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Antioxidant and Antibacterial Activities of *Spondias pinnata* Kurz. Leaves

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Authors' contributions

This work was carried out in collaboration of all the authors. Author PJ planned and designed the work, interpreted the results and drafted the manuscript. Authors KRH and TRM performed experiments. Author HMR discussed the analyses, presentation of data and critically reviewed the manuscript. All the authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To evaluate the antibacterial and antioxidant potential of various extracts of *S. pinnata* leaves.
Study Design: Cold extraction of the leaves using various solvents followed by *in vitro* antioxidant and antibacterial assays.
Place and Duration of Study: Department of Pharmacy, North South University, Dhaka, Bangladesh, August 2011 to February 2012.
Methodology: Dried leaves were powdered and extracted with hexane, ethyl acetate and ethanol followed by screening for the presence of phytochemicals. The antioxidant potential of the extracts was evaluated using multiple *in vitro* models which included 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide and superoxide radical scavenging assays and reducing power estimation. Ascorbic acid and quercetin were used as the reference antioxidants. Total phenolic and total flavonoid contents were determined using Folin–Ciocalteu and aluminium chloride colorimetric method respectively. Antibacterial property of the extracts was evaluated against four gram-positive and four gram-negative bacteria using disc diffusion technique followed by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination.
Results: The extracts obtained in this study showed a varied level of scavenging activities. Ethanol extract exhibited the highest free radical scavenging activity at tested

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concentrations. High scavenging activity was also observed with ethyl acetate extract while hexane extract showed poor antioxidant activity. The reducing power increased with increasing amounts of extract in all cases. Ethanol extract displayed the highest total phenolic content (27.76±1.11 mg GAE/g extract) while the highest flavonoid content was shown by ethyl acetate extract (86.53±1.95 mg QE/ g extract). Again, all the extracts showed antibacterial activity with zone of inhibition ranging from 8.33±1.53 to 28.67±0.58 mm. The Lowest MIC and MBC values were found with ethanol extract which was 2.0 and 3.5 mg/ml respectively against *Staphylococcus aureus*.
Conclusion: The results suggest that extracts of *S. pinnata* leaf may serve as a potential source of natural antioxidant and antibacterial agents for pharmaceutical application.

Keywords: Antioxidant; antibacterial activity; S. pinnata; phytochemical screening.

1. INTRODUCTION

Natural products, particularly of plant origin, are gaining much importance in therapeutical applications due to their fewer side effects and lesser toxicity as compared to synthetic drugs. Moreover, long-term use of synthetic drugs often leads to development of resistance [1]. World Health Organization estimates that around 65 - 80% of the world population relies on plant based preparations for maintaining health and combating diseases [2]. Therapeutic properties of plant based natural products are attributed to the presence of a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, etc. To date, many plants have been evaluated for possible antimicrobial and antioxidant activities and compounds isolated from them have shown great potential in the treatment of various infectious and degenerative diseases [3]. Still, extensive search for natural antioxidants that could minimize free radical induced damage to biomolecules like lipids, proteins and nucleic acids is going on world over. These natural antioxidants might play an important role in combating oxidative stress associated diseases such as cancer, atherosclerosis, aging, immunosuppression, inflammation, ischemic heart disease, diabetes, hair loss, and neurodegenerative disorders such as Alzheimer's and Parkinson's disease [4-6]. Again, the development of antibiotic resistance by pathogenic organisms to conventional drugs has necessitated the search for novel bioactive molecules with antimicrobial properties [7]. From about 250,000 species of plants worldwide, all with potential to obtain natural products for the development of new drugs, only 1% of tropical species have been studied for their pharmaceutical or therapeutic potential [8].

