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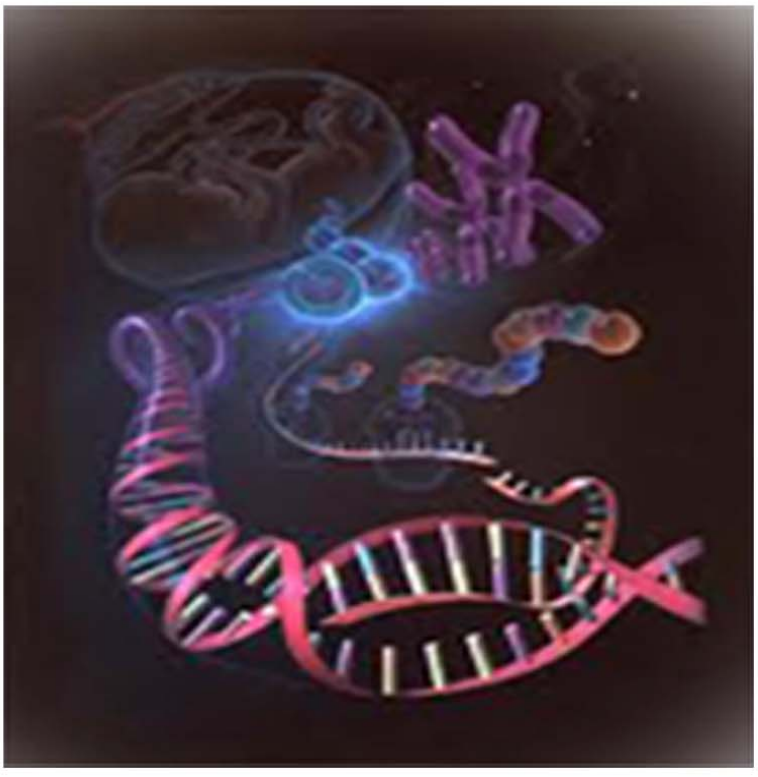

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Research Paper

ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF LEAF AND BARK EXTRACTS OF SEABUCKTHORN (*HIPPOPHAE SALICIFOLIA* D. DON) OF NORTH EAST INDIA

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The present study was conducted to evaluate the antioxidant and antibacterial activities of different solvent extracts of leaf and bark of Seabuckthorn (*Hippophae salicifolia*) species found in North East India. Antioxidant activity was measured using total phenolics content, reducing power and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assays. Methanolic extract of both leaf and bark samples showed highest antioxidant activities compared to the extracts in other solvents. Antibacterial activity was tested against six pathogenic strains by agar diffusion and broth macrodilution methods. The extracts were found to exert low to moderate antibacterial activity compared to a standard antibacterial agent. Both antioxidant and antibacterial activities were found to be higher in methanolic extract. Strong correlation ($P < 0.05$) was observed between total phenolic content versus antioxidant and antibacterial activity from the extracts under study.

Keywords: *Hippophae salicifolia*, Leaf, Bark, Antioxidant activity, Antibacterial activity, Methanolic extract

INTRODUCTION

Natural products, such as plant extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched chemical diversity they can provide (Cos *et al.*, 2006). These compounds are significant in therapeutic application against human and animal pathogens, including bacteria, fungi and viruses (Khan *et al*., 2003; and Pavrez *et al*., 2005). The uninhibited production of oxygen-derived free radicals is involved in the onset of many diseases such as arthritis, atherosclerosis, rheumatoid and cancer as well as in many degenerative diseases related with aging (Halliwell and Gutteridge, 1984). To face these situations, efforts are being on at various levels to develop herbal based drugs and therapeutic agents. The present investigation is a part of such efforts on a less reported plant resource of North East India.

