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Antioxidant and antibacterial activities of various seabuckthorn (*Hippophae rhamnoides* L.) seed extracts

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Abstract

Seabuckthorn (*Hippophae rhamnoides* L.) seeds were successively extracted with chloroform, ethyl acetate, acetone and methanol (MeOH) using a Soxhlet extractor for 8 h each. The crude extracts were screened for antioxidant and antibacterial activities. The reducing power and antioxidant activities evaluated in various in vitro models (1,1-diphenyl-2-picrylhydrazine and liposome model system) showed the highest activity for MeOH extract. The MeOH extract was also found to posses maximum antibacterial activity. The MIC values, with respect to MeOH extract for *Bacillus cereus*, *Bacillus coagulans*, *Bacillus subtilis*, *Listeria monocytogenes*, *Yersinia enterocolitica*, were found to be 200, 300, 300, 300, and 350 ppm, respectively. These results indicated the possibility of using seabuckthorn seeds for medicinal uses and food preservation.
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Keywords: *Hippophae rhamnoides*; Antioxidant activity; Antibacterial activity; Seed extract; Methanolic extract

1. Introduction

Seabuckthorn (*Hippophae rhamnoides* L., Elaeagnaceae) is a native of Eurasia and has been domesticated in several countries including India, China, Nepal, Pakistan, Myanmar, Russia, Britain, Germany, Finland, Romania and France at a high altitude of 2500–4300 m. It is known as the wonder plant and bears small orange yellow- to red-coloured fruits on two-year-old thorny twigs. The berry-like fruit develops from an ovary, or calyx tube connected to an ovary. Seabuckthorn (SBT) berries are known to be acidic with a mild sweet unique aroma. High amounts of vitamin C, flavonoids, oils and oil-soluble compounds, as well as minerals, are present in the berry (Kallio, Yang, Tahvonen, & Hakala, 2000). Berries also contain many bioactive substances and can be used in the treatment of several diseases, such as cardiovascular disease, cancer, and acute mountain sickness. For the past 50 years several medicinal preparations of SBT have been clinically used to treat radiation damage, burns, oral inflammation and gastric ulcers in China and the former Soviet republic, and more than 300 preparations have been reported in literature (Lu, 1992). In addition to medicinal use, the berries are processed into various products such as juice and marmalade, and used for flavouring of dairy products because of their unique taste (Gao, Ohlander, Jeppsson, Bjork, & Trajkovski, 2000).

Spoilage of foods due to the presence of bacterial and fungal infection has been a major concern for decades and it causes a considerable loss worldwide. The demand for non-toxic, natural preservatives has been rising with increased awareness and reports of ill-effects of synthetic chemicals present in foods. Furthermore, emergence of food-borne pathogens has lately become a major public health concern. Many compounds

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present in plants have been reported to be biologically active, antimicrobial, allopathic antioxidants and have bioregulatory properties. SBT oils, juice, leaves and bark are well known for their medicinal properties, and they have been used to treat high blood lipid symptoms, gingivitis, eye and skin ailments, and cardiovascular diseases (Liu, Wu, & Liu, 1980; Yang et al., 2000).

Proximate composition of SBT has been well documented (Gao et al., 2000). Also, there have been reports on chemical composition, characterization and health benefits of SBT fruit and its oil (Chen et al., 1990; Yang et al., 2000). But, SBT seed, a byproduct of the berry processing industry, is not yet exploited. To our knowledge, no information is available on the antioxidant and antibacterial properties of SBT seeds. Therefore, the objective of the present study was to investigate the antioxidant activity of various SBT seed extracts in in vitro models, and determine their antibacterial activity against different food-borne pathogens for their potential as a natural preservative and for nutraceutical formulations.

2. Materials and methods

2.1. Materials

SBT berries were collected from the Lahaul Spiti region (Himachal Pradesh, India) and seeds were obtained after deseeding the berries by passing through stainless steel sievers (Mesh pore size 0.042 in.). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), and tannic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All solvents/chemicals used were of analytical grade and obtained from Merck, Mumbai, India.

2.2. Preparation of the SBT seed extracts

Dried seeds of *Hippophae rhamnoides* were powdered and successively extracted in a Soxhlet extractor for 8 h each with chloroform, ethyl acetate, acetone, and methanol according to the procedure of Chauhan, Negi, and Ramteke (2003). The extracts were used as such for determination of reducing power, dissolved in methanol: water (6:4 v/v) for evaluation of antioxidant capacity by the liposome model system, in dimethyl sulphoxide for scavenging activity by DPPH method; and in propylene glycol for evaluation of antibacterial activity.

