In vitro Antioxidative, Fibrinolytic and Phytochemical Effects of Different Extracts of Sterculia villosa Barks

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Abstract: To investigate the phytochemical, in vitro antioxidative and fibrinolytic activity of methanol extract and its ethyl acetate fraction of Sterculia villosa barks. Methanol extract of Sterculia villosa was obtained by cold extraction method and then ethyl acetate fraction was obtained by modified Kapchan method from methanol extract. Methanol extract and its ethyl acetate fraction were then subjected to phytochemical investigation. For phytochemical investigation different phytochemical group tests were performed. In vitro antioxidant activity was performed by using DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay. An in vitro thrombolytic model was used to check the fibrinolytic activity of Sterculia villosa by using enzyme streptokinase as a positive control and water as a negative control. Maximum percent scavenging activity was obtained by methanol extract and that was about 72.56% at concentration 800 μg/ml. Ethyl acetate fraction showed highest scavenging activity of 91.34% at the same concentration (800 μg/ml). Percent (%) of scavenging activity (% inhibition) was plotted against log concentration and from the graph IC₅₀ (Inhibition Concentration 50) value was calculated by linear regression analysis. The ethyl acetate fraction of Sterculia villosa showed least IC₅₀ values (23.99 ± 1.21) by DPPH assay. Significant (P < 0.05) IC₅₀ values compared to respective standard (Ascorbic acid) was recorded in DPPH radical scavenging. Methanol extract showed 32.34% fibrinolytic activity whereas ethyl acetate fraction of Sterculia villosa showed 47.88% which was significant (P < 0.05) compared to the reference drug streptokinase (65.56 ± 2.42%). The results indicate that Sterculia villosa has significant antioxidant activity and moderate fibrinolytic activity and hence can be used as a natural source of antioxidant.

Keywords: Sterculia villosa, DPPH, Ascorbic acid, Streptokinase, Antioxidative, Fibrinolytic.

1. INTRODUCTION

An antioxidant is a type of compound or molecule which is capable of slowing or preventing the oxidation of other molecules. Oxidation reactions can produce free radicals like reactive oxygen species (ROS), e.g., superoxide, hydroxyl, peroxyl and alkoxy radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. Under non-stressed conditions, production and scavenging of reactive oxygen species (ROS) in the organisms is in equilibrium. Free radicals damage DNA, RNA, proteins and enzymes, lead to the formation of (a) Tumors and cause cancers (b) Cardiovascular diseases (c) Nervous disorders (d) Premature ageing (e) Parkinson's and Alzheimer's diseases (f) Rheumatic and Pulmonary disorders [1]. In order to avoid the oxidative stress, prevention and treatment of complex diseases antioxidant based drug formulations are used. Fruits and vegetables are rich in natural oxidants and have been health promoting effects and these positive effects have been related with their antioxidant activity [2]. Flavonoids and alkaloids which are usually found in medicinal plants have been accounted to have high antioxidant activity [3]. Synthetic antioxidants are toxic to man. Therefore the use of natural antioxidant has gained much attention from the consumers because they are considered safer than synthetic antioxidants. Recently there has been a worldwide trend towards the use and ingestion of natural antioxidants present in different parts of plants due to their phytochemical constituents [4, 5]. Formation of blood clots is one of the vital reasons of blood circulation problem. Thrombi or emboli can lodge in a blood
vessel and block the flow of blood in that location depriving tissues of normal blood flow and oxygen. This can result in damage, destruction (infarction), or even death of the tissues (necrosis) in that area [6]. A blood clot (thrombus) is formed from fibrinogen by thrombin and is lysed by plasmin which is activated from plasminogen by tissue plasminogen activator (tPA). Fibrinolytic drugs have been used to dissolve thrombi in acutely occluded coronary arteries there by restoring blood supply to ischemic myocardium to limit necrosis and to improve prognosis [7]. Streptokinase is an antigenic fibrinolytic agent used for the treatment of acute myocardial infarction. It reduces mortality as effectively as the non-antigenic alteplase in most infarct patients while having the advantages of being much less expensive. Tissue-type plasminogen activator (tPA) is generally preferred as being effective and safer than either urokinase or streptokinase type activators. All available fibrinolytic agents still have significant shortcomings, including the need for large doses to be maximally effective, limited fibrin specificity and a significant associated bleeding tendency. Because of the shortcomings of the available fibrinolytic drugs, attempts are underway to develop improved recombinant variants of these drugs [8, 9, 10, 11, 12]. Sterculia villosa (local name: Udal; Chala) under the family sterculiaceae is a medium sized, deciduous tree with white bark and is widely distributed in forests of Chittagong, Chittagong hill tracts, Cox’s bazaar, Gazipur, Tangail, Comilla and Habiganj. The plant possesses diuretic, cooling and aphrodisiac properties traditionally [13]. Since there is no scientific report for anti-oxidant and fibrinolytic activities of S. villosa barks extract, the present study was an attempt to evaluate the antioxidative and fibrinolytic effect by in vitro analysis.