Spondias pinnata (Linn.f.) Kurz (Family– Anacardiaceae) is a medium-sized deciduous tree with imparipinnate leaves, polygamous flowers and ovoid greenish-yellow fruits. This plant is found mainly in Bangladesh, India, Sri Lanka and South-East Asian countries. People in these regions have been using this plant traditionally in the treatment of infectious diseases like bronchitis, ulcer, dysentery and skin diseases [9, 10]. Its roots, bark, leaves and fruits are used in traditional medicine [11]. The bark is aromatic, astringent and refrigerant and used in dysentery, diarrhea, vomiting and muscular rheumatism. Fruits are useful in bilious dyspepsia, diarrhea and general debility. Leaves of this plant are aromatic, acidic and astringent and find use in dysentery. Ethanolic extract of pulp of *S. pinnata* has been reported to show antimicrobial activity [12]. Antioxidant effects of methnolic extract of *S. pinnata* bark has been studied by Hazra et al. [13]. A crude extract of *S. pinnata* has been reported to show antibacterial activity [14]. Gupta et al. [15] reported antimicrobial activity of resin of *Spondias pinnata.* However, no extensive report is found on the antioxidant and antibacterial properties of the leaves of this plant. The objective of the present study was to

investigate the antioxidant and antibacterial properties of *S. pinnata* leaves using various extracts *in vitro*.

2. MATERIALS AND METHODS

2.1 Sample Collection

Fresh leaves of *Spondias pinnata* plant were collected from a farmland area in Faridpur, Bangladesh. Authentication was done at Bangladesh National Herbarium, where voucher specimen (Accession No.35357) was deposited.

2.2 Extract Preparation

The leaves were separated from undesirable materials and washed thoroughly with distilled water to remove debris and dust particles. Cleaned leaves were air-dried under shade at room temperature to constant weight. Dried samples were then powdered using a laboratory scale mill and blender. Ground material (100 g) was extracted independently with 500 ml of hexane, ethyl acetate and ethanol. The extraction was done for 5 days with occasional shaking. The obtained extracts were filtered using filter paper (Whatman No. 1) and each filtrate was then evaporated to dryness in a rotary evaporator. All the extracts were kept stored at 4ºC in a refrigerator until required for further analyses.

2.3 Phytochemical Screening

To identify the chemical constituents present in the extracts, standard phytochemical screening was carried out [16,17]. Alkaloids test was performed with Wagner's reagent, flavonoids with conc. HCl, gum and carbohydrate with Molisch's reagent, saponin by frothing test, tannins by $FeCl_3$ and terpenoids by Salkowski test.

2.4 Determination of Total Phenolic Content

Total phenolic content (TPC) in the extracts was determined spectrophotometrically according to the Folin–Ciocalteu procedure [18]. Briefly, 1.5 ml Folin–Ciocalteu's reagent (diluted 1:10) and 1.2 ml of 7.5% (w/v) Na_2CO_3 were added to 0.3 ml of the extract. After incubation at room temperature for 1 h in dark, the absorbance was measured at 765 nm against blank. The total phenolic content was evaluated from a gallic acid standard curve and the results were expressed as gallic acid equivalents (GAE) in milligrams per gram extract.

2.5 Determination of Total Flavonoid Content

Total flavonoid content of the extracts was determined by aluminium chloride colorimetric method [19]. 1.5 ml of methanol, 0.1 ml of aluminium chloride (10%), 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water were mixed with 0.5 ml of extract solution. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with spectrophotometer. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Quercetin was used to make the calibration curve and the results were expressed as quercetin equivalents (QE) in milligrams per gram extract.

2.6 Antioxidant Activity

2.6.1 DPPH radical scavenging assay

The free radical scavenging activity of the extracts was measured in terms of hydrogen donating or radical scavenging ability of the stable 1, 1-diphenyl-2- picrylhydrazyl (DPPH) free radical [20]. Briefly, 0.1 ml of plant extract at various concentrations (0-200 µg/ml) was added to 3 ml of a 0.002% methanolic solution of DPPH. The reaction mixtures were incubated for 30 min at room temperature and the absorbance at 517 nm was measured against a blank. A low absorbance of the reaction mixture indicated a high free radical scavenging activity. Ascorbic acid was used as standard in the experiment. The radical scavenging activity was calculated using the following formula:

$$\text{Percentage of inhibition} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$$