Hippophae salicifolia D. Don. (Commonly known as Seabuckthorn or Seaberry), is a

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deciduous tree, which belongs to the family *Elaeagnaceae* and is naturally distributed over Asia and Europe (Yang *et al*., 2000). In India, *Hippophae* grows naturally in high-altitude areas of Jammu and Kashmir, Himachal Pradesh, Uttar Pradesh and Sikkim (Singh, 1998). In Sikkim, *H. salicifolia* grows along the riverside, torrential slides, vertical hills and slopes, mostly on the south-east aspect of the Lachen and Lachung valley at an altitudinal range between 2,391 and 3,111 m (Basistha *et al*., 2010).

Seabuckthorn 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 *et al*., 1980; and Yang *et al*., 2000). Leaves were also reported to possess anti-inflammatory properties (Padwad *et al*., 2006). In recent years, extracts from this plant have been used increasingly in the US as a dietary supplement. Seabuckthorn contains a series of compounds including carotenoids, tocopherols, sterols, flavonoids, lipids, ascorbic acid, and tannins. These compounds are of interest not only from the chemical point of view, but also because many of them possess biological and therapeutic activity including antioxidant, antitumor, hepato-protective, and immunomodulatory properties (Cheng *et al*., 2003). The leaves of *H. rhamnoides* were considered for their antioxidant potential correlated to flavonoides and phenolic acids derivatives (Sharma *et al.,* 2008; Kim *et al.,* 2011; and Upadhyay *et al*., 2011). Antimicrobial activities have also been reported for Seabuck thorn leaves (Upadhyay *et al*., 2011). However, there is no information available on antioxidant and antibacterial activity of *Hippophae salicifolia* found in NE India, except a preliminary report on antioxidant activity by Goyal *et al.* (2011). In this work attempt has been made to evaluate the antioxidant and antibacterial properties of *H. salicifolia* found in Sikkim, India.

MATERIALS AND METHODS

Chemicals and Reagents

DPPH (2, 2-diphenyl-1-picryl-hydrazyl) and catechin were purchased from Sigma-Aldrich (USA). Gallic acid was obtained from HiMedia Laboratories (India). Sodium carbonate (Na_2CO_3), phosphate buffer, potassium ferricyanide, trichloroacetic acid, ferric chloride, ascorbic acid, Mueller Hinton agar, DMSO and methanol were purchased from Merck (India). Folin-Ciocalteu reagent was from SRL (India). All chemicals and solvents were of analytical grade.

Plant Material

Leaf and bark of *Hippophae salicifolia* were collected from Lachung valley of North Sikkim district lying between 27º41'00" N and 88º44'00" E. The species was authenticated with the voucher specimen at the Botanical survey of India, Shillong. The samples collected were washed using tap water and dried in an incubator at 40ºC. Dried samples were ground to produce fine homogenous powder using an electric blender and the powder (10 g) was soaked in 100 mL of selected solvents (methanol, acetone, chloroform and petroleum ether) at room temperature in the dark for three days. Each sample was filtered through Whatman no. 1 filter paper (Whatman International, England) and the filtered solutions were then evaporated to dryness using water bath at 40 ºC overnight. The plant extracts were then stored at 4 ºC.

Determination of the Total Phenolic contents

The amounts of phenolic compounds in the extracts were determined according to the

method of Waterman and Mole (1994) with certain modifications using Folin-Ciocalteu method, and Gallic acid was used as the standard phenolic compound. The extract solution in appropriate solvent (0.1 mL) was transferred to a volumetric flask containing 3 mL of distilled water. After that, 0.5 mL of Folin-Ciocalteu reagent was added. Three minutes later, 2 mL of 20% sodium carbonate solution was added. Subsequently, the shaken mixture was placed in boiling water for exactly 1 min, cooled and then measured at 650 nm. The experiment was carried out in triplicate and the content of total phenolic compounds was calculated using a standard curve prepared with gallic acid.

