Investigation of In Vitro Antioxidant, Antimicrobial and Thrombolytic Activity of the Exocarp of Spondias pinnata (Anacardiaceae)

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Received: August 15, 2013 Revised: September 17, 2013 Accepted: September 17, 2013 Published: September 17, 2013

Abstract: The aim of the current study was to evaluate the antioxidant, antimicrobial and thrombolytic potentials of the exocarp of Spondias pinnata fruits. The crude ethanolic extract of the exocarp of S. pinnata fruit was partitioned successively by solvents of different polarity. All fractions were then investigated for qualitative preliminary phytochemical screening by specific standard procedure. The antioxidant potential of all fractions were then evaluated in terms of total phenolic content, total flavonoid content, DPPH free radical scavenging potential, reducing potential and total antioxidant capacity by specific standard procedure. The disc diffusion method was incorporated to evaluate the in vitro antimicrobial activity on nutrient agar medium. The highest total phenolic content was found in aqueous fraction (570.20±0.48 mg GAE/g of dried extract) while the lowest in the n-Hexane fraction (337.51±0.21 mg GAE/g of dried extract). However, ethyl acetate fraction exhibited the highest flavonoid content which amounted to 132.27±0.25 mg quercetin equivalents/g of dried extract. Likewise, ethyl acetate fraction showed the highest antioxidant capacity (21.61±0.11 g of L-ascorbic acid equivalents/g of dried extract) along with the lowest IC₅₀ (1.72±0.39 µg/ml) & EC₅₀ (2.25±0.75 µg/ml) value. However, DPPH free radical scavenging activity and reducing power of all fractions were found to be concentration dependent. Nonetheless, comparatively more polar fractions (ethyl acetate and aqueous fraction) were found to be ineffective against all the microbial strains except S. dysentery and P. aeruginosa while non-polar fractions (n-hexane and dichloromethane fraction) showed variable antimicrobial activity. In addition, all fractions produced statistically significant (P<0.05 for ethyl acetate and aqueous fraction, P<0.001 for others) thrombolytic activity. To conclude, our present study suggested that exocarp of S. pinnata fruit exhibits antimicrobial activity against a wide variety of strains while it produces noteworthy...
antioxidant and significant thrombolytic activity.

**Keywords:** *S. pinnata*, Antioxidant, Folin-Ciocalteu reagent, DPPH, Antimicrobial, Thrombolytic.

### 1. INTRODUCTION

The interest in investigating the medicinal values of plants in order to explore new arsenals against the threat of new and recent diseases is mounting constantly throughout the world especially in Asia. Various features of plant i.e. economic feasibility, low toxicity, fewer side effects and potent pharmacological activity contribute to their increasing popularity [1]. However, numerous lead compounds were discovered from plants till now. In addition, more than 30% of global pharmaceutical preparations are plant based [2].

Although oxidation is essential to many living organism to furnish energy to biological processes, continuous generation of oxygen-centered free radicals and other reactive oxygen species *in vivo* causes cell death and tissue damage [3]. Oxidative damage done by free radicals causes the pathogenesis of many deadly diseases like cancer, Alzheimer’s & diabetes [4]. Along with the nutritional value, plants contribute to the protection from free radical deterioration by hindrance of lipid peroxidation via numerous mechanisms including scavenging free radicals, inducing antioxidant enzymes, modulating protein kinase, and lipid kinase signaling pathway, inhibiting cyclooxygenase-2 (COX 2) and matrix metalloproteinases (MMP 2 and MMP 9) enzymatic activity, influencing phytoestrogenic and nuclear transcription factor NF-κB, inducing cell cycle arrest and phase 1 or phase 2 metabolizing enzymes [5].

