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Evaluation of Antioxidant, Thrombolytic and Cytotoxic Potentials of Methanolic Extract of *Aporosa wallichii* Hook.f. Leaves: An Unexplored Phytomedicine

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ABSTRACT

Aporosa wallichii Hook.f. is a medicinal plant, which belongs to Phyllanthaceae family. This plant was used in this study for the determination of several pharmacological activities. This study involved experiments including determination of antioxidant property by performing 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and total phenolic content (TPC) test, determination of cytotoxic activity by brine shrimp lethality assay and thrombolytic property analysis. In this study, it was observed that for DPPH free radical scavenging assay; standard (ascorbic acid) provided the IC_{50} value of 75.6 µg/mL, whereas the extract provided the IC_{50} value of 58.7 µg/mL. In case of TPC test, plant extract showed 308.97 mg of gallic acid equivalents/g of extract. Brine shrimp lethality bioassay revealed the cytotoxic property of the plant and the LC_{50} value of the plant extract was 26.7 µg/mL. Then again, the standard vincristine sulfate provided the LC_{50} value of 2.0 µg/mL. Moreover, the thrombolytic analysis showed for standard (clopidogrel) 59.3% clot lysis and for extract 24.5 % clot lysis. This study revealed that this plant has significant antioxidant activity, moderate level of cytotoxic activity and thrombolytic activity.

INTRODUCTION

The medicinal plants serve as a vital source of new pharmacologically active compounds (Atanasov *et al*., 2015). These agents are derived from different parts of plants and used directly as drug or as semi-synthesized or synthesized drug (Ghani, 2003). Origin of *Aporosa wallichii* Hook.f is dry evergreen forests of Meghalaya and Tripura in India, Bangladesh, Myanmar and Thailand (Schot, 2004). The local name of this plant is Kokra in Bangladesh and commonly known as Castoma (Hossain *et al*., 2015). The height of the tree can be up to 15 m and the diameter is about 15 cm. It has thick, rough, grooved and brownish color bark. Plant's stipules are narrowly ovate and the size is about 5-7 mm × 1.5-2.8 mm. Leafstalk is cylindrical, smooth and size up to 6-19 mm × 0.8-1.2 mm. Leaves narrowly egg-shaped to narrowly elliptic and the size is up to 9-17.5 cm × 3.5-6.5 cm, the base is thick and small basal glands is black in color. The color of the upper part of the leaves is bright to yellowish green and color of lower part of the leaves is reddish green or brownish. Cuspidate of *Aporosa wallichii* Hook.f. is sharp, smooth, a little shiny and not fragile. On the surface of the plant's leaf, irregular dense dots are present. Fruits are smooth and ovoid. Young fruits are striped and the size is 9-11 mm × 6-9 mm and the color is brown to black. Each fruit contains either 1 or 2 seeds (Schot, 2004). *Aporosa wallichii* Hook.f. is one of the plants of a Phyllanthaceae family. Different medicinal plants contain many therapeutic activities to treat cancer, skin diseases, inflammation, diarrhea, dysentery, jaundice and headache etc. (Rahman and Akter, 2013). A literature review of *Aporosa wallichii* Hook.f. plant showed that no significant previous study

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has been conducted on this plant (Fabricant and Farnsworth, 2001). However, previous studies on various species of Phyllanthaceae family showed powerful antioxidant, anthelmintic, antimicrobial, anti-diarrheal, anti-tumor, anti-inflammation and insecticidal properties (Li, 2000). *Aporosa lindleyana* is also from the same genus and has antioxidant, anti-amylase and lipid-lowering properties (Kathirgamanathar *et al*., 2018). This plant also has antimicrobial, analgesic (Srikrishna *et al*., 2008) and antidiuretic activity (Ganegamage *et al*., 2014). Other species like *Baccaurea parviflora, Antidesma tomentosum, Aporosa aurea, and Mallotus paniculatus* have various pharmacological properties including cytotoxicity and antitrypanosomal activity (Mohmod *et al*., 2015). *Croton gratissimus* also has an acceptable rate of antiplasmodial activity. On the other hand, *Croton argyratus* has excellent antiprotozoal activity (Abdullah *et al*., 2007). So, there is a high possibility of the presence of different types of pharmacological properties in *Aporosa wallichii* Hook.f. plant. Free radicals are responsible not only for aging but also for many age-related diseases (Harman, 2009). Evidence from various sources suggests that free radicals trigger cell death mechanisms in the body such as apoptosis and necrosis (Chatterjee *et al*., 2011). Blockage of veins is a thrombosis which affects different organs and this thrombosis can cause various pathological conditions (Bekker *et al*., 2009). Antithrombotic and thrombolytic therapies have a great effect on thrombosis and they play crucial roles in the management of thromboembolic disorders (Hirsh *et al*., 2008). Cytotoxic property is very important to destroy cancer cells. Therefore, this plant *Aporosa wallichii* Hook.f. can be a potential source of medicinal properties and for this purpose, this study was focused on to find out the antioxidant, cytotoxicity and thrombolytic activity of the plant.