Determination of Antioxidative Activity

Reducing power: The reducing power was based on the method described previously by Yildirim *et al.* (2001). Different concentrations of extracts and ascorbic acid (50-200 mg/mL) in 1 mL of methanol were mixed with 2.5 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of 10 g/L potassium ferricyanide. The mixture was incubated at 50 ^{o}C for 30 min. An aliquot (2.5 mL) of 100 g/L trichloroacetic acid was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. Finally, 2.5 mL of the upper layer solution was mixed with 2.5 mL of distilled water and 0.5 mL of 1 g/L $FeCl_3$, and the absorbance of the resulting solution was measured at 700 nm.

DPPH-radical scavenging activity: The stable free radical scavenging activity was determined by the 1, 1-diphenyl-2-picryl-hydracyl (DPPH) method of Shyur *et al.* (2005) with minor modifications. The assay was performed in 3ml reaction mixture containing 2 mL of 0.1 mM DPPH methanol solution, 0.9 mL of 50 mM tris-HCl buffer (pH 7.4) and 0.1 mL of test extract at different concentrations or catechin and ascorbic acid (standard reference). The mixture was incubated at room temperature for 30 min and then the absorbance was measured at 517 nm. Radical scavenging activity is represented as % inhibition of DPPH radical which is calculated by the following formula:

$$\% \text{ Inhibition} = [(\text{Absorbance}_{control} - \text{Absorbance}_{sample}) / \text{Absorbance}_{control}] \times 100$$

IC_{50} is defined as the concentration of substrate that causes 50% loss of the DPPH activity (color) Molyneux (2004). IC_{50} of reference antioxidant compound, catechin was used for comparison to IC_{50} of the extracts.

Determination of Antibacterial Activity

Test Microorganisms: All the microbial cultures, used for antimicrobial screening were produced from MTCC, Chandigarh, India. A total of six bacterial species were tested including *Staphylococcus aureus (MTCC 96), Bacillus subtilis (MTCC 441), Escherichia coli (MTCC 739), Klebsiella pneumonia (MTCC 432), Enterobacter aerogenes (MTCC III)* and *Pseudomonas aeruginosa (MTCC 424)*. The bacterial culture was maintained on nutrient agar slants which were stored at –4^{o}C.

Antibacterial Assays: The antibacterial activity was based on agar well diffusion method using bacterial cell suspension whose concentration was equilibrated to a 0.5 McFarland standard. A 100 µL of each bacterial suspension was spread on a Mueller Hinton agar plate. Well (6 mm diameter) were impregnated with 10, 30 and 50 µL of each extract dissolved in DMSO at a concentration of 100 mg/mL. The wells were allowed to dry and then placed in the incubated at 37 ^{0}C for 24 h. Wells with the solvent used for dissolution were used as negative control and 1 mg/mL amoxicillin were used as positive controls.

After incubation time, zone of inhibition was measured. The experiment was performed in triplicate.

Determination of Minimum Inhibitory Concentration: Minimum inhibition concentrations of the extracts were evaluated for the bacterial strains which were determined as being sensitive to the extracts in the well diffusion assay. A broth macrodilution method was used, as previously described by Nakamura *et al*. (1999) with a slight modification. Serial twofold dilutions of each extract were prepared in dimethyl-sulfoxide (DMSO) at a concentration of 2 mg/mL, and 2 mL of each dilution was added to 2 mL of nutrient broth. These were inoculated with 50 µL of culture of the test bacterial strains. After incubation of the cultures at 37°C, the MIC value was determined as the lowest concentration of the extract that demonstrated no visible growth.

Statistical Analysis: All experimental results were expressed as means ± SD analysis of variance was performed by ANOVA procedures. Correlation coefficient (R) was used to determine two variables. The results with $P < 0.05$ were regarded to be statistically significant.

RESULTS AND DISCUSSION

In this study, phenolic content, antioxidant activity and antibacterial activity of various *Hippophae salicifolia* leaf and bark extracts were determined.