Despite of the availability of several synthetic antioxidants e.g. butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), their use is restricted because of their carcinogenicity and other side effects. Therefore, nowadays, natural products and their potential as antioxidants are of considerable interest for researchers [6]. Nonetheless, jeopardous multiple drug resistance occurring from unorganized and irregular administration of commercial antimicrobial drugs, and adverse effects like hypersensitivity, allergic reaction, immune suppression, abdominal pain, anorexia etc drive the scientists to explore new and effective antimicrobial agents that could be a better alternative of the current regimens [7-9]. However, the role of plant secondary metabolites in defending the plant against microorganisms made plant a potential source of active compounds against microorganism [10]. Thrombosis can block the normal blood circulation and thereby can cause infarction and necrosis. However, more or less all the currently available thrombolytic regimens have few vital shortcomings including limited fibrin specificity, noteworthy bleeding tendency and large dose requirement. Therefore, currently researchers shift their focus on natural resources to find more effective alternative regimen which can counter the dilemma [11-13].

*Spondias pinnata* (Linn. F.) Kurz, also known as *S. mangifera* is a small aromatic, deciduous tree (Anacardiaceae) which is found wild or cultivated throughout the Indian subcontinent and in the Andamans up to an altitude of 1500 m in Himalayas [14]. However, *S. pinnata* is widely available in Bangladesh especially wild in the forests of Chittagong, Cox’s Bazar, Tangail, Sylhet and Dinajpur. The plant is familiar in several names in different languages like hog-plum, wild mango (English), amra (Bengali), mambulichi (Tamil), jangali aam (Hindi) etc. Indian Ayurveda called it as amrataaka, markatamra [14,15]. The plant is known to have several medicinal properties. Resin extract was found to be active against gram positive bacteria [16]. The fruit, leaf and bark of the tree exhibit astringent,
antidysenteric, antiseptic, antiscorbutic properties [15]. In ethnomedicine, equal quantities of bark juice of *S. pinnata* and *Syzygium cumini* are prescribed as a remedy for dysentery [17]. Both bark and root are used in menstrual disorder [15,18]. The Ayurvedic pharmacopeia of India also recommends the use of stem bark in hemorrhagic disease. Wetwitayaklung *et al* reported that young shoot are eaten as vegetable, fruits are used as vitamin C supplement and antithirst, and bark is used as anti-vomit and diuretic [19]. The report also revealed that bark is used in diarrhea and root is used as anti-thirst [19]. The fruit contains β-amyrin, oleanolic acid and some amino acids including glycine, cysteine, serine, alanine and leucine. Aerial parts contain lignoceric acid, 24-methyleneclactonone, stigmast-4-en-3-one, beta-sitosterol and its glucoside [15].

Because of its copious and widespread availability, the objective of the study was to investigate the potential of the exocarp (peel) of *Spondias pinnata* fruit extract as antioxidant, antimicrobial and thrombolytic agent. Furthermore, the report of previous studies [19-21] on antioxidant and antibacterial activities conducting using fruits of *S. pinnata* influenced us to explore similar type of activities from the peel of the fruit. Prior to the evaluation of antioxidant, antimicrobial and thrombolytic activity, preliminary phytochemical screening of the extract was done.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH), L-Ascorbic acid, Gallic acid, Quercetin, TCA (trichloroacetic acid), ferric chloride and Folin-Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Analytical grade n-Hexane, dichloromethane, methanol, ethanol and ethyl acetate were purchased from BDH, E Merck. Cephradin and Nystatin were purchased from Square Pharmaceuticals Ltd., Bangladesh. Streptokinase was purchased from Beacon pharmaceutical Ltd, Bangladesh. All other chemicals and reagents including ammonium molybdate were Merck, Germany.

