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

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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-pircyl-hydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. Further, both extracts were observed to have cytoprotective activity against hydrogen peroxide and hypoxantine-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.

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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; Saggu et al., 2007; Upadhyay et al., 2009).
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 SB10-2006) was characterized by Dr. O.P. Chaurasia, an ethnobotanist at the Defence Institute of High Altitude Research (DI-HAR), 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 reaction mixture was pooled, filtered through muslin cloth and centrifuged at 5000 g for 10 min at 4°C. The supernatant was filtered through a 0.22 μm filter (Millipore), and appropriately diluted (0.01–100 μg/ml) before use. The solution was then diluted with 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 added to react with 2850 μl of the ABTS solution and the absorbance was measured at 734 nm after 2 h using the spectrophotometer. The working standard curve was linear between 25 and 150 ppm Trolox.

2.3. Determination of 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 390 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 1% 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 column 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: 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 reaction mixture was pooled, filtered through muslin cloth and centrifuged at 5000 g for 10 min at 4°C. The supernatant was filtered through a 0.22 μm filter (Millipore), and appropriately diluted (0.01–100 μg/ml) before use. The solution was then diluted with 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 added to react with 2850 μl of the ABTS solution and the absorbance was measured at 734 nm after 2 h using the spectrophotometer. The working standard curve was linear between 25 and 150 ppm Trolox. Results were expressed in mg of Trolox equivalents/g of dry leaf.

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

The 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 mM stock solution of ABTS+ (2,2’-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) was mixed with 0.2 M phosphate buffer, 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 added 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.

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 6.8 (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 a density of 1 × 104 cells/well in Dulbecco’s Modified Eagle’s Medium containing the gradient flow rate 0.75 ml/min of the mobile phase-high performance liquid chromatography (RP-HPLC). After 24 h, the cells were treated with various concentrations 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 percent cell viability was calculated with the following equation:

\[ \text{Cell viability} (%) = \left( \frac{A_{t}}{A_{0}} \right) \times 100\% \]
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 inoculums. 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.

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).

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total phenols (mg gallic acid equivalents/g dry leaf)</th>
<th>Total flavonoids (mg rutin equivalents/g dry leaf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous leaf extract</td>
<td>40.49 ± 2.10</td>
<td>14.90 ± 1.10</td>
</tr>
<tr>
<td>Hydroalcoholic leaf extract</td>
<td>56.28 ± 2.30</td>
<td>20.76 ± 1.35</td>
</tr>
</tbody>
</table>

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

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

### 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$_2$O$_2$ ($2 \times 10^{-4}$ mol/l) or HX–OX ($2 \times 10^{-2}$ units/ml) as a cytotoxic agent and oxidative stress inducer. The results demonstrated that this concentration of H$_2$O$_2$ 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 µg/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.
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-gluco side, 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 activities 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 Fe3+/Ferricyanide complex to the ferrous form. Therefore, the Fe2+ can be monitored by measurement of the formation of Perl’s Prussian blue at 700 nm (Zou et al., 2004). The reducing potential decreased in the order, ascobic 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 H2O2 or HX–XO 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 H2O2 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; Klankin 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 in 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.

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