2. MATERIALS AND METHODS

2.1. Collection and Identification of Plant

The plant S. villosa was selected by Talha Bin Emran, Lecturer, Department of Pharmacy, BGC Trust University Bangladesh, Chittagong-4000, Bangladesh. The plant was collected from hilly forest region of Chokoria and Cox’s Bazar in summer season. The plant was identified by Dr. Shaikh Bokhtear Uddin, Taxonomist and Associate Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh.

2.2. Extraction of Plant and its Fractionation

The shade dried ground powder (1.2 kg) was extracted with 5 L methanol (HPLC Grade, Sigma Chemical Company Ltd.). The extract was evaporated in rotary evaporator at 40-45 °C under vacumm to yield the residue (79.2 gm), which was then dissolved in distilled water (1 L) and then portioned with ethyl acetate (1 L). The ethyl acetate fraction was then concentrated on rotary evaporator at 45 °C under vacumm.

2.3. Chemicals

1. 1-diphenyl-2-picrylhydrazyl (DPPH), methanol, ethyl acetate were purchased from Sigma chemicals Ltd. (USA). Streptokinase (Durakinase) was purchased from Dongkook Phama. Co. Ltd. (South Korea). Sulphuric acid, hydrochlooric acid, ammonium molydate, ferric chloride and acetic acid were purchased from Merck (Pvt.) Ltd. (Germany). Ascorbic acid was purchased from BDH, England. All the chemicals used were analytical grade.

2.4. Phytochemical Screening

Different phytochemical group tests were done to investigate the presence or absence of phytochemical constituents like alkaloids, terpenoids, glycosides, steroids, tannins, flavonoids, saponins and reducing sugar by following standard procedures [14, 24].

2.4.1. Test for Alkaloids

**Mayer’s Test**

Stir 0.5 gm of extracts with 5 ml 1% HCl on a steam bath and filter. Treat 1 ml of filtrate with a few drop of Mayer’s reagent. White or creamy white precipitate indicates the presence of alkaloid.

**Wagner’s Test**

Stir 0.5 gm of extracts with 5 ml 1% HCl on a steam bath and filter. Treat 1 ml of filtrate with a few drop of Wagner’s reagent for brown or deep brown precipitate.
2.4.2. Test for Cardiac Glycoside

Baljet’s Test

Add one drop of Baljet’s reagent (picric acid + NaOH) to a portion of the extracts. A yellow orange color is produced due to the presence of five member lacton ring at C-17 of the aglycone in cardiac glycoside.

2.4.2.2. Legal’s Test

Dissolve 0.1 gm of extracts in 2 ml of sodium nitroprusside solution (0.5%). Make the mixture alkaline with NaOH (0.2 N) solution. Pink to red color indicates the presence of cardiac glycosides due to lactone ring.

2.4.3. Test for Flavonoids

Take about 0.5 ml of extracts in a test tube. Add a small piece of magnesium or zinc ribbon. Then add 5-10 drops of concentrated HCl. Boil the solution for few minutes. Developments of orange to red, red to crimson, crimson to magenta indicate flavones, flavanols and flavanones respectively.

2.4.4. Test for Terpenoids

Five (5 ml) of each extract was mixed with 2 ml of chloroform and concentrated H2SO4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed to indicate the positive results for the presence of terpenoids.