2.2 Collection and Preparation of Plant Material

Fresh fruits of *Spondias pinnata* were collected from Mirzapur (Tangail) in August 2011 and identified by Bangladesh National Herbarium. A voucher specimen was submitted to both Bangladesh National Herbarium and East West University for future reference. The separated, air dried and powdered exocarps of *S. pinnata* fruits were macerated in ethanol for one week. The extracts were then filtered through a cotton plug followed by the filtration through Whatman no. 1 filter papers. The filtrate of the plant material was dried by using rotary evaporator (RV10 Basic, IKA, Germany) at low temperature and pressure. The dried crude extract (14 gm) was then partitioned successively with n-Hexane, dichloromethane and ethyl acetate by using modified Kupchan partitioning method [22]. The resultant partitionates i.e. n-hexane (SPH), dichloromethane (SPD), ethyl acetate (SPE) and aqueous (SPA) soluble fractions were stored at 4°C until used for experimental process.

2.3 Phytochemical Screening

All the extracts were qualitatively tested for the presence of alkaloids (Dragendorff’s reagent), flavonoids (cyanidine reaction), steroids (Salkowski test), reducing sugars (Fehling reagent), saponins (Frothing test), tannins (iron chloride) and cardiac glycosides (Keller-Killani test) [23-25].

2.4 In vitro Antioxidant Activity

2.4.1 Total Phenolic Content Assay

Folin-Ciocalteu method [26,27] was used to determine the total phenolic content of the partitionates; 0.5 ml of a methanol solution of the fractions having concentration of 1 mg/ml was mixed with 5 ml Folin ciocalteu reagent (1:10 v/v distilled water) and 4 ml (75g/L) of Sodium carbonate. The
mixtures were vortexed for 15 seconds and allowed to stand for 30 min at room temperature in dark place for color development and the absorbance was measured at 760 nm against methanol as blank by using a UV-visible spectrophotometer. All the tests were carried out in triplicate and average absorption was noted for each time. The total phenolic content was calculated by using the standard Gallic acid calibration curve and expressed as mg of GAE (gallic acid equivalents)/gm of the dried extract.

2.4.2 Total Flavonoid Content Assay

The total flavonoid content in each fraction of *S. pinnata* was measured by Aluminum chloride (AlCl₃) colorimetric method [28]. 0.5 ml of methanol solution of each extract of concentration of 10 mg/ml was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. The blank was prepared in similar fashion by replacing aluminum chloride with distilled water. Both sample and blank solution were filtered through double rings filter paper. After an incubation period of 30 min, the absorbance was measured at 415 nm against the blank by using a UV-visible spectrophotometer. The total flavonoid contents were expressed as quercetin equivalents in mg/g of dry weight.

2.4.3 DPPH Free Radical Scavenging Activity:

The free radical scavenging activity of each fraction of *S. pinnata* was estimated using stable free radical of DPPH [29]. 2.0 ml of methanol solution of each extract at different concentration (2, 4, 6, 8, 10μg/ml) were mixed with 3.0 ml of DPPH methanol solution (20 μg/ml). After an incubation period of 30 min, the absorbance was measured at 517 nm against methanol as blank by using a UV-visible spectrophotometer. The radical scavenging activity (%) was calculated based on the following formula:

\[
\text{DPPH scavenging activity} \% = \left( \frac{A_B - A_T}{A_B} \right) \times 100
\]

where \(A_B\) and \(A_T\) are the absorbance of blank and plant material respectively.

The percentage scavenging activity of each extract was compared with L-Ascorbic acid, the positive control. IC₅₀ value of each extract was determined from the plotted graph of percentage DPPH neutralization versus concentration of extract, which is defined as the amount of antioxidant required to reduce the initial DPPH free radical concentration by 50%.

2.4.4 Reducing Power Assay:

The reducing power of all the extracts of *S. pinnata* were measured by the method previously described by Oyaizu [30]. 1 ml of each extract of varying concentrations (1, 5, 10, 50, 100μg/mL) in double distilled water was mixed with 2.5 ml phosphate buffer and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min after which, 2.5 ml of 10% trichloroacetic acid (TCA) was added and centrifuged at 3000 rpm for 10 min. From each tube, 0.5 ml of the supernatant upper layer was mixed with 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride. Incubation with water in place of additives was used as blank while L-Ascorbic acid was used as positive control. The absorbance was measured at 700 nm by using a UV-visible spectrophotometer. Increased absorbance of the reaction mixture suggests increasing reducing power. The reducing power (%) was calculated based on the following formula:

\[
\text{Reducing power} \% = \left( \frac{A_T - A_B}{A_B} \right) \times 100
\]

where \(A_B\) and \(A_T\) are the absorbance of blank and plant material respectively. Effective concentration, EC₅₀ value of each extract was estimated from the plotted graph of percentage reducing power versus concentration of extract.