2.6.2 Nitric oxide radical scavenging activity

Nitric oxide radicals generated in aqueous sodium nitroprusside solution at physiological pH interact with oxygen to produce nitrite ions, which were measured by the Griess Illosvoy reaction [21]. Briefly, 3 ml of the reaction mixture containing 10 mM sodium nitroprusside and the extract solutions (0 –200 µg/ml) in phosphate buffer were incubated at 25°C for 150 min. This was followed by addition of 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) to 0.5 ml of the incubated solution. The mixture was allowed to stand for 5 min followed by mixing with 1 ml of 0.1% naphthyl ethylene diamine dihydrochloride (NED) and incubation at 25°C for 30 min. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against the corresponding blank solutions. Ascorbic acid was used as a standard. The ability to scavenge the nitric oxide radical is expressed as % inhibition and calculated using the following equation:

$$\text{Percentage of inhibition} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$$

2.6.3 Scavenging of superoxide anions

The superoxide anion radical scavenging activity of the extracts was assayed by the inhibition of nitro blue tetrazolium (NBT) reduction by NADH in the presence of phenazine methosulfate (PMS) [22]. Reaction mixtures containing 73 mM NADH, 15 mM PMS, 50 mM NBT in 20 mM phosphate buffer, pH 7.4 and the samples at various concentrations were incubated for 5 min at room temperature. Finally, absorbance at 560 nm was measured against blank samples. Quercetin was used as a positive control. Decreased absorbance of the reaction mixture indicates increased superoxide radical scavenging activity. The % inhibition of superoxide radical generation was calculated using the following formula:

$$\text{Percentage of inhibition} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$$

2.6.4 Total reducing power

Total reducing capacity of the prepared extracts was determined according to the method of Oyaizu [23]. Briefly, 1ml of the extract at different concentrations was added with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% (w/v) solution of potassium ferricyanide. The resulting mixtures were incubated in a water bath at 50°C for 20 min followed by the

addition of 2.5 ml of trichloroacetic acid (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min. A 2.5 ml aliquot of the upper layer was combined with 2.5 ml of distilled water and 0.5 ml of a 0.1% (w/v) solution of ferric chloride. The absorbance was measured at 700 nm with a spectrophotometer. Ascorbic acid was used as positive control. The higher the absorbance of the reaction mixture the greater is the reducing power. All tests were carried out in triplicates.

2.7 Antibacterial Activity

2.7.1 Antibacterial susceptibility test: disc diffusion assay

Antibacterial activity of the extracts was determined by paper disc diffusion (Kirby-Bauer) method [24] against four gram-positive (*Bacillus subtilis, Bacillus cereus, Staphylococcus aureus and Sarcina lutea*) and four gram-negative (*Salmonella typhi*, *Pseudomonas aeruginosa, Shigella boydii and Vibrio mimicus*) bacteria. The strains were maintained on agar slant at 4°C and activated at 37°C for 24 h on nutrient agar prior to any screening. For all the bacterial strains, overnight cultures grown in broth were adjusted to an inoculum size of approximately 10^6CFU/ml following a 0.5 McFarland turbidity standard. Using a sterile cotton swab, the nutrient broth cultures were swabbed on the surface of sterile Müeller-Hinton agar plates and the plates were allowed to dry for 5 min. Sterile filter paper discs (6 mm in diameter) impregnated with different test extracts (1 mg/disc) were then placed on the surface of seeded agar plate. The plates were then incubated at 37°C for 24 h. Antibacterial activity was evaluated by measuring the zone of inhibition in millimeter. Commercially available kanamycin discs (30 µg/disc) were used as positive control while the discs prepared using the solvent (DMSO) only served as negative control.

2.7.2 Determination of MIC and MBC

The minimum inhibitory concentration (MIC) considered as the lowest concentration of the sample which inhibits the visible growth of a microbe was determined by the broth dilution method in Mueller Hinton broth supplemented with 10% glucose and 0.5% phenol red. Various dilutions were prepared from the stock solution of crude extracts to give concentrations ranging from 10 to 0.5 mg/ml. Each tube was inoculated with an overnight culture of strains diluted to give a final concentration of 10^6 CFU/ml. The culture tubes were then incubated aerobically at 37°C for 24 h and the MIC values were recorded as the lowest concentration that inhibits the visible bacterial growth or changed in color from red to yellow due to the formation of acidic metabolites corresponding to microbial growth [25]. Culture medium without sample extract and the others without microorganisms were used as controls in the study. The minimum bactericidal concentration (MBC) was determined by spreading a loop full of the culture medium from broth MIC assay sample on fresh Müeller-Hinton Agar (MHA) plates. After incubation at 37°C for 24 h, the MBC was recorded as the lowest concentration of the test sample showing no bacterial growth on the MHA plates [26].

