



# Journal of Biological Sciences

ISSN 1727-3048

**science**  
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## Phytochemical Analysis and Bioactivities of *Aphanamixis polystachya* (Wall.) R. Parker Leaves from Bangladesh

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**Abstract:** The purpose of the study was to investigate the crude n-hexane, ethyl acetate and methanol extracts of *Aphanamixis polystachya* leaves for their antimicrobial, antioxidant, cytotoxic and thrombolytic activities. The leaves extracts were screened for major phytochemical compounds using *in vitro* established procedures. Antimicrobial and cytotoxic studies of the leaves extracts were conducted using disc diffusion and brine shrimp lethality bioassay methods, respectively, while an *in vitro* thrombolytic model was used to assess the clot lysis effect of the extracts with streptokinase as positive control. Antioxidant activity was evaluated by free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide assay as well as total phenolic and flavonoid content. The leaves extracts were found to be a rich source of phytochemicals and among the extracts methanol extract was the most active against tested micro-organisms-*Shigella dysenteriae*, *Staphylococcus aureus* and *Candida albicans*. Compared to potassium permanganate with a median lethal concentration ( $LC_{50}$ ) of  $11.27 \mu\text{g mL}^{-1}$  in the brine shrimp lethality assay, the  $LC_{50}$  of n-hexane, ethyl acetate and methanol extracts were 20.09, 36.33 and  $60.12 \mu\text{g mL}^{-1}$ , respectively. All the extracts showed significant clot lysis activity with reference to control. Notable antioxidant activity of the methanol extract was observed unlike the other extracts. The results of the study demonstrated bioactivities of the leaves extracts of *A. polystachya* and therefore further studies on the isolation and identification of active principles are required.

**Key words:** *Aphanamixis polystachya*, antimicrobial, antioxidant, cytotoxic, thrombolytic, phytochemical screening

### INTRODUCTION

Currently more than half of drugs in clinical uses and about 50% of the world's 25 best-selling pharmaceutical agents are natural-product origin. As a source of chemical substances with potential therapeutic effects, medicinal plants have been used in all cultures, since times immemorial. *Aphanamixis polystachya* (Wall.) R. Parker is one of the traditional medicinal plants of the Meliaceae family (Common name: mahogany family) (Chan *et al.*, 2011). It is an evergreen timber tree with bunches of rounded subglobose fruits and glossy deep brown seeds, mainly grows in the tropical areas of Asia. The vernacular names of *A. polystachya* (Synonym: *Aphanamixis grandifolia* Blume, *Amoora rohituka*) include Roina, Pittraj (Bengali); Rohitak, Pithraj (English, Hindi), etc (Chan *et al.*, 2011).

The bark of this plant is employed traditionally as an astringent. Ayurveda recommends the boiling of *A. polystachya* root bark in abdominal complaints like enlargement of glands, liver and spleen disorders and

corpulence. Seeds have refrigerant, laxative, anthelmintic activities; used against the diseases of the blood and scale back muscular pain. Oil of the seeds is used to treat rheumatism and conjointly has pesticidal character. Bark and seeds of the plant are useful for ulcer (Hossain *et al.*, 2009).

Various parts of the plant have been reported to possess analgesic (Hossain *et al.*, 2009), antimicrobial (Chowdhury *et al.*, 2003a; Yadav *et al.*, 2010; Apu *et al.*, 2013a), antioxidant (Krishnaraju *et al.*, 2009; Sikder *et al.*, 2010; Apu *et al.*, 2013a), antitumor (Chan *et al.*, 2011), CNS depressant (Hossain *et al.*, 2009), cytotoxic (Sikder *et al.*, 2010; Apu *et al.*, 2013a), hepatoprotective (Gole and Dasgupta, 2002), insecticidal (Talukder and Howse, 1993), laxative (Chowdhury and Rashid, 2003), membrane stabilizing (Sikder *et al.*, 2010) and thrombolytic (Apu *et al.*, 2013a) activities.