2.4.5 Total Antioxidant Capacity:
The total antioxidant capacity of all the extracts of S. pinnata were estimated by phosphomolybdenum method [31]. 0.2 ml of each extract of concentration of 0.5 mg/ml was mixed with 2 ml of reagent solution (600 mM Sulfuric acid, 28mM Sodium Phosphate and 4mM Ammonium molybdate). The reaction mixtures were then incubated at 95°C for 1 hour. The absorbance was measured at 695 nm against a blank containing 3 ml of reagent solution by using a UV-visible spectrophotometer. The total antioxidant activity of the crude extract was expressed as L-ascorbic acid equivalents in g/g of dry weight.

2.5 Antimicrobial Assay:

Disc diffusion method [32-34] was used to determine the antimicrobial activity of the extracts on nutrient agar medium. Thirteen reference microbial strains (four gram positive, seven gram negative and two fungi) were used as the test organisms which were collected from East West University microbiology lab. The test organisms were inoculated on 10 ml previously sterilized nutrient agar media, mixed thoroughly and transferred immediately to the sterile petri dish under an aseptic condition using a sterile loop. Two doses of each extract (400 µg/disc and 800 µg/disc) were infused in dried and sterilized filter paper discs, which were then placed gently on the previously marked zones in the agar plates. Standard discs of cephradine (30 µg/disc) and nystatine (30 µg/disc) were used as the positive controls for bacteria and fungus respectively while distilled water was used as the negative control. After incubation at 37°C for 24 hours, the antimicrobial activity of the test materials were determined by measuring the diameter of clear zone of inhibition around the point of application of sample solution expressed in mm.

2.6 Thrombolytic Activity:

The in vitro thrombolytic potential of each extract of S. pinnata was evaluated by the method developed by Daginawala using streptokinase as the standard substance [35,36]. 5 mL venous blood drawn from each of the six healthy volunteers (three male and three female) without a history of oral contraceptive or anticoagulant therapy was transferred to 6 pre-weighed sterile eppendorf tubes (500 µL/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completely aspirated out without disturbing the clot formed and the weight of clot in each tube was measured. To each eppendorf tube containing pre-weighed clot, 100µl aqueous solution of different extracts of concentration of 10 mg/ml was added separately. Then, 100µl of streptokinase (SK) and 100µl of distilled water were separately added to the control tube as positive and negative controls respectively. All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, the released fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

\[
\text{% of clot lysis} = \left( \frac{\text{wt of released clot}}{\text{clot wt}} \right) \times 100
\]

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening:

Preliminary phytochemical screening, described in Table 1 revealed the absence of reducing sugar and saponin in all the fractions whereas other phytochemical constituents are present.

Table 1: Phytochemical Screening of Different Fractions of the Exocarp of Spondias Pinnata Fruit
3.2 Total Phenolic Content Assay:
The total phenolic contents of various fractions of the exocarp of *S. pinnata* fruit were expressed as mg of gallic acid equivalent/gm of dried extract and are presented in Table 2. The Folin-Ciocalteu reagent used in the total phenolic content assay reacts with phenols and nonphenolic reducing substances to form chromogens that can be detected spectrophotometrically. In total phenolic content assay, the amount of phenolic and polyphenolic antioxidants required to inhibit the oxidation of the Folin-Ciocalteu reagent is measured [37]. Among all the fractions, the aqueous fraction showed the highest amount of phenolic compounds followed by ethyl acetate, dichloromethane and n-hexane soluble fractions. The total phenolic content varied widely from fractions to fractions in the range of 337.51 to 570.20 mg of gallic acid equivalents/gm of dried extract.