MATERIALS AND METHODS

Collection of plant materials

The leaf part of *Aporosa wallichii* Hook.f. plant was collected in May 2017 from Moulovibazar district of Sylhet division; after that, it was sent to the National Herbarium Bangladesh (NHB), Mirpur, Dhaka for verification. It was authenticated by NHB and the plant accession number was also provided (DACB-44996).

Preparation of the extract

The leaves were washed with clean water to remove the plant scrap and dust particles. Then the cleaned leaves were allowed to dry under the sun for a day after which the leaves were dried for 1 hour at 30-40°C in a hot air oven. After that, the dry and crusty leaves were ground with coarse dust using a high capacity grinding machine. Approximately 900 g of powder was soaked in 2.5 L of methanol for a period of 2 days at a normal ambient temperature (22-25°C) with occasional stirring. After that filtration was done by using a cotton filter (pore size: 110 mm), then the maximum amount of solvent was evaporated by using rotary evaporator at 100 rpm at 30°C. Then the extract of the leaves was placed under laminar airflow cabinet to vaporize the solvent completely from the extract and it was used to avoid any possibility of microbial growth in the extract while drying. Finally, 22.4 g of plant leaf extract was obtained and it was kept in a dry and cool place with proper labeling. Antioxidant, brine shrimp lethality assay and thrombolytic studies were conducted with this extract.

Chemicals

The chemicals were gallic acid [Sigma-Aldrich, USA], sodium chloride [Sigma-Aldrich, USA], Folin-Ciocalteu reagent [Sigma-Aldrich, USA], vincristine sulphate [Sigma-Aldrich, USA], clopidogrel [Sigma-Aldrich, USA], 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) [Sigma-Aldrich, USA]., sodium carbonate [Merck, India] and ascorbic acid (ASA) [Merck, India], dimethyl sulfoxide (DMSO) [Fisher Scientific, UK]. All the chemicals used in this study were of analytical grade.

Total phenolic content (TPC)

Folin-Ciocalteu chemical easily oxidizes the phenols when these chemicals added in this ionic phenolic solution, the phenols easily oxidizes by reagents (Singleton *et al*., 1999). When the oxidation procedure completed in the solution yellow color of Folin-Ciocalteu chemical turned into dark blue. This color changed mixture measured in a 760 nm in UV spectrophotometer. Value of the absorbance plotted in the gallic acid calibration curve and data was evaluated as gallic acid equivalents (GAE) (Wolfe *et al*., 2003).

DPPH free radical scavenging assay

DPPH free radical scavenging assay was performed to determine the antioxidant activity of *Aporosa wallichii*. Methanol plant extracts showed free radical scavenging activities on stable 2,2-diphenyl-1-picrylhydrazyl radicals were measured for this test (Braca *et al*., 2001). At different concentrations, plant extracts were mixed with 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution. In methanol or aqueous solution, it generated stable free radicals by the delocalization of the free electrons; which in turn produced a deep purple colored solution. Absorbance values of these concentrations were calculated at 517 nm in UV spectrometer and the decreasing value of DPPH at 517 nm is directly proportional to the radical scavenging activity (Brand-Williams *et al*., 1995).