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

Qualitative phytochemical analysis of *S. pinnata* leaf extracts revealed the presence of various bioactive constituents in hexane, ethyl acetate and ethanol extracts (Table-1).

Table 1. Phytochemical analysis of the various extracts of *S. pinnata* leaves

Extract	Flavonoids	Tannins	Gum & Carbohydrates	Alkaloids	Saponins	Terpenoids
Hexane	+	-	+	+	-	+
Ethyl acetate	+	+	+	+	-	+
Ethanol	+	+	+	+	+	+

(+) indicates presence and (-) indicates absence of phytochemicals

3.2 Total Phenol and Flavonoid Content

Different studies suggest that different types of polyphenolic compounds such as flavonoids, phenolic acids which are found in plants have multiple biological activities, including antibacterial and antioxidant activities. Antioxidant activity of phenolic compounds is attributed to their ability to donate hydrogen atoms to free radicals [27]. In addition, they possess the structural properties of free radical scavengers which enable them to serve as potential antioxidants [28]. Total phenolic content of the extracts in this study was evaluated using Folin-Ciocalteu method which measured redox properties of polyphenols [29]. Total phenolic content present in the extracts ranged from 12.43 ± 0.08 to 27.76 ± 1.11 mg GAE/g extract and total flavonoid content ranged from 39.11 ± 1.42 to 86.53 ± 1.95 mg QEE / g extract (Table 2). Ethanol extract showed the highest phenolic content whereas the highest flavonoid content was obtained with ethyl acetate extract. The variation in the extraction capacity of the solvents could be due to the polarity of the solvents used as also reported in some previous studies [30,31].

Table 2. Total phenolic and flavonoid content of various extracts of *S. pinnata* leaves

Extract	Total Phenolic Content mg GAE / g extract	Total Flavonoid Content mg QEE / g extract
Hexane	12.43 ± 0.08	39.11 ± 1.42
Ethyl acetate	21.97 ± 0.95	86.53 ± 1.95
Ethanol	27.76 ± 1.11	76.03 ± 2.54

Results are expressed as mean± SD (n=3)

3.3 Antioxidant activity

3.3.1 DPPH free radical scavenging

The antioxidant activity of the extracts was determined using DPPH method because it is one of the most effective methods for evaluating radical-scavengers. The DPPH radical contains an odd electron which is responsible for the absorbance at 517 nm and also for a visible deep purple color. The decolourisation of the purple reaction solution is stoichiometric with respect to number of electrons donated by antioxidant compounds [7]. Fig. 1 shows the dose- response curve of DPPH radical scavenging activity of different extract, compared with standard. The results showed that ethanol extract has the highest scavenging activity, which was followed by ethyl acetate extract at all the tested concentrations. The lowest scavenging activity was shown by hexane extract. At a concentration of 150 µg/ml, the scavenging activity of the ethanol extract reached 85.3±3.05%, while at the same concentration, that of the hexane extract was 66.0±1.0%.

