 0.55

(–), indicates zone of inhibition less than 8 mm. Data are presented as the mean ± standard deviation of three determinations.

4. Discussion

Polyphenols are the major plant compounds with antioxidant activity. This activity is believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Long et al., 2000). In the present study, as a part of analysis of chemical composition, total phenolics and flavonoids contents of aqueous and hydroalcoholic extracts of SBT leaves were determined. Results showed that phenolic compounds were present in considerable amount in both extracts. However, higher total phenolics and flavonoids contents were observed in the hydroalcoholic SBT leaf extract. A simple and gradient elution-based RP-HPLC method was developed for analysis of quercetin-3-O-galactoside, quercetin-3-O-glucoside, kaempferol and isorhamnetin in both extracts. For the development of an effective mobile phase, various solvent systems, including different combinations of acetonitrile, methanol and water with ortho phosphoric acid were standardized. Finally, a solvent system consisting of 0.3% ortho phosphoric acid in water and acetonitrile: methanol (75:25) was followed as it allows separation of maximum compounds with optimum resolution. Quercetin-3-O-galactoside, quercetin-3-O-glucoside, kaempferol and isorhamnetin were identified in both aqueous and hydroalcoholic SBT leaf extracts. There was higher quercetin-3-O-galactoside and quercetin-3-O-glucoside content in the aqueous extract, whereas higher kaempferol and isorhamnetin content was observed in hydroalcoholic extract.

The free radical scavenging activity of SBT leaf extracts was studied by their ability to bleach the stable ABTS and DPPH free radicals, which provides information on the reactivity of compounds with a stable free radical (Badami et al., 2003). The results shows that SBT leaf extracts are effective in scavenging ABTS and DPPH radicals. Though the ABTS and DPPH radical scavenging abilities of the extracts were significantly less than those of trolox, the study showed that the extracts have the proton-donating or scavenging ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. In FRAP assay, the antioxidant potentials of SBT leaf aqueous and hydroalcoholic extracts were estimated from their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II). Results obtained from this assay further supported the findings of the DPPH and ABTS assay and reconfirms the antioxidant potential of the SBT leaf extracts. Preliminary phytochemical screening of the SBT leaf aqueous and hydroalcoholic extracts gave positive results for the presence of flavonoids and polyphenols which could be responsible for the antioxidant potential of this plant.

The reducing potential of SBT leaf aqueous and hydroalcoholic extracts was determined using a modified iron (III) to iron (II) reduction assay. In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the

reducing power of extracts or compounds. The presence of reductants in solution causes reduction of Fe^{3+} /Ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measurement of the formation of Perle's Prussian blue at 700 nm (Zou et al., 2004). The reducing potential decreased in the order, ascorbic acid > SBT hydroalcoholic leaf extract > SBT aqueous leaf extract. Further, it was observed that reducing activity of the extracts was concentration dependent.

In the present study, addition of H_2O_2 or HX-OX to BHK-cells resulted in a significant increase in cytotoxicity and decrease in mitochondrial integrity while simultaneous treatment of cells with aqueous and hydroalcoholic extracts of SBT leaves significantly inhibited cytotoxicity and maintained mitochondrial integrity. It may be postulated that both extracts have marked cytoprotective activity against H_2O_2 and HX-XO induced cytotoxicity. The fact that simultaneous treatment gave the protective effect to cells indicated that the direct interaction of extracts with peroxide and superoxide play a major role in the protection observed *in vitro*.

Antibacterial activity of aqueous and hydroalcoholic extracts of SBT leaves was compared with ampicillin as a standard antibiotic. Both extracts had shown marked antibacterial activity against *B. cereus*, *P. aeruginosa*, *S. aureus* and *E. faecalis*, however extracts were less potent than ampicillin. Phenol constituents of the plant extracts have shown potent antimicrobial properties (Kaur and Arora, 2009; Klanckin et al., 2009). The observed antibacterial activity could be attributed to the phenol constituents of the SBT leaf extracts, especially quercetin derivatives. Further, antibacterial activity observed in the present study justified the traditional uses of SBT for wound healing, skin disorders and other infectious conditions.

In conclusion, the antioxidant activity here reported, along with the cytoprotective and antibacterial activity, give a scientific support to the modern studies which reported the positive influence of Sea buckthorn leaf in stress management, radioprotection, wound healing, inflammatory and free radical mediated diseases. Further investigation on the isolation and identification of active component(s) may lead to chemical entities with potential clinical use.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors are thankful to Director, Defence Institute of Physiology and Allied Sciences, Delhi, India, for his keen interest and providing support to the study. One of the authors (NKU) is grate-

ful to the Council of Scientific and Industrial Research (CSIR), New Delhi, India for the Senior Research Fellowship.