2.4.5. Test for Steroids

Libermann-Burchard’s Test

Take small amount (0.1 gm) of plant extracts in a test tube. Dissolve in 1 ml of chloroform. Add 2 ml of acetic anhydride and then 1 ml of concentrated H2SO4. A greenish color is formed indicating the presence of steroid.

Salkowski Test

Five (5 ml) of each extract was mixed in 2 ml of chloroform and concentrated H2SO4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed to show positive results for the presence of steroids.

2.4.6. Test for Tannins

About 0.5 gm of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

2.4.7. Test for Saponins

About 2 gm of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

2.5. Evaluation of Antioxidant Activity

DPPH free radical scavenging method was used for the assay of each extract of S. villosa and the scavenging activity was compared with the standard antioxidant ascorbic acid (Vitamin C). Each plant extract and ascorbic acid with different concentrations (20, 40, 60, 80, 100, 200, 400 and 800 µg/ml) were prepared in methanol. Then 0.004% DPPH solution was prepared in methanol. Then 3 ml of this DPPH solution was mixed with 5 ml of each extract solutions and standard solution separately. These solution mixtures were kept in dark for 30 minutes. The degree of DPPH purple decolorization to DPPH yellow indicated the scavenging efficiency of the extract. The absorbance of the mixture was determined at 517 nm by using UV-Visible Spectrophotometer (Shimadzu, Japan). Ascorbic acid was served as a positive control. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. The scavenging activity against DPPH was calculated using the following equation:

\[
\text{Scavenging Activity (\%)} = \left(\frac{(A - B)}{A}\right) \times 100
\]
Where A was the absorbance of control (DPPH solution without the sample), B was the absorbance of DPPH solution in the presence of the sample (extract/ ascorbic acid). Then, % scavenging activity or % inhibition was plotted against log concentration and from the graph IC50 (Inhibition concentration 50) value was calculated by linear regression analysis [15].

2.5.1. Total Antioxidant Activity

The total antioxidant activities of methanol extract and its ethyl acetate fraction of *S. villosa* were performed by phosphomolybdenum method [16]. Here, 500 µg/ml of each crude extract was mixed with 4 ml of reagent solution (0.6 mol/l sulphuric acid, 28 mmol/l sodium phosphate and 4 mmol/l ammonium molybdate) in sample vials. The blank solution contained 4 ml of reagent solution. The vials were capped and incubated in water bath at 95 °C for 90 min. After the samples had been cooled to room temperature, the absorbance of mixture was measured at 695 nm against blank. The antioxidant activity was expressed relative to that of ascorbic acid.

2.6. Evaluation of Fibrinolytic Activity

Here, 4 ml venous blood drawn from healthy volunteers was distributed in three different pre-weighed sterile microcentrifuge tube (0.5 ml/tube) and incubated at 37 °C for 45 minutes. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube-weight of tube alone). To one microcentrifuge tube containing pre-weighed clot, 100 µl of each extract of *S. villosa* were added. As a positive control, 100 µl of SK and as a negative non-fibrinolytic control, 100 µl of distilled water were separately added to the control tubes numbered. All the tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, fluid released 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. The experiment was repeated three times with the blood samples of 20 volunteers. The significance between % clot lysis by Streptokinase, herbal extract, water by means of weight difference was tested by the Student’s t-test analysis calculated by SPSS version 18.0 software [17].

2.7. Statistical Analysis

All data are presented as mean ± standard deviation (SD). All the measurements were done in triplicate. The data were analyzed by a statistical software package (SPSS, version 18.0, IBM Corporation, NY, USA) using Tukey’s multiple range post hoc tests. The values were considered significantly different at P < 0.05.

3. RESULTS

3.1. Phytochemical Investigation

Phytochemical investigation of methanol extract and its ethyl acetate fraction of *S. villosa* under this study explored the presence of medicinally active secondary metabolites alkaloids, glycoside, steroids, tannins, terpenoids and flavonoids. This investigations are also indicated the absence of reducing sugar and saponins. These findings with their corresponding results are summarized in Table1.