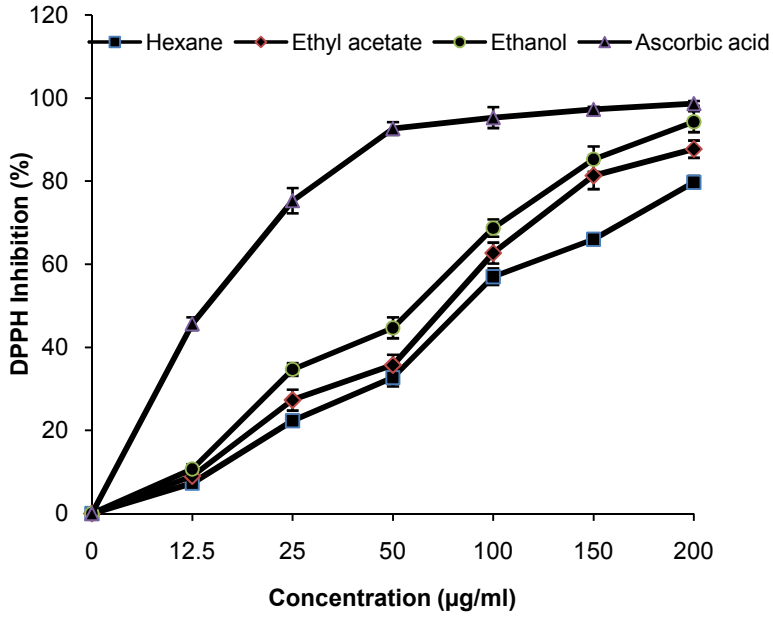

Fig. 1. DPPH radical scavenging activity of the various extracts of *S. pinnata* leaves in comparison to ascorbic acid.
Results are expressed as mean± SD (n=3)

3.3.2 Nitric oxide radical scavenging activity

$NO^{\bullet}$ radicals play an important role in inducing inflammatory response and their toxicity multiplies only when they react with $O_2^{\bullet-}$ radicals to form peroxynitrite, which damages biomolecules like proteins, lipids and nucleic acids [32]. Nitric oxide is generated when sodium nitroprusside reacts with oxygen to form nitrite. Fig. 2 shows the $NO^{\bullet}$ scavenging activity of the extracts tested. All the extracts exhibited $NO^{\bullet}$ scavenging effect in a dose-dependent manner. Out of the three extracts investigated, ethanol extract showed the highest percentage of nitric oxide inhibition. This was followed by ethyl acetate extract and hexane extract. At concentration of 100 µg/ml, the scavenging activity of the ethanol extract was comparable to that of ascorbic acid. The results suggest that *S.pinnata* leaf extract particularly ethanol extract might serve as a potent and novel therapeutic agent for scavenging of $NO^{\bullet}$ and the regulation of pathological conditions caused by excessive generation of $NO^{\bullet}$ and its oxidation product, peroxynitrite.

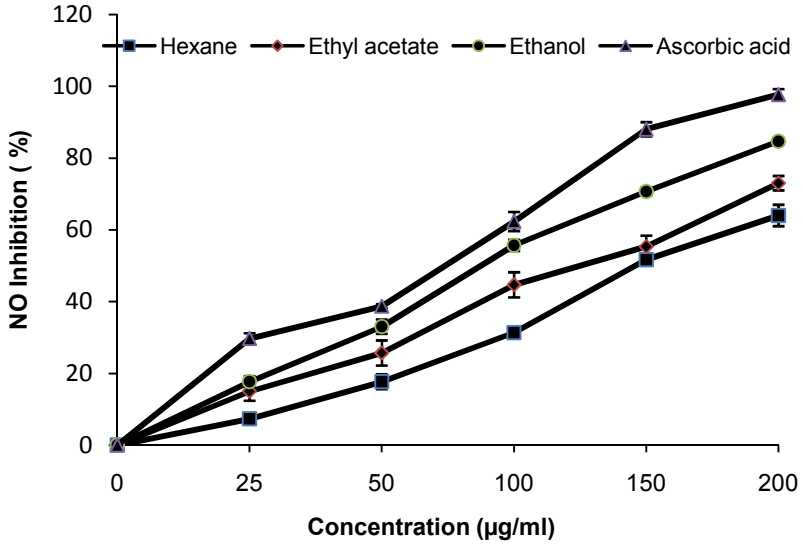

Fig. 2. Scavenging effect of *S. pinnata* leaf extracts and ascorbic acid on Nitric Oxide radical.