Percentage of inhibition of DPPH free radical (I%) was calculated by using the following equation:

$$(I\%) = ((\text{Absorbance of blank} - \text{Absorbacne of sample})/(\text{Absorbance of blank})) \times 100$$

50% of inhibition (IC_{50}) of extract concentration was calculated from the graph; where the percentage of inhibition (I%) was plotted against extract concentration.

Brine shrimp lethality assay

Artemia salina shrimp used in this assay. Its offspring was hatched in replicated seawater to grow nauplii (Meyer *et al*., 1982). By adding the calculated amount of dimethylsulfoxide (DMSO), the sample was prepared in desired concentration by dilution. The nauplii were counted with visual examination and were placed in vials which contained around 5 mL simulated seawater. Subsequently, various concentrations of samples were added to the tubes by micropipette and vincristine sulfate was used as positive control. These tubes were then left in a dry place for 24 hours at room temperature. Finally, survivors were counted after 24 hours (Hossain *et al*., 2012). Percentage (%) of mortality

was calculated by using following equation:

Percentage (%) of mortality = ((Number of nauplii taken − Number of nauplii alive)/(Number of nauplii taken)) × 100

50% of lethal concentration (LC_{50}) of extract concentration was calculated from the graph plotted percentage of mortality against concentration.

Thrombolytic activity

Thrombus hampers the normal flow of blood to cells and tissues by blocking the blood vessel which can lead to lack of blood and oxygen. So, thrombolytic medications such as urokinase, clopidogrel, and streptokinase remove this thrombus and help to keep cells and tissues in normal condition (Prasad *et al*., 2006). In this study, fresh human blood was collected and blood samples were taken in three different pre-weighed sterile microbes and allowed to incubate at 37°C for 45 min. When the clot was formed, the upper fluid was entirely discharged from all micro-tube lines. Clopidogrel was used as positive control and water (distilled) was used as negative control. Each test tube was filled with 100 µl of plant extract and then microtubes were incubated for 90 min at 37°C. Afterward, the liquid was removed which was released from the clot and again the tubes were weighed to see the weight difference when the clot disruption occurred (Ali *et al*., 2014).

Percentage of clot lysis was calculated by the following equation:

(%) of clot lysis = ((released clot weight)/(clot weight after clot disruption)) × 100.

RESULTS AND DISCUSSION

Antioxidant property

In this experiment, methanol extract of *Aporosa wallichii* Hook.f. leaves were tested properly through DPPH assay and TPC to determine the antioxidant property of this plant (Pavithra *et al*., 2009). Antioxidant activity is very important in preventing free radical reactions because they can neutralize free radical by their reducing ability.

Determination of DPPH radical scavenging activity

Here, the percentage of inhibition of ascorbic acid and methanol extract in different concentrations were obtained (Table 1). It was found that DPPH free radical scavenging activity was increasing along with increasing concentration of the methanol extract (Figure 1). As the reference standard, ascorbic acid was used in this experiment for which IC_{50} value was 75.6 µg/mL. On the other hand, the IC_{50} value of the methanol extract of *Aporosa wallichii* Hook.f. leaves were 58.7 µg/mL (Table 1). This result indicates the presence of DPPH free radical scavenging activity; which specifies the presence of antioxidant activity in *Aporosa wallichii* Hook.f.

Table 1: Evaluation of DPPH free radical scavenging activity of methanol extract of *Aporosa wallichii* Hook.f. leaves.

Concentration (µg/mL)	Absorbance of ascorbic acid	Absorbance of plant extract	Ascorbic acid inhibition (%)	Methanol extract inhibition (%)	IC_{50} value in µg/mL (Ascorbic acid)	IC_{50} value in µg/mL (Methanol extract)
500	0.031	0.058	94.9	90.6		
250	0.043	0.077	93.0	87.5		
125	0.065	0.091	89.4	85.2		
62.5	0.184	0.113	70.2	81.6		
31.25	0.292	0.274	52.6	55.6		
15.625	0.385	0.314	37.6	49.1	75.6	58.7
7.813	0.418	0.368	32.2	40.3		
3.906	0.464	0.418	24.7	32.2		
1.953	0.481	0.465	22.0	24.6		
0.977	0.497	0.574	19.4	7.0		
Blank	Absorbance = 0.617					