<table>
<thead>
<tr>
<th>Secondary Metabolites</th>
<th>Test Performed</th>
<th>Methanol Extract</th>
<th>Ethyl Acetate Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Meyers Test</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>Wagners Test</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>Legal Test</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Baljits Test</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Salkowski Test</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Libermann-Burchred Test</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Concentrated HCl and Alcoholic Test</td>
<td>+ +</td>
<td>+++</td>
</tr>
<tr>
<td>Tannins</td>
<td>Ferric Chloride Test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>Froth Test</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>Salkowski Test</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>Reducing Sugar</td>
<td>Benedicts Test</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*NB: + = Mild, ++ = Moderate, +++ = Highly Present, - = Absent*
In vitro Antioxidative, Fibrinolytic and Phytochemical Effects of Different Extracts of Sterculia villosa Barks

3.2. DPPH Radical Scavenging Assay

DPPH free radical scavenging method was used for the assay of methanol extract and its ethyl acetate fraction of S. villosa and the scavenging activity was compared with the standard antioxidant ascorbic acid (Vitamin C). The free radical scavenging activity of the ascorbic acid and each extracts of S. villosa is shown in Figure 1. Both ascorbic acid and S. villosa extracts showed dose dependent activity. Among the eight different concentrations used in the study (20, 40, 60, 80, 100, 200, 400 and 800 µg/ml) maximum % scavenging activity was obtained at concentration 800 µg/ml. At this concentration methanol extract showed 72.56% of scavenging activity, ethyl acetate fraction showed 91.34% scavenging activity and ascorbic acid showed 95.95% scavenging activity. % of scavenging activity or % of inhibition was then plotted against log concentration and from the graph IC_{50} (Inhibition concentration 50) value was calculated by linear regression analysis. IC_{50} value of methanol extract, ethyl acetate fraction and ascorbic acid was found 23.99 µg/ml, 60.25 µg/ml and 16.1 µg/ml respectively.

![Figure 1](image)

Figure 1. Comparative antioxidative effect of ascorbic acid, methanol extract and ethyl acetate fraction of Sterculia villosa. Data are presented as mean ± SD for triplicate. Data leveled letters a, b and c shown on the graph lines indicate that the values are significantly different (Tukey’s post hoc test for multiple comparisons, SPSS for windows, version 18.0, P < 0.05) from each other

3.2.1. Total Antioxidant Activity

Total antioxidant activities of methanol extract and its ethyl acetate fraction were evaluated photometrically by phosphomolybdenum procedure. The free radical scavenging activities were compared with reference standard antioxidant ascorbic acid (Vitamin C). The ethyl acetate fraction showed the highest total antioxidant activity (0.91 ± 0.05) and methanol extracts showed moderate total antioxidant activity (0.64 ± 0.37) which is shown in Table 2 and Figure 2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC_{50} (µg/ml) by DPPH Radical Scavenging Activity</th>
<th>Total Antioxidant Activity (Absorbance at 690 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>16.14 ± 0.88</td>
<td>0.96 ± 0.02</td>
</tr>
<tr>
<td>Ethyl Acetate Fraction</td>
<td>23.99 ± 1.21</td>
<td>0.91 ± 0.05</td>
</tr>
<tr>
<td>Methanol Extract</td>
<td>60.25 ± 2.46</td>
<td>0.64 ± 0.37</td>
</tr>
</tbody>
</table>

NB: All results are presented as mean ± SD (n=3)
Figure 2. Comparative IC₅₀ values of ascorbic acid, methanol extract and ethyl acetate fraction of Sterculia villosa. Data are presented as mean ± SD for triplicate. Data leveled letters a, b and c shown on the graph lines indicate that the values are significantly different (Tukey’s post hoc test for multiple comparisons, SPSS for windows, version 18.0, P < 0.05) from each other

3.3. In Vitro Fibrinolytic Activity

Addition of 100 µl Streptokinase (Durakinase, Dongkook Phama.Co.Ltd, South Korea), a positive control (30,000 I.U.) to the clots along with 90 minutes incubation at 37 °C, showed 65.56% clot lysis.