Results are expressed as mean± SD (n=3)

3.3.3 Superoxide radicals scavenging assay

It is well known that superoxide anions damage biomolecules directly or indirectly by forming peroxide, hydroxyl, peroxy nitrite or singlet oxygen during aging and other pathological events. Superoxide has also been observed to directly initiate lipid peroxidation [33]. Figure 3 illustrates the % inhibition by all the extracts in the following order: ethanol > ethyl acetate > hexane. The superoxide radical scavenging activities of the extracts and the reference compound increased markedly with increasing concentrations (Fig. 3). At a concentration of 200 µg/ml, ethanol extract exhibited the highest inhibition of about 91.67±1.53% but this is lower than the standard quercetin whose % of inhibition is 97±1.0%.

3.3.4 Reducing power

The reducing power of a compound is related to its electron transfer ability and may therefore; serve as an indicator of its potential antioxidant activity [34]. In the reducing power assay, a higher absorbance indicates a stronger reducing power. In this study we observed a concentration-dependent increase in the absorbance of reaction mixture for all the solvent extracts and the standard compound ascorbic acid (Fig. 4). Ethanol extract showed the highest reducing power at all the tested concentrations. This was followed by ethyl acetate extract, while the hexane extract showed the lowest reducing power. The results show that, at concentration of 250 µg/ml, reducing capacity of ethanol extract (absorbance 0.789±0.007) was comparable to that of the standard ascorbic acid (absorbance 0.856±0.015). The observed reducing ability of the plant extracts could be attributed to the presence of hydrophilic polyphenolic compounds [31]. These results are in line with the previous studies

which reported that the reducing power of plant extracts correlate with the phenolic content [31,35].

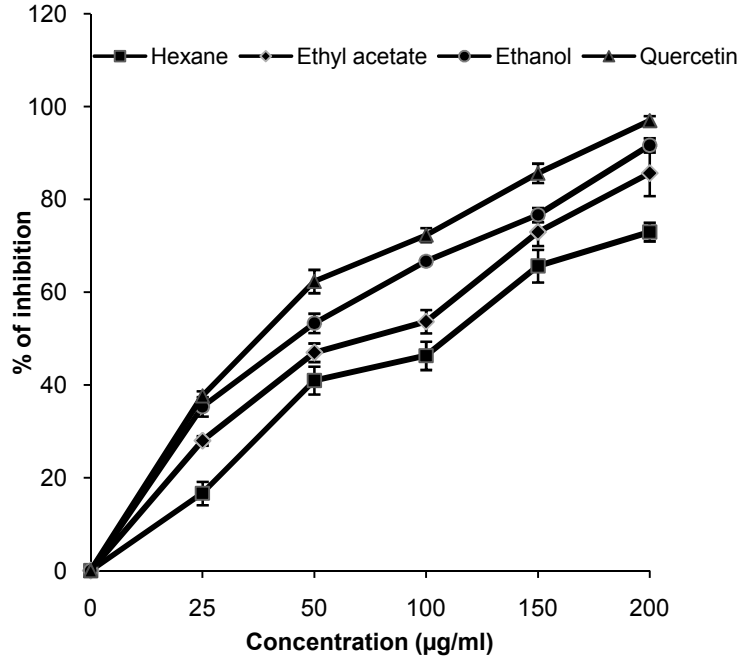

Fig. 3. Superoxide radical scavenging activity of *S. pinnata* leaf extracts and standard quercetin.

Results are expressed as mean± SD (n=3)