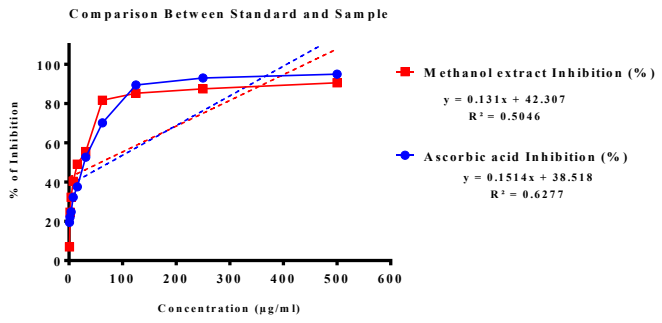

Fig. 1: Comparison of % inhibition between ascorbic acid and methanol extract.

Total phenolic content (TPC)

In total phenolic content test methanol extract showed a significant level of reducing power. In this test, the gallic acid used as standard and methanol extract's absorbance plotted in gallic acid calibration curve (Figure 2). The absorbance of the plant extract was high for that reason the obtained TPC value was 308.97 mg of GAE/g of extract (Table 2). This TPC value indicated that *Aporosa wallichii* Hook.f. has antioxidant activity.

Cytotoxic property

Brine shrimp lethality assay

The brine shrimp lethality assay was used to assess the cytotoxic property of methanol extract (Runa *et al*., 2013). At

different concentrations; standard and extract provided different percentages of mortality (Table 3). Vincristine sulfate was used in this experiment as a standard (positive control), in which 2.0 µg/mL was obtained as the value of LC_{50}, compared to the standard methanol extract of the *Aporosa wallichii* Hook.f. leaves gave 26.7 µg/mL as the value of LC_{50} (Table 3). Percentage of mortality was found to increase with increasing concentrations of vincristine sulfate and methanol extract (Figures 3 and 4). This study indicated the methanol extract of *Aporosa wallichii* Hook.f. leaves have cytotoxic activity.

Table 2: Total phenolic content (TPC) profile of the plant extract of *Aporosa wallichii* Hook.f. leaves.

Name of extract	Plant part	The absorbance of methanol plant extract	Total phenolic content (mg of GAE/g of extract)
Methanol extract	Leaves of *Aporosa wallichii*	2.5	308.97

Table 3: Brine shrimp lethality profile of the plant extract of *Aporosa wallichii* Hook.f. leaves.

Concentration (µg/mL)	% of Mortality (Vincristine sulphate)	LC_{50} µg/mL (Vincristine sulphate)	Concentration (µg/mL)	% of Mortality (Methanol extract of leaves)	LC_{50} µg/mL (Methanol extract of leaves)
0.039	20		0.781	20	
0.078	30		1.562	20	
0.156	30		3.125	30	
0.312	40		6.25	50	
0.625	50	2.0	12.5	60	26.7
1.25	60		25	70	
2.5	70		50	70	
5	80		100	80	
10	90		200	90	
20	100		400	100	

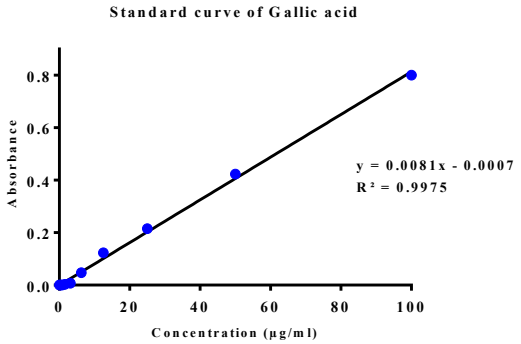

Fig. 2: Gallic acid's standard curve for the total phenolic content test.