Table 3. Fibrinolytic activity of methanol extract and its ethyl acetate fraction of Sterculia villosa, streptokinase and water

<table>
<thead>
<tr>
<th>Effectors</th>
<th>% of Clot Lysis Activity</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptokinase (Positive Control)</td>
<td>65.56 ± 0.07*</td>
<td>-</td>
</tr>
<tr>
<td>Water (Negative Control)</td>
<td>11.75 ± 0.01*</td>
<td>-</td>
</tr>
<tr>
<td>Methanol Extract of Sterculia villosa</td>
<td>32.34 ± 0.05</td>
<td>1.339</td>
</tr>
<tr>
<td>Ethyl Acetate Fraction of Sterculia villosa</td>
<td>47.88 ± 0.03*</td>
<td>0.002</td>
</tr>
</tbody>
</table>

NB: All results are presented as mean ± SD (n=3). *P < 0.05 indicates that the result is significant compared to positive control

Figure 3. Comparative fibrinolytic activity of water, streptokinase, methanol extract and ethyl acetate fraction of Sterculia villosa. Data are presented as mean ± SD for triplicate. *P < 0.05 indicates that the result is significant compared to positive control (Streptokinase)
In vitro Antioxidative, Fibrinolytic and Phytochemical Effects of Different Extracts of Sterculia villosa Barks

On the other hand, clots when treated with 100 µl sterile distilled water (negative control) showed only negligible clot lysis which was only 11.75%. The mean difference in clot lysis percentage between positive and negative control was very significant. But when 100 µl ethyl acetate fraction of S. villosa was added to 20 different clots, 47.88% clot lysis were obtained and when compared with the negative control (water) the mean clot lysis percentage differences was significant (P < 0.05). Again methanol extract showed 32.34% fibrinolytic activity which was not significant (P = 1.339). All results for fibrinolytic activity are shown in Table 3 and Figure 3.

4. DISCUSSION

The phytochemical investigation explored the presence of medicinally active secondary metabolites alkaloids, glycoside, steroids, tannins, terpenoids and flavonoids in the methanol and ethyl acetate fraction of S. villosa. This investigation also indicates the absence of reducing sugar and saponins. DPPH scavenging activity is based on the ability of sample to donate hydrogen which reacts with the DPPH radical. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom or transfer electron to DPPH, thus neutralize the free radical character and then this gives rise to the reduced form DPPH (non-radical) with the loss of the violet color. Radical scavenging activity increases with increasing percentage of the free radical inhibition [18]. The color change from violet to yellow and fall in absorbance of the stable radical DPPH was measured for different concentrations. In the present study the methanol extract and polar fraction (ethyl acetate) showed moderate and higher antioxidant activities as compared to the standard ascorbic acid. This is due to most bioactive compounds such as polyphenols including tannins, flavonoids existed in higher polar fraction [19].

The total antioxidant activity was analyzed by phosphomolybdenum method which is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate Mo (V) complex at acidic pH. Electron transfer occurs in this assay which depends upon the structure of the antioxidant [20]. In our study the polar fraction showed highest antioxidant activity, whereas the non-polar fraction didn’t reduce Mo (VI) to Mo (V) when compared with ascorbic acid. This shows that ethyl acetate fraction which contains more polar compounds than methanol possess more antioxidant activity.

Platelets play a significant role in the formation of clot by adhering to the broken regions of the epithelial tissue surface. Most fibrinolytic agents work by regulating the enzyme plasminogen, which cuts the cross-linked fibrin mesh. This makes the clot soluble and leads to further proteolysis by other enzymes, and thus causes blood flow over occluded blood vessels. Thus fibrinolytic agents are useful for the treatment of myocardial infarction, thromboembolic strokes, deep vein thrombosis etc [21]. The comparison of the positive management (streptokinase) with negative management clearly showed that clot dissolution didn’t occur when water was added to the clot. In the present study ethyl acetate fraction of S. villosa showed (47.88 ± 0.03)% fibrinolytic activity where as methanol extract showed (32.34 ± 0.05)% fibrinolytic activity. Since phytochemical analysis showed that the crude extract contains flavonoids, triterpenes, saponins, phenols, diterpenes, proteins and tannins. It can be predicted that these phyto-constituents could also be liable for its clot lysis activity [22, 23].

5. CONCLUSION

In sum, we can say that S. villosa has a good antioxidant activity and moderate fibrinolytic activity and hence can be used as a natural source of antioxidant and this may be of pharmaceutical importance.

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