Extraction Yield and Total Phenolics: The extraction yields (g/100 g) from various extractants, i.e., methanol, acetone, chloroform and petroleum ether were presented in Table 1. The extraction yield depends on solvents, time and temperature of extraction as well as the chemical nature of the sample. Under the same time and temperature conditions, the solvent used and the chemical property of the sample are the two most important factors (Shimada *et al.*, 1992). In the present study the obtained extraction yields of

Table 1: Extraction Yield and Total Phenolics Content of *Hippophae salicifolia* Extracts of Leaf and Bark

S. No.	Plant Extract	Extraction Yield (g/100g)	Total Phenolics (mg/g)GAE
A. Leaves			
1.	Methanol	25.0	98.5 ± 0.2[a]
2.	Acetone	14.3	65.2 ± 1.2[c]
3.	Chloroform	9.70	42.9 ± 1.7[d]
4.	Pet. Ether	1.74	27.3 ± 0.2[a]
B. Bark			
1.	Methanol	21.0	84.4 ± 1.8[e]
2.	Acetone	16.7	45.6 ± 0.2[a]
3.	Chloroform	7.45	26.2 ± 0.5[b]
4.	Pet. Ether	0.94	3.50 ± 0.2[a]

Note: Mean values ± standard deviations (n = 3) with the same letter are not significantly different (P < 0.05).

bark and leaf for the different tested solvents ranked in the following order: Methanol > Acetone > Chloroform > Petroleum ether.

It is well known that plants contain many phenolic compounds which contain a hydroxyl group on an aromatic ring. These phenolic compounds interrupt chain oxidation reactions by donation of a hydrogen atom or chelating metals. So they act as reducing agents and antioxidants (Bursal and Koksal, 2010).

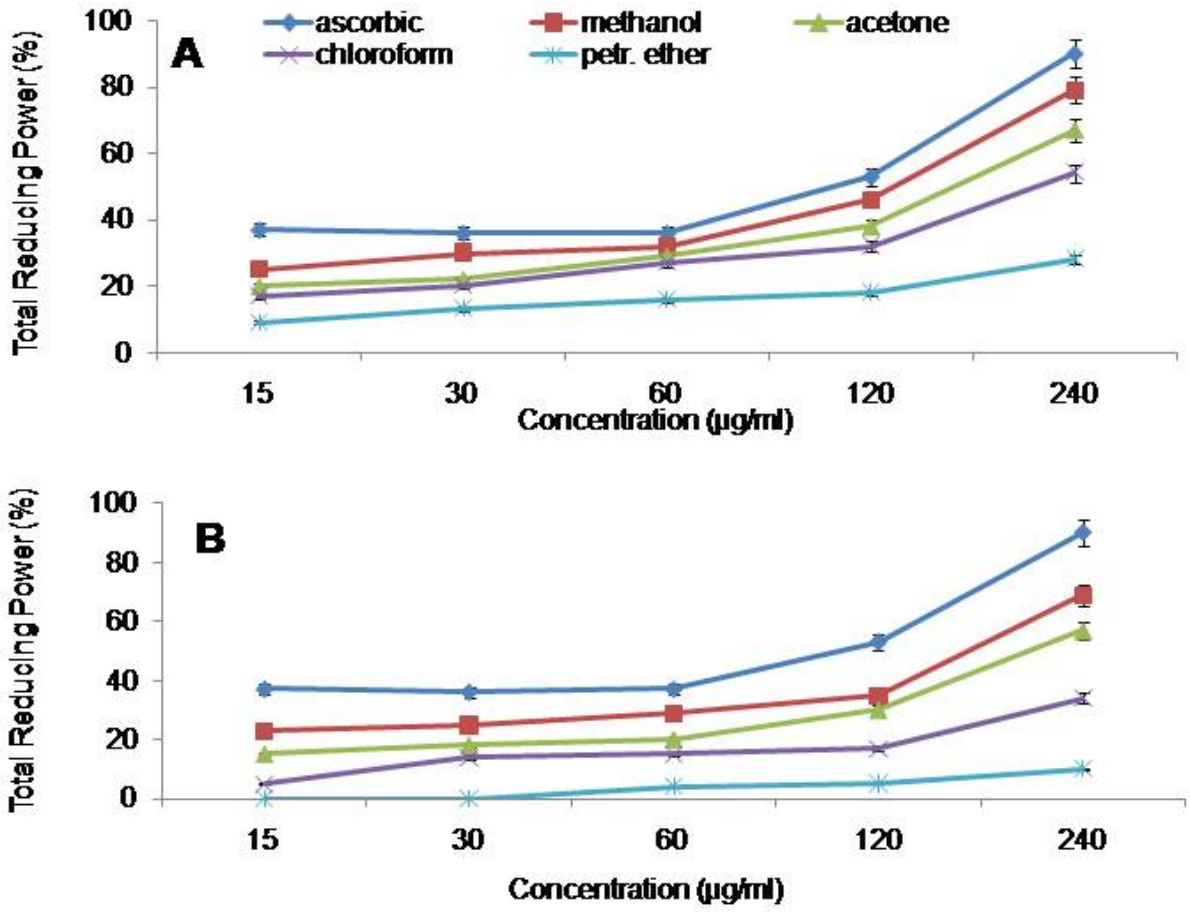
The total phenolic contents of the extracts were determined by Folin-Ciocalteu method. The high amounts of phenolic compounds of the leaf and bark extract were found to be in the following order: methanol extract > acetone extract > chloroform extract > petroleum ether extract (Table 1). The assays were performed in the whole extracts, as that could be more beneficial than isolated constituents because of the additive and synergistic effects, and considering that a bioactive individual component can change its properties in the presence of other compounds present in the extracts (Liu, 2003). A significant variation of phenolic content was observed in different extracts. The phenolic content of the leaf was found to be highest in methanolic extract with 98.5 mg/g followed by acetone, chloroform and petroleum ether extract. The methanolic extract of bark also shows high phenolic content with 84.4 mg/g followed by acetone and chloroform extract. The petroleum ether extract of bark was found to be lowest with 3.5 mg/g. Variations in the yields and phenolic contents of various extracts are attributed to polarities of different compounds present in the leaf and bark, and such differences have been reported in the literature for other fruit seeds (Jayaprakasha *et al.*, 2001).