3.3 Total Flavonoid Content Assay:
Total flavonoid contents of various fractions of the exocarp of *S. pinnata* fruit were expressed as mg of quercetin equivalents/gm of dried extract and are also presented in Table 2. Total flavonoid content by AlCl3 method is based on the determination of the flavonoid-aluminium complex between flavonoid of the crude extract and aluminium chloride. Since all the fractions exhibit positive results for flavonoid in the preliminary phytochemical screening, the same reflects in the analysis of total flavonoid content. However, ethyl acetate fraction exhibited the highest flavonoid content while dichloromethane fraction was found to contain the lowest flavonoid content.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Phenolic content (mg of GAE/gm of dried extract)</th>
<th>Total Flavonoid content (mg of quercetin equivalents/gm of dried extract)</th>
<th>DPPH radical scavenging activity (IC50 µg/ml)</th>
<th>Reducing power (EC50 µg/ml)</th>
<th>Total antioxidant capacity (gm of L-ascorbic acid equivalents/g of dried extract)</th>
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<tbody>
<tr>
<td>SPH</td>
<td>337.51±0.21</td>
<td>67.27±0.47</td>
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<td>SPE</td>
<td>513.63±0.53</td>
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<td>SPA</td>
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<td>Ascorbic acid</td>
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<td>--</td>
<td>4.94±0.21</td>
<td>5.10±0.37</td>
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</table>

3.4 DPPH Free Radical Scavenging Activity:
DPPH radical scavenging activity was found to be dose dependent and all the fractions exhibited very good free radical scavenging activity with low IC50 values (Table 2). DPPH, purple colored stable nitrogen centered free radical turns yellow in color in presence of antioxidants through using its odd...
electron to pair with a hydrogen from a free radical scavenging antioxidant [38]. The IC_{50} value of all fractions except dichloromethane soluble fraction was lower than that of ascorbic acid (positive control). However, ethyl acetate soluble fraction exhibited the lowest IC_{50} value. The DPPH free radical neutralization potential of all fractions is illustrated in Figure 1.

![Figure 1: DPPH Neutralization Potential of the Exocarp of S. Pinnata Fruit](image1)

3.5 Reducing Power Assay:

Like DPPH radical scavenging activity, reducing potential of all fractions was increased in a dose

![Figure 2: Reducing Potential of The Exocarp of S. Pinnata Fruit](image2)
dependent manner and all the fractions exhibited remarkable reducing power with substantially low \( EC_{50} \) values (Table 2). Reducing power assay is based on the principle that substances which have reduction potential react with potassium ferricyanide \([K_3Fe(CN)_6]\) to form potassium ferrocyanide \([K_4Fe(CN)_6]\), which then reacts with ferric chloride to form ferrous complex that has an absorption maximum at 700nm [39]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [40]. The presence of reducing agent in the crude extract can exhibit antioxidant activities through the action of breaking the free radical chain by donating a hydrogen atom. All extracts possess lower \( EC_{50} \) value than the positive control. Nevertheless, like IC\(_{50}\) value, ethyl acetate soluble fraction demonstrated the lowest \( EC_{50} \) value. The reducing potential of all fractions is illustrated in Figure 2.

### 3.6 Total Antioxidant Capacity:

The total antioxidant capacities of various fractions of the exocarp of \( S. pinnata \) fruit were expressed as gm of L-ascorbic acid equivalents/gm of dried extract and are also presented in Table 2. Total antioxidant capacity by Phosphomolybdenum assay is based on the reduction of Molybdenum (VI) to Molybdenum (V) by the sample analyte which has reduction potential and the subsequent formation of a stable green Mo (V) phosphate complex at acidic pH with the chromogenic reagent [38]. Among all fractions, like DPPH radical scavenging activity, ethyl acetate fraction exhibited the highest antioxidant activity while dichloromethane fraction was found to exhibit the lowest antioxidant activity. The total antioxidant activity varied widely from fractions to fractions in the range of 9.74 to 21.61 g of L-ascorbic acid equivalents/gm of dried extract.