2.3. Determination of total phenolics

The concentration of phenolics in the extracts was determined by the method of Negi, Jayaprakasha, and Jena (2003) and results were expressed as (+) tannic acid equivalents. Five milligrammes of each dried SBT seed extract were mixed with 10 ml mixture of acetone and water (6:4 v/v). An aliquot of the samples was mixed with 1.0 ml of 10-fold diluted Folin–Ciocalteu reagent and 2 ml of 10% sodium carbonate solution. After standing for 30 min at room temperature, the absorbance was measured at 765 nm using a GBC UV–Vis Spectrophotometer (Model Cintra-10, Victoria, Australia).

2.4. Evaluation of antioxidant activity by liposome model systems

Egg lecithin (300 mg) was sonicated with 30 ml phosphate buffer (10 mM, pH 7.4) in an ultrasonic sonicator for 10 min to ensure proper liposome formation. The various dried extracts (25–100 μg) of each sample were mixed with the sonicated solution (0.5 ml, 10 mg/ml), $FeCl_3$(0.5 ml, 400 mM), and ascorbic acid (0.5 ml, 400 mM). The antioxidative action was measured by the method of Buege and Aust (1978). The absorbance of the samples was determined at 535 nm after incubation for 1 h at 37 °C. The results were expressed as nmol of malondialdehyde (MDA) formed per mg lipid and were calculated by using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.5. Determination of reducing power

The determination of reducing power was performed as described by Yen and Duh (1993). Various extracts (0.48, 1.2, 2.4, 3.6, and 4.8 mg) were mixed with phosphate buffer (5.0 ml, 2.0 M, pH 6.6) and 1% potassium ferricyanide (5 ml), and incubated at 50 °C for 20 min; 5 ml of 10% trichloroacetic acid were added, and the mixture was centrifuged at 2500 rpm for 10 min. The upper layer of the solution (5 ml) was mixed with distilled water (5 ml) and 0.1% ferric chloride (1 ml) and the absorbance was read at 700 nm. Increase in the absorbance of the reaction mixture indicated increase in the reducing power.

2.6. Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical

Free radical-scavenging activity of each antioxidant was assayed using a stable free radical, DPPH, according to the method of Blois (1958). The reaction mixture contained 0.5 ml of 0.5 mM DPPH and 0.1 ml of dimethyl sulphoxide containing the antioxidant extract at different concentrations (10–50 μg). Finally, the total volume of the reaction mixture was adjusted to 1.0 ml by adding 100 mM Tris–HCl buffer (pH 7.4). The reaction mixture was allowed to stand for 20 min at room temperature in the dark and the radical-scavenging activity of each antioxidant was quantified by decolorization at 517 nm.

2.7. Antibacterial test by pour plate method

Bacterial cultures, namely, *Bacillus cereus*, *B. coagulans*, *B. subtilis*, *Listeria monocytogenes* and *Yersinia enterocolitica*, were obtained from the Department of Food Microbiology of this Institute. *Bacillus cereus*, *B. coagulans* and *B. subtilis* were grown in nutrient agar (HiMedia, Mumbai, India), and *L. monocytogenes* and *Y. enterocolitica* were grown in Brain heart infusion agar (HiMedia, Mumbai, India) at 37 °C. Each bacterial strain was transferred from stored slants at 4–5 °C to 10 ml broth and cultivated overnight at 37 °C. A preculture was prepared by transferring 1 ml of this culture to 9 ml broth and cultivated for 48 h. The cells were harvested by centrifugation (1200*g*, 5 min), washed and suspended in saline.

The SBT seed extracts were tested against different bacteria by the method of Negi, Jayaprakasha, Rao, and Sakariah (1999). To flasks containing 20 ml melted cool agar, different concentrations of test material in propylene glycol were added. Equivalent amounts of propylene glycol were used as controls. One hundred μl (about 10^3 cfu/ml) of each bacterium to be tested were inoculated into the flasks under aseptic conditions. The media were then poured into sterilized Petri plates, in duplicate, and incubated at 37 °C for 20–24 h. The colonies, developed after incubation, were counted and expressed as colony forming units per ml of culture (cfu/ml). The minimum inhibitory concentration (MIC) was reported as the lowest concentration of the compound capable of inhibiting the complete growth of the bacterium being tested.