Reducing Power: The extracts obtained by various solvent extractions were determined for their antioxidant activities. The extracts were investigated for the reductive capabilities by using the potassium ferricyanide reduction method. The reducing ability may serve as a significant indicator of potential antioxidant activity (Meir *et al.*, 1995). It appears that antioxidative activity may have a mutual correlation with the reducing effect. The leaf and bark extracts increased in reducing powers with increasing concentration (Figure 1) and the leaf extracts exhibited higher reducing ability than the bark extracts. The methanolic extract showed the highest activity, followed by the acetone, chloroform and petroleum ether extracts. When compared to the ascorbic acid, the methanolic fraction showed higher activity ($P< 0.05$) at all concentrations. Yen and Chen (1995) reported that the extract which showed a reducing power could function as an electron donor and also could reduce the oxidized intermediates generated from the lipid peroxidation reaction. Therefore, the marked antioxidative activity in methanolic extract of leaf and bark may be associated with its higher reducing power.

DPPH Radical Scavenging Activity: The free radical scavenging capacity of the extracts against common free radicals (DPPH) in vitro were further determined. The results indicated that the extracts exhibited a potential free radical scavenging activity. The inhibition percentage of the radical scavenging activity of the leaf and bark extracts were calculated and are illustrated in Figure 2. The results revealed that the extract with the highest effective radical scavenging activity was the methanol extract, followed by the acetone and chloroform extract, while lower activities were found in the petroleum ether extracts.

The total phenolic contents could be regarded as an important indication of antioxidant properties of plant extracts (Wang *et al.*, 2010).