### 3.7 Antimicrobial Assay:

The antimicrobial activity of different fractions of the peel of \( S. pinnata \) fruit was found to be dose dependent and is illustrated in Table 3. Ethyl acetate and aqueous fraction were found to be ineffective against all the microbial strains except \( S. dysentery \) and \( P. aeruginosa \) while n-hexane and dichloromethane extract showed variable antimicrobial activity. However, n-hexane extract exhibited the most prominent inhibitory activity against \( B. subtilis \) with the zone of inhibition of 15 mm at 400 µg/disc and 18 mm at 800 µg/disc while dichloromethane extract showed the most remarkable antimicrobial activity against \( S. aureus \). Nevertheless, dichloromethane extract showed the most promising antimicrobial activity against \( S. aureus \) since the zone of inhibition it exhibited at a dose of 800 µg/disc was greater than the standard. However, the antimicrobial activities of ethyl acetate extract against \( S. dysentery \) and aqueous extract against \( P. aeruginosa \) at a dose of 800 µg/disc were very close to that of standard. However, the presence of very minute concentrations of bioactive compounds in the plant extract may contribute to the poor antimicrobial activity [41].

### 3.8 Thrombolytic Activity:

Thrombolytic activity of all fractions is presented as mean percentage of clot lysis ± standard error of mean in Table 4. However, all fractions demonstrate statistically very significant thrombolytic activity (P<0.05 for ethyl acetate and aqueous fraction, P<0.001 for others). Among all fractions, n-hexane fraction exhibited maximum thrombolytic activity while aqueous extract showed minimum activity. Nevertheless, the mean difference in the percentage of clot lysis between standard (Streptokinase) and blank (sterile distilled water) was found statistically very significant.

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**Table 3: Results of Antimicrobial Assay by Disc Diffusion Method**

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**Table 4: Results of Thrombolytic Activity**

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Microorganisms

<table>
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<th>SPH 800</th>
<th>SPD 400</th>
<th>SPD 800</th>
<th>SPE 400</th>
<th>SPE 800</th>
<th>SPA 400</th>
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The zone of inhibition in mm is expressed as the result.

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<th>Sample</th>
<th>% Clot lysis</th>
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<td>Blank</td>
<td>2.61±0.36***</td>
</tr>
<tr>
<td>Streptokinase (Standard)</td>
<td>50.01±0.28***</td>
</tr>
<tr>
<td>SPH</td>
<td>27.12±0.04***</td>
</tr>
<tr>
<td>SPD</td>
<td>25.5±0.11***</td>
</tr>
<tr>
<td>SPE</td>
<td>10.43±0.18**</td>
</tr>
<tr>
<td>SPA</td>
<td>10.18±0.09**</td>
</tr>
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</table>

Values are expressed as mean ± SEM (Standard error of Mean). **P<0.05, and ***P<0.001.

4. CONCLUSION

To conclude, the present study demonstrates that the exocarp of S. pinnata fruit can be considered as a valuable source of therapeutic agents for human health, as an antioxidant, antimicrobial and thrombolytic agent. In brief, all fractions exhibited good antioxidant and significant thrombolytic activity. The higher total antioxidant capacity and lower IC50 and EC50 value could be the reflection of higher total phenolic content. Nevertheless, the antioxidant and antimicrobial activity were found to be concentration dependent. Furthermore, the plant extract showed good antimicrobial activity against some strains. However, continuation of further extensive phytochemical and pharmacological investigations is essential not only to evaluate this preliminary experiment but also to characterize and isolate the unknown underlying compounds in order to establish their pharmacological properties.

REFERENCES


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