2.8. Statistical analysis

All the experiments were repeated three times and the data were calculated as means ± SD. The data on the antioxidant effect of different extracts were analyzed by a two factor experiment in a randomized complete block design with an experiment involving 4 extracts, 5 concentrations and 3 replications for the liposome model system, 3 extracts, 5 concentrations and 3 replications for the radical-scavenging activity on DPPH, and 4 extracts, 6 concentrations and 3 replications for reducing power studies. One-way ANOVA was used to determine the differences in yields and phenolic contents of different extracts. The Duncan's multiple range test (DMRT) was used for making comparisons (Gomez & Gomez, 1984).

3. Results and discussion

The SBT seeds were extracted successively with chloroform, ethyl acetate, acetone and methanol using a Soxhlet extractor for 8 h each. The yields of ethyl acetate, acetone and chloroform extracts were significantly lower than methanol extracts (Fig. 1). The phenolic contents in different extracts also varied significantly. Then methanolic extract had the highest phenolic content, followed by ethyl acetate and acetone extract. Variations in the yields and phenolic contents of various extracts are attributed to polarities of different compounds present in the seeds and such differences have been reported in the literature for other fruit seeds (Jayaprakasha, Singh, & Sakariah, 2001).

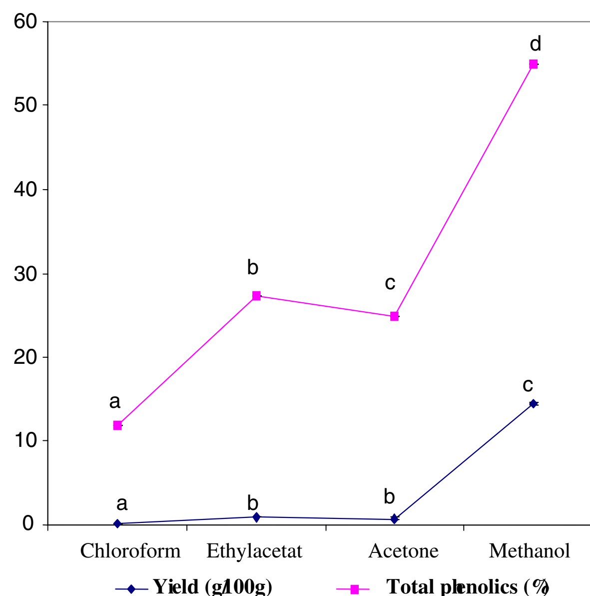

Fig. 1. Yield and total phenolics in SBT seed extracts: –◆– Yield (g/100 g), –■– Total phenolics (%). Mean values ± SD followed by same letter are not significantly different ($P < 0.05$).

Table 1
Antioxidant activity of SBT seed extracts by the liposome model system [A]

Concentration (μg/ml)	Chloroform	Ethyl acetate	Acetone	Methanol
0	3.03 ± 0.17^a	3.03 ± 0.17^a	3.03 ± 0.17^a	3.03 ± 0.17^a
25	2.86 ± 0.00^b	1.83 ± 0.03^f	1.48 ± 0.035^g	1.29 ± 0.00^h
50	2.57 ± 0.00^c	1.51 ± 0.04^g	1.14 ± 0.02^h	0.98 ± 0.02^i
75	2.33 ± 0.04^d	0.93 ± 0.04^i	0.76 ± 0.05^j	0.68 ± 0.04^{jk}
100	2.15 ± 0.03^e	0.58 ± 0.00^k	0.55 ± 0.02^k	0.34 ± 0.02^l

[A] Antioxidant activity as nmol MDA/mg lipid.Mean values ± standard deviations (n = 3) with the same letter are not significantly different ($P < 0.05$).

The antioxidative actions of SBT seed extracts in the liposome system, induced by $FeCl_3$plus ascorbic acid and determined by the thiobarbituric acid method, are shown in Table 1. Significant differences ($P < 0.05$) were found among control and liposome-containing SBT seed extracts. The chloroform, ethyl acetate, acetone and methanol extracts at 100 μg showed 2.15, 0.58, 0.54 and 0.35 nmol MDA/mg lipid formation, respectively. However, the formation of the MDA for the control was 3.02 nmol MDA/mg lipid, indicating that the chloroform, ethyl acetate, acetone and methanol extracts inhibited the peroxidation of lecithin by 29.1%, 80.8%, 82.2% and 88.5%, respectively. A similar trend of variable antioxidant activity for different solvent extracts of grape seeds was also observed by Jayaprakasha et al. (2001).