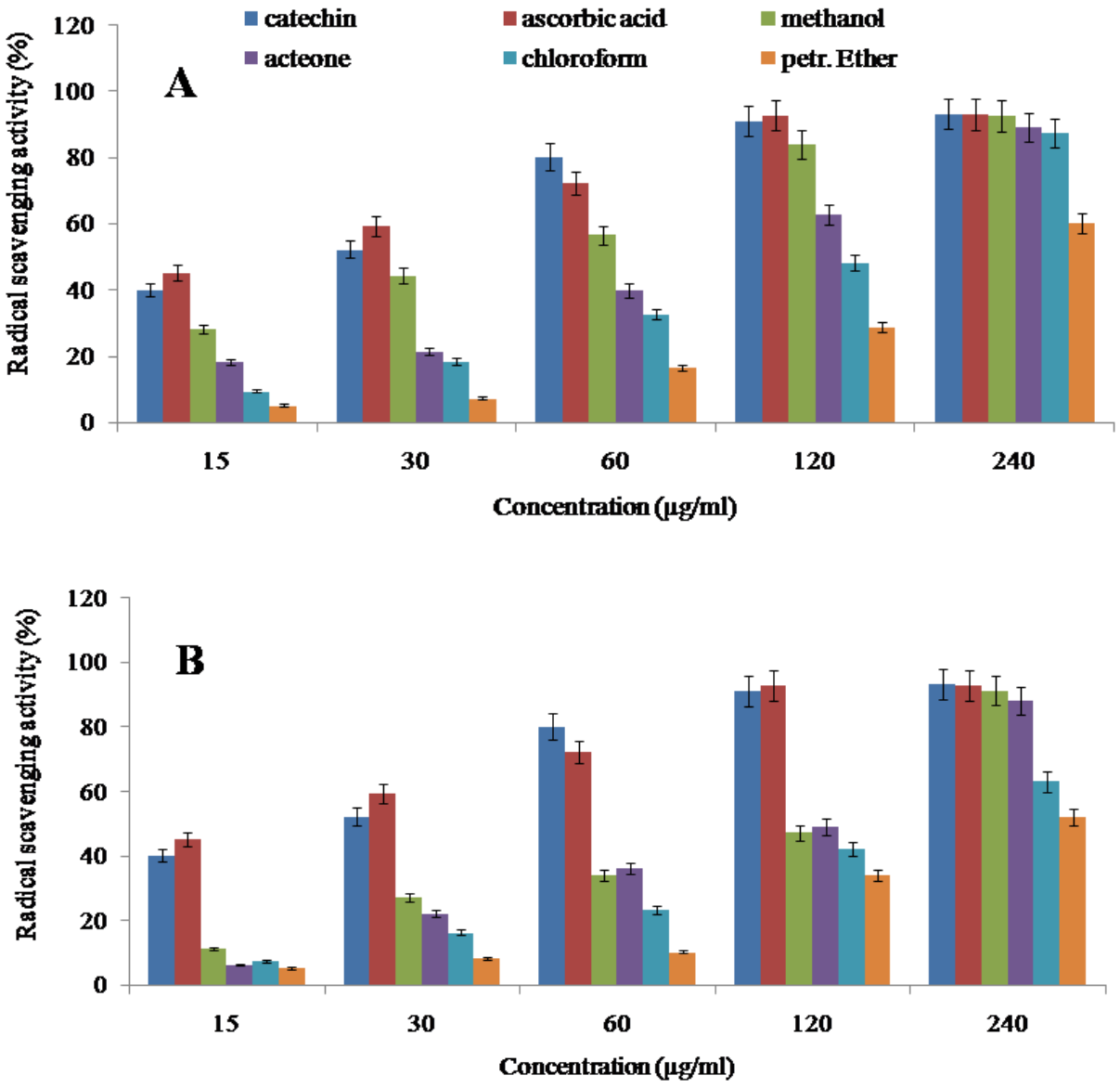
Figure 1: Total Reducing Power of Leaf (A) and Bark (B) Extracts of *Hippophae salicifolia* D. Don. Data are Presented as Mean ± SD of Triplicate Determinations