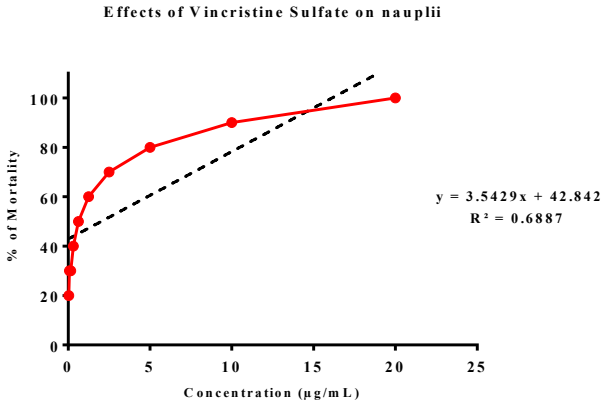

Fig. 3: Percentage (%) of mortality and predicted regression line of vincristine sulfate.

Thrombolytic activity

Plasminogen enzyme is usually activated by thrombolytic agents and it also removes fibrin bonds in blood, as a result, the clot becomes soluble and blood flow is restored. Here, methanol extract showed much lower level of thrombolytic activity than standard (Figure 5). In thrombolytic activity test, clopidogrel was used as a positive control; because it is a blood thinning agent (Maegdefessel *et al.*, 2010). Clopidogrel gave 59.3% clot lysis, distilled water was used as a negative control, which provided 2.5% clot lysis and methanol extract of *Aporosa wallichii* Hook.f. leaves showed 24.5% clot lysis (Table 4). After comparing the clots lysis value of plant extract with the positive control value, it was observed that *Aporosa wallichii* Hook.f. revealed thrombolytic activity.

Table 4: Evaluation and results of the thrombolytic activity.

Name of samples	W1	W2	W3	W4	W5	% of clot lysis
Plant extract	0.789	1.547	1.398	0.609	0.149	24.5
Clopidogrel (Anti-platelet agent) as standard	0.799	1.559	1.276	0.477	0.283	59.3
Blank	0.787	1.533	1.515	0.728	0.018	2.5

Here, W1 = Micro-tube weight, W2 = Clot with micro-tube weight, W3 = Clot with micro-tube weight after clot disruption, W4 = Clot weight after clot disruption, W5 = Released clot weight.

CONCLUSION

The methanol extract of the *Aporosa wallichii* Hook.f. leaf was investigated to evaluate the therapeutic properties. In this study, it was clearly observed that *Aporosa wallichii* Hook.f. has various therapeutic potentials. This study showed that this plant has an acceptable level of antioxidant and thrombolytic property along with the moderate level of the cytotoxic property. Furthermore, additional investigations on *Aporosa wallichii* Hook.f. to find out

unidentified biological properties; will help in the development of new and effective therapeutic agent in the field of medicine.

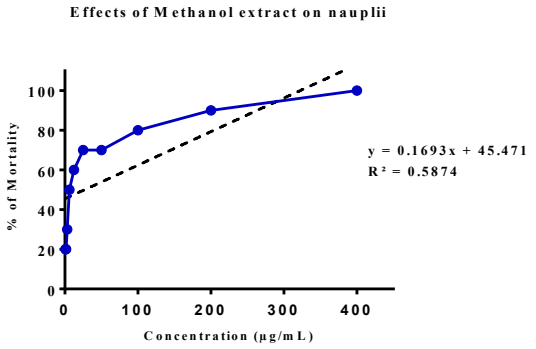

Fig. 4: Percentage (%) of mortality and predicted regression line of methanol extract of *Aporosa wallichii* Hook.f.

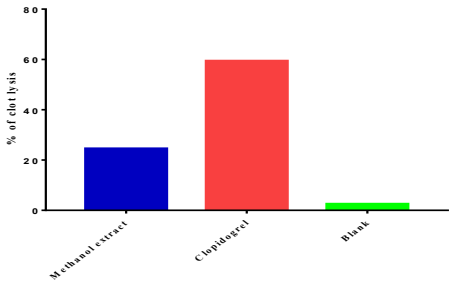

Fig. 5: Percentage (%) of clot lysis of sample, standard, and blank.

CONFLICT OF INTERESTS

The authors proclaim that they have no conflicts of interest.

ABBREVIATIONS

ASA	Ascorbic acid
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
NHB	National herbarium Bangladesh
TPC	Total phenolic content
DMSO	Dimethylsulfoxide
GAE	Gallic acid equivalents

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