It is well known that free radicals play an important role in autooxidation of unsaturated lipids in foodstuffs. Oxidation of muscle cholesterol may be initiated by free

Table 2
% Radical-scavenging activity on DPPH by various SBT seed extracts[A]

Concentration (μg/ml)	Ethyl acetate	Acetone	Methanol
10	13.5 ± 1.44[b]	9.51 ± 0.02[a]	40.4 ± 2.24[e]
20	26.8 ± 2.18[c]	14.8 ± 0.73[b]	71.2 ± 0.80[h]
30	40.0 ± 1.94[e]	31.4 ± 1.27[d]	85.2 ± 2.21[i]
40	43.6 ± 1.39[e]	41.3 ± 2.75[e]	92.2 ± 0.68[j]
50	50.1 ± 5.50[f]	59.0 ± 4.14[g]	93.5 ± 0.32[j]

[A] Mean values ± standard deviations (n = 3) with the same letter are not significantly different ($P < 0.05$).

Table 3
Reducing power of various SBT seed extracts[A]

Amount (mg)	Chloroform	Ethyl acetate	Acetone	Methanol
0.0	0.03 ± 0.00[a]	0.03 ± 0.00[a]	0.03 ± 0.00[a]	0.03 ± 0.00[a]
0.48	0.17 ± 0.01[b]	0.38 ± 0.05[d]	0.62 ± 0.002[e]	0.74 ± 0.02[g]
1.20	0.26 ± 0.00[c]	0.68 ± 0.01[f]	1.21 ± 0.00[j]	1.50 ± 0.00[k]
2.40	0.58 ± 0.01[e]	0.98 ± 0.01[i]	1.87 ± 0.01[m]	2.14 ± 0.00[n]
3.60	0.65 ± 0.00[f]	1.23 ± 0.02[j]	2.61 ± 0.00[o]	2.87 ± 0.00[p]
4.80	0.81 ± 0.00[h]	1.64 ± 0.01[l]	3.10 ± 0.00[q]	3.62 ± 0.00[r]

[A] Absorbance at 700 nm. Mean values ± standard deviations (n = 3) with the same letter are not significantly different ($P < 0.05$).