In the present study, the correlation coefficient (R^2) between the total phenolic content versus reducing power of *H. salicifolia* leaf and bark extracts was found to be 0.93 and 0.95, respectively and free radical scavenging activity was found to be 0.90 and 0.92, respectively. This correlation coefficient suggests that the phenolic compounds of *H. salicifolia* extracts contributed by 90-95% to their antioxidant activities. Thus, it can be noted that the strong antioxidant properties may be attributed to the phenolic components in the extracts. However, the antioxidant activity of plant extracts is not limited to phenolics. Activity may also come from the presence of other antioxidant secondary metabolites, such as volatile oils, carotenoids, and vitamins, among others (Javanmardi *et al.*, 2003).

Antibacterial Activity: The results of the antibacterial activity of methanol, acetone, chloroform and petroleum ether extracts of *H. salicifolia* leaf and bark are given in Table 2. The obtained antibacterial activities were categorized as follows: (a) strong: for inhibition P 70%; (b) moderate: for inhibition 50-70%; or (c) weak: for inhibition <50% (Chan *et al.*, 2007). The antimicrobial activity of the tested extracts showed different selectivity for each microorganism. The results revealed that petroleum ether extracts of

Figure 2: Percentage Inhibition Concentration For DPPH Radical Scavenging Activity of Leaf (A) and Bark (B) Extracts of Hippophae Salicifolia D. Don. Data are Presented As Mean ± SD of Triplicate Determinations



leaf and bark was found to have no activity against all tested organisms. Chloroform leaf extract exhibited moderate inhibition against *E. aerogenes*, while it showed weak inhibition against *S. aureus*, *B. subtilis, P. aeruginosa* and *K. pneumonia*. However, no activity was found against *E.coli*. Acetone leaf extract exhibited moderate antibacterial activity against *S. aureus* and *B. subtilis*, whereas chloroform and acetone bark extract showed moderate activity only against *S. aureus.* Methanolic extracts of leaf and bark showed moderate antibacterial activity against *S. aureus*, whereas showed weak inhibition against all other tested organisms.

Table 2: Antimicrobial activity of the extracts of *Hippophae salicifolia* leaf and bark (50µl/well) against the tested microorganisms based on agar well diffusion method

S.No.		Plant ExtractsInhibition zone diameter in mm (inhibition %)					
		S. aureus	*B. subtilis*	*E. aerogenes*	*P. aeruginosa*	*K. pneumonia*	*E. coli*
A. Leaves							
1.	Methanol	24 (75) ++	15 (44) +	14 (46) +	14 (42) +	12 (37) +	10 (27) +
2.	Acetone	22 (68) ++	18 (52) ++	13 (43) +	13 (39) +	12 (37) +	-
3.	Chloroform	14 (43) +	10 (29) +	20 (66) ++	14 (42) +	11 (34) +	-
4.	Pet. Ether	-	-	-	-	-	-
B. Bark							
1.	Methanol	21 (65) ++	16 (47) +	14 (46) +	14 (42) +	11 (34) +	9 (24) +
2.	Acetone	20 (62) ++	15 (44) +	13 (43) +	11 (33) +	12 (37) +	-
3.	Chloroform	13 (40) +	11 (32) +	14 (46) +	12 (36) +	9 (28) +	-
4.	Petr. Ether	-	-	-	-	-	-
C. Standard							
1.	Amoxicillin	32	34	30	33	32	37

Note: Values in parentheses are the inhibition percentages compared to standard antibacterial agent.+ Weak inhibition; ++ Moderate inhibition; - No inhibition zone.

The result of the Minimum Inhibitory Concentration (MIC) were found to be the most effective against methanolic extracts, followed by acetone, chloroform and petroleum ether extracts. *B. subtilis* and *S. aureus* was the most resistant to all the extracts, and higher MIC values were presented in Table 3.