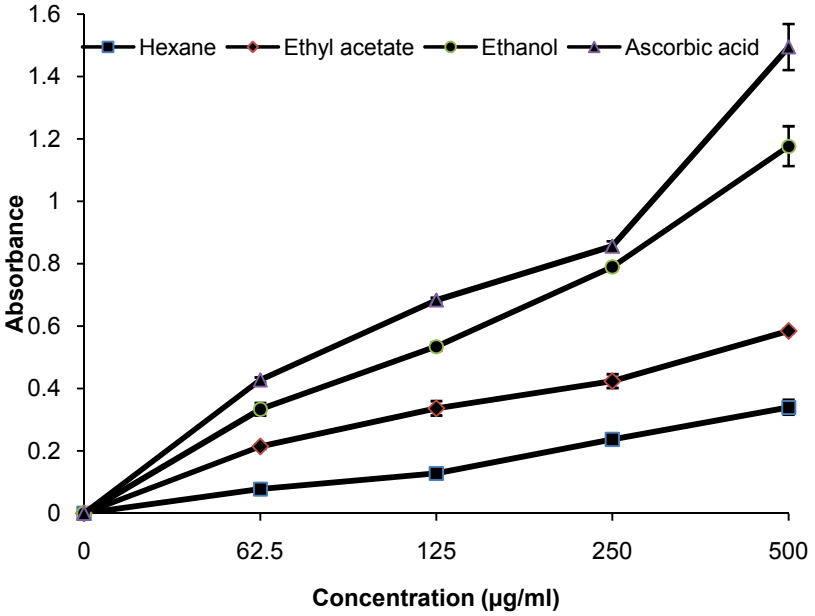

Fig. 4. Reducing ability of different *S. pinnata* leaf extract and standard ascorbic acid.

Results are expressed as mean± SD (n=3)

3.4 Antibacterial Activity

Antibacterial activity of all the extracts of *S.pinnata* leaf has been evaluated *in vitro* against gram-positive and negative bacteria by disc diffusion method. All the extracts exhibited significant antibacterial activity against a number of pathogenic bacteria (Table-3). Maximum antibacterial activity was recorded by hexane extract with 28.67±0.58 mm of clear inhibition zone against *S. aureus*. The standard drug, kanamycin showed zones of inhibition ranging from 26.67±1.15 to 34.00±0.00 mm against all the test organisms. All the extracts were found to be less effective than the standard antibiotic kanamycin. In a negative control, DMSO had no inhibitory effect on the tested organisms.

MIC and MBC values were determined taking different extracts that showed the highest zone of inhibition in disc diffusion assay against respective organisms. Hexane extract showed MIC and MBC values of 3 and 4.5 mg/ml respectively against *Staphylococcus aureus* while ethyl acetate extract exhibited MIC and MBC values of 3.5 and 5 mg/ml respectively against *Salmonella typhi.* MIC and MBC value of ethanol extract obtained from *S.pinnanta* leaf was found to be 2 and 3.5 mg/ml respectively against *S. aureus.* Findings from this study suggest that *S.pinnata* leaf extracts can be exploited as a natural drug for the treatment of several infectious diseases. The results of this study and some previous reports [12, 14, 15] support the traditional uses of *S.pinnata* as a therapeutic agent for diarrhea, dyspepsia and gastrointestinal disorders. However, further studies are needed to evaluate the antibacterial activity of the specific molecules present in the extract against pathogenic bacterial strains.

Table 3. Antibacterial activity of the various extracts of *S. pinnata* leaves using disc diffusion method

Bacteria	Zone of Inhibition(mm)			
	Hexane	**Ethyl acetate**	**Ethanol**	**Standard (Kanamycin)**
Gram-positive				
Bacillus subtilis	8.33±1.53	11.00±0.00	19.0±1.73	33.0±0.00
Bacillus cereus	-	-	12.67±2.31	30.0±2.0
Staphylococcus aureus	28.67±0.58	18.67±1.15	24.67±1.53	30.67±1.53
Sarcina lutea	25.00±1.73	17.00±0.00	9.33±1.15	26.67±1.15
Gram-negative				
Salmonella typhi	25.33±1.15	23.00±0.00	18.67±1.15	32.67±1.00
Pseudomonas aeruginosa	22.00±0.00	16.00±1.73	21.0±1.00	28.67±1.53
Shigella boydii	23.00±0.00	21.33±1.15	21.33±1.15	34.00±0.00
Vibrio mimicus	21.33±1.15	17.00±0.00	18.33±1.53	29.00±0.00

Values are mean± SD (n=3), (-) indicates no inhibition

4. CONCLUSION

Based on our results, it can be concluded that *S. pinnata* leaves possess significant antibacterial and antioxidant activity which could be attributed to the high phenolic and flavonoid contents. Further research is needed towards isolation and identification of active principles present in the extracts. Such screening of various natural compounds and purification of potential lead molecules could possibly be exploited for pharmaceutical use.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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