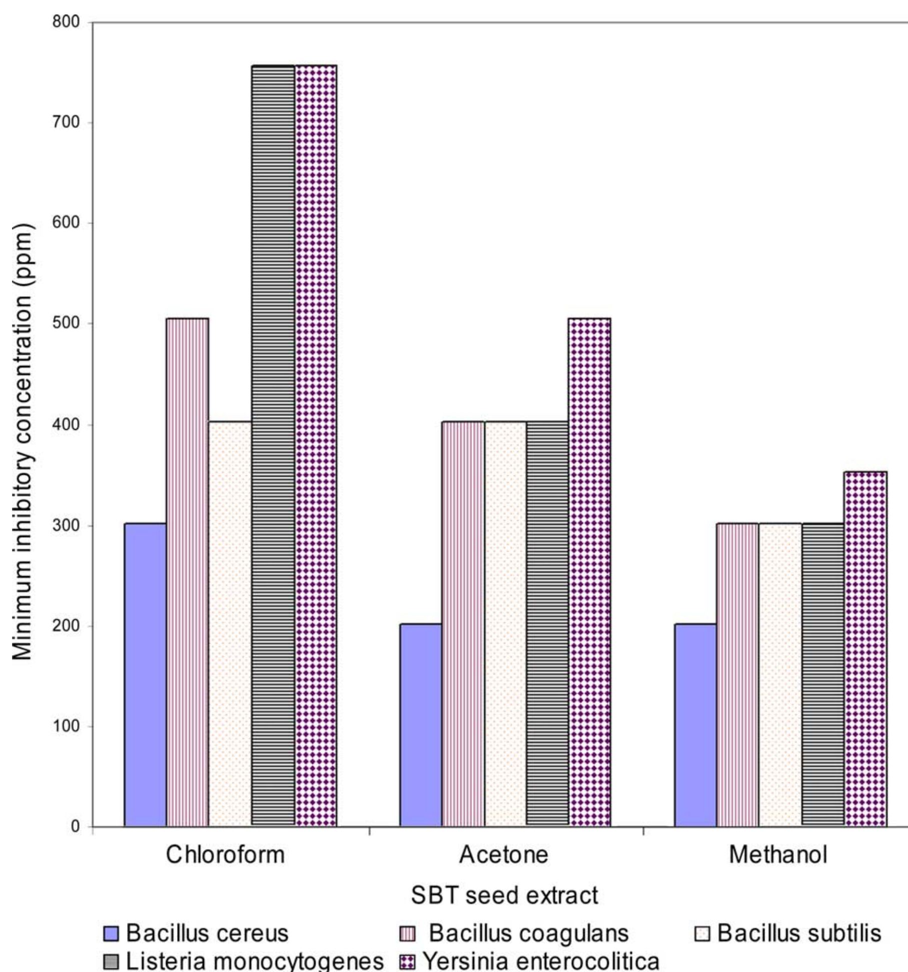

Fig. 2. Effects of SBT seed extracts on growth of different bacteria: *Bacillus cereus*, *Bacillus coagulans*, *Bacillus subtilis*, *Listeria monocytogenes*, *Yersinia enterocolitica*.

radicals generated during the oxidation of polyunsaturated fatty acids (Hoelscher, Savell, Smith, & Cros, 1988). DPPH is used as a free radical to evaluate antioxidative activity of some natural sources, and the degree of its discoloration is attributed to hydrogen-donating ability of test compounds, which is indicative of their scavenging potential (Shimada, Fujikawa, Yahara, & Nakamura, 1992). In the present work, also, the comparison of scavenging effects of ethyl acetate, acetone and methanol extracts on DPPH radical showed the consistently higher radical-scavenging activity for methanolic extract at all the concentrations (Table 2). The data show that the extracts are free radical inhibitors and act as primary antioxidants. These results confirm earlier studies, where inhibition of chromium-induced free radicals was reported by SBT leaf and fruit methanolic extracts (Geetha, Sai Ram, Singh, Ilavazhagan, & Sawhney, 2002).

Table 3 shows the reducing powers of different SBT seed extracts using the potassium ferricyanide method at various extract concentrations. It appears that antioxidative activity may have a mutual correlation with the reducing effect. Chloroform extract showed the least reducing power and methanol extract had the highest activity. Jayaprakasha et al. (2001) also noted differences in reducing power of various extracts of grape seed. The reducing properties are generally associated with the presence of reductones and Gordon (1990) reported that the antioxidant activity of reductones is believed to break radical chains by donation of a hydrogen atom, indicating that the antioxidative properties are concomitant with the development of the reducing power. Therefore, the marked antioxidative activity in MeOH extract may be associated with its higher reducing power.

The effect of SBT seed extracts on growth of different bacteria is presented in Fig. 2. Different extracts inhibited growth to variable extents, depending on the bacterium in question. Methanolic extract was found to be the most effective, followed by chloroform and acetone extracts. *Yersinia enterocolitica* was the most resistant to all the extracts, and higher MIC values were obtained for it. Higher resistance of Gram-negative bacteria to external agents has been earlier reported, and it is attributed to the presence of lipopolysaccharides in their outer membranes, which make them inherently resistant to antibiotics, detergent and hydrophilic dyes (Nikaido & Vaara, 1985). Similar trend for inhibition of bacterial growth have been observed in earlier studies with SBT berry extracts (Puupponen-Pimla et al., 2001) and other plant extracts (Negi & Jayaprakasha, 2003). The reason for higher sensitivity of the Gram-positive bacteria than negative bacteria could be ascribed to the differences between their cell wall compositions. The Gram-positive bacteria contain an outer peptidoglycone layer, which is an ineffective permeability barrier (Scherrer & Gerhardt, 1971).

4. Conclusions

We propose that the higher antibacterial and antioxidant activities of methanol extract of SBT seeds may be due to the higher phenolic content. Patro et al. (2001) also concluded that higher antioxigenic activity of SBT berry is due to higher contents of flavonoids. Recently Rosch, Bergmann, Knorr, and Kroh (2003) observed that, although a few phenolic compounds present in SBT fruit juice have good antioxidant capacity, their contribution to the antioxidant effect is meagre in comparison to ascorbic acid. As our extraction procedure has eliminated the presence of ascorbic acid, we suppose that selective extraction and concentration of phenolic compounds in methanolic extract of SBT seed might have resulted in higher biological activity. Thus, the results indicate that selective extraction from natural sources, by an appropriate solvent, is important for obtaining fractions with high antioxidant and high antibacterial activity. The antibacterial and antioxidant properties of SBT seeds can add value to this wonder plant as, at present, except for the seeds, all parts of the plant are being utilized. This is a preliminary report on the isolation of antioxidants and antibacterial fractions from SBT seed, and further studies are needed for the characterization of individual phenolic compounds to elucidate the mechanisms underlying bioactive properties and the existence of possible synergism, if any, among these compounds.

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