References

- Badami, S., Gupta, M.K., Suresh, B., 2003. Antioxidant activity of the ethanolic extract of *Striga orobanchioides*. *J. Ethnopharmacol.* 85, 27–30.
- Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M., 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 43, 493–496.
- Begnami, A.F., Duarte, M.C.T., Furelletti, V., Rehder, V.L.G., 2010. Antimicrobial potential of *Coriandrum sativum* L. against different *Candida* species in vitro. *Food Chem.* 118, 74–77.
- Benzie, I.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. *Anal. Biochem.* 239, 70–76.
- Bernath, J., Foldesi, D., 1992. Sea-buckthorn (*Hippophae rhamnoides* L.): a promising new medicinal and food crop. *J. Herbs Spices Med. Plants* 1, 27–35.
- Bland, J.S., 1995. Oxidants and antioxidants in clinical medicine: past, present and future potential. *J. Nutr. Environ. Med.* 5, 255–280.
- Blois, M.S., 1958. Antioxidant determination by the use of stable free radicals. *Nature* 181, 1199–1200.
- Chawla, R., Arora, R., Singh, S., Sagar, R.K., Sharma, R.K., Kumar, R., Sharma, A., Gupta, M.L., Singh, S., Prasad, J., Khan, H.A., Swaroop, A., Sinha, A.K., Gupta, A.K., Tripathi, R.P., Ahuja, P.S., 2007. Radioprotective and antioxidant activity of fractionated extracts of berries of *Hippophae rhamnoides*. *J. Med. Food* 10, 101–109.
- Ganju, L., Padwad, Y., Singh, R., Karan, D., Chanda, S., Chopra, M.K., 2005. Anti-inflammatory activity of Sea buckthorn (*Hippophae rhamnoides*) leaves. *Int. Immunopharmacol.* 12, 1675–1684.
- Geetha, S., Singh, V., Ram, M.S., Ilavazhagan, G., Banerjee, P.K., Sawhney, R.C., 2005. Immunomodulatory effects of Sea buckthorn (*Hippophae rhamnoides* L.) against chromium (VI) induced immunosuppression. *Mol. Cell Biochem.* 278, 101–109.
- Gupta, A., Kumar, R., Pal, K., Banerjee, P.K., Sawhney, R.C., 2005. A preclinical study of the effects of sea buckthorn (*Hippophae rhamnoides* L.) leaf extract on cutaneous wound healing in albino rats. *Int. J. Low. Extrem. Wounds* 4, 88–92.
- Gupta, A., Upadhyay, N.K., Sawhney, R.C., Kumar, R., 2008. A poly-herbal formulation accelerates normal and impaired diabetic wound healing. *Wound Rep. Regen.* 16, 784–790.
- Halliwell, B., Gutteridge, J.M.C., 1990. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol.* 186, 1–85.
- Kaur, G., Arora, D.S., 2009. Antibacterial and phytochemical screening of *Anythum graveolens*, *Foeniculum vulgare* and *Trachyspermum ammi*. *BMC Complement. Altern. Med.* 9, 30–39.
- Klanckin, A., Guzy, B., Kolar, M.H., Abramovic, H., Mozina, S.S., 2009. In vitro antimicrobial and antioxidant activity of commercial rosemary extract formulation. *J. Food Prot.* 72, 1744–1752.
- Long, L.H., Kwee, D.C., Halliwell, B., 2000. The antioxidant activities of seasonings used in Asian cooking. Powerful antioxidant activity of dark soy sauce revealed using the ABTS assay. *Free Rad. Res.* 32, 181–186.
- Negi, P.S., Chauhan, A.S., Sadia, G.A., Rohinishree, Y.S., Ramteke, R.S., 2005. Antioxidant and antibacterial activities of various Sea buckthorn (*Hippophae rhamnoides* L.) seed extracts. *Food Chem.* 92, 119–124.
- Phan, T.T., Patrick, S., Grayer, R.J., Chan, S.Y., Lee, S.T., 2001. Phenolics compounds of *Chromolaena odorata* protect cultured skin cells from oxidative damage: implication for cutaneous wound healing. *Biol. Pharm. Bull.* 24, 1373–1379.
- Re, R., Pellegrini, N., Proteggeente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad. Bio. Med.* 26, 1231–1237.
- Rousi, A., 1971. The genus *Hippophae* L., a taxonomic study. *Ann. Bot.* 8, 177–227.
- Saggu, S., Divekar, H.M., Gupta, V., Sawhney, R.C., Banerjee, P.K., Kumar, R., 2007. Adaptogenic and safety evaluation of Sea buckthorn (*Hippophae rhamnoides*) leaf extract: a dose dependent study. *Food Chem. Toxicol.* 45, 609–617.
- Singleton, V.L., Rossi, J.A., 1965. Calorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16, 144–158.
- Thomson, M.J., 1995. The role of free radicals and antioxidants: how do we know that they are working. *Crit. Rev. Food Sci. Nutr.* 35, 21–39.
- Upadhyay, N.K., Kumar, R., Siddiqui, M.S., Gupta, A., 2009. Mechanism of wound-healing activity of *hippophae rhamnoides* l. leaf extract in experimental burns. evid. based complement. *Altern. Med.* doi:10.1093/ecam/nep189.
- Zou, Y., Lu, Y., Wei, D., 2004. Antioxidant activity of a flavonoid-rich extract of *Hypericum perforatum* L. in vitro. *J. Agric. Food Chem.* 52, 5032–5039.