It is worth noting that all of the extracts showed greater potent antibacterial activity against Gram-positive bacteria than Gram-negative. This result was supported by the fact that Gram-negative bacteria have an outer membrane consisting of lipoprotein and lipopolysaccharide, which is selectively permeable and thus regulates access to the underlying structures (Chopra and Greenwood, 2001). This renders the Gram-negative bacteria generally less susceptible to plant extracts than the Gram-positive bacteria (Chan *et al.*, 2007).

The antibacterial activity of the plant extracts might be attributed to the presence bioactive plant compounds such as tannins, phenolic compounds, polyphenols and flavonoids (Ouattara *et al.*, 2011). Among these bioactive compounds, Fernandez *et al.* (1996), Shoko et al. (1999) and Baydar *et al.* (2004) confirmed that phenolics were the most important active compounds against bacteria. Thus the results of antibacterial activities obtained in the present study for each of the *H. salicifolia* leaf and bark extracts were correlated to their total phenolic contents.

Table 3: The Minimum Inhibition Concentration (MIC) Values of the Extracts of *Hippophae salicifolia* Leaf and Bark Extract

S.No.	Plant Extracts	MIC with Concentration of Extract (μg/ml)					
		S. aureus	*B. subtilis*	*E. aerogenes*	*P. aeruginosa*	*K. pneumonia*	*E. coli*
A. Leaves							
1.	Methanol	250	250	250	250	500	250
2.	Acetone	250	250	250	500	500	500
3.	Chloroform	250	250	500	500	500	500
4.	Pet. Ether	ND	ND	ND	ND	ND	ND
B. Bark							
1.	Methanol	250	250	250	250	500	250
2.	Acetone	250	250	250	500	500	500
3.	Chloroform	250	250	500	500	500	500
4.	Pet. Ether	ND	ND	ND	ND	ND	ND

Note: ND- Not determined.

Positive correlations were obtained between the concentrations of phenolic compounds in the different *H. salicifolia* extracts and inhibition of all of the tested bacteria. The correlation coefficients (R^2) values of leaf extracts were found to be: 0.88 and 0.90 for the Gram positive bacteria *S. aureus and B. subtilis*; respectively whereas for the Gram negative bacteria *E. aerogenes, P. aeruginosa, K. pneumonia* and *E. coli*, the corresponding (R^2) values were 0.88, 0.71, 0.71 and 0.86. The correlation coefficient (R^2) values of bark extracts for *S. aureus and B. subtilis* were found to be 0.87 and 0.85, respectively, whereas for *E. aerogenes, P. aeruginosa, K. pneumonia* and *E. coli*, the corresponding (R^2) values were 0.74, 0.83, 0.83 and 0.86, respectively. Strong antibacterial properties may be attributed to the phenolic components in the extracts. Thus, it may be concluded that the phenolic compounds in the *H. salicifolia* extracts could be the main components which possess the antioxidant and antibacterial properties.

CONCLUSION

This study has demonstrated the antioxidant and antibacterial activities of various extracts from *H. salicifolia* leaf and bark. Methanol was a better solvent for extraction of antioxidant and antibacterial substances compared to the other solvents by providing high extraction yields and also strong antioxidant and antibacterial activities. The study revealed that the leaf and bark of *H. salicifolia* contain a considerable quantity of phenolic compounds that were found to be the major contributor for their antioxidant and antibacterial activities. Thus, the *H. salicifolia* can be considered as an easily accessible source of natural antioxidants and antibacterial agents and may be considered in future to replace synthetic preservatives in food and pharmaceutical

products. We are continuing our efforts to identify the antioxidant and antibacterial phenolic compounds in the methanolic fraction of *H. salicifolia* by further fractionation and analysis.

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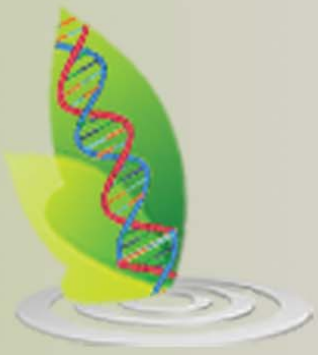

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