The presence of variety of compounds is responsible for the diverse activities of *A. polystachya*. Stem bark contains aphanamixin and aphanamixinin (Chatterjee *et al.*, 1970), amoorinin (Agnihotri *et al.*, 1987),

sesquiterpenes (Chowdhury *et al.*, 2003b), lignin-polystachyol, two lignan glycosides- lyoniside and nudiposide and a sterol- ergosta-4,6,8(14),22-tetraen-3-one, with stigmasterol, and oleic and linoleic acids (Sadhu *et al.*, 2006). Saponin (Bhatt *et al.*, 1981), limonoids- rohituka-15 and Dregeana-1 (Zhang *et al.*, 2002), aphanamolides A and B (Yang *et al.*, 2011) have been isolated from the seeds of *A. polystachya*. Fruits of the plant are the source of triterpene- aphananin (Kundu *et al.*, 1985), limonoids- aphapolynins A and B (Zhang *et al.*, 2011), ring A-seco limonoids, aphanalides A-H (Wang *et al.*, 2012). A flavone glycoside has been reported to present in roots (Jain and Srivastava, 1985). Moreover, the plant is a source of rohitukine which is a chromane alkaloid (Harmon *et al.*, 1979).

Numbers of research works have been published, but there are no scientific report on phytochemical screening and *in vitro* bioactivities of the leaves of *A. polystachya* collected from Bangladesh. The aim of the present investigations was to assess the phytochemicals and *in vitro* antimicrobial, antioxidant, cytotoxic and thrombolytic potentiality of leaves of *A. polystachya* available in Bangladesh.

## MATERIALS AND METHODS

**Collection of plant material:** The leaves of *A. polystachya* were collected from Mymensingh, Bangladesh in February, 2011. The leaves were taken to the Bangladesh National Herbarium, Mirpur, Bangladesh, where their identity was confirmed by a taxonomist (Dr. Bushra Khan, Principal Scientific Officer) and a voucher specimen (Accession no. DACB-35449) deposited for future reference.

**Preparation and extraction of plant material:** Thoroughly clean leaves were shade-dried and crushed into coarse powder using a mechanical grinder. The powdered material (150 g×2) was successively extracted with solvents from nonpolar to polar like n-hexane (APHE), ethyl acetate (APEA) and methanol (APME) by continuous hot extraction for 6 h using Soxhlet apparatus at a temperature not exceeding the boiling point of the solvent. The extracts were concentrated with a rotary evaporator (IKA, Germany) at low temperature (40-50°C) and reduced pressure.

**Phytochemical screening:** A small portion of the dry extracts were subjected to preliminary phytochemical screening (Ayoola *et al.*, 2008) to detect the presence of various phytoconstituents in the leaves of *A. polystachya*.

**Antimicrobial screening:** Disc diffusion method (Jelodarian *et al.*, 2012) was used to evaluate the antimicrobial potential of *A. polystachya* extracts on Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Shigella dysenteriae*) and fungus (*Candida albicans*). Sterile 6 mm filter paper discs (Whatman No. 1) were impregnated with 500 and 1000 µg of the extracts of each solvent system and dried in open air to evaporate the residual solvent. Standard ciprofloxacin disc (5 µg disc<sup>-1</sup>) was used as the positive control. Two sample discs of two different concentrations, standard antibiotic disc and blank disc (impregnated with solvents followed by evaporation) were placed gently on previously marked zones in the agar plates pre-inoculated with 100 µL of suspension containing 10<sup>8</sup> CFU mL<sup>-1</sup> of bacteria and 10<sup>4</sup> spore mL<sup>-1</sup> of fungus on the nutrient agar. After incubation of bacteria plates at 37°C for 24 h and fungus plate at 25°C for 48 h, the antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm (Jelodarian *et al.*, 2012). The screening of antimicrobial activity was carried out in triplicate.

## Antioxidant activity

**Estimation of total phenolic content:** The amount of total phenolic compounds in the leaves extracts was measured as a way of determining antioxidant activity according to Folin-Ciocalteu procedure (Ainsworth and Gillespie, 2007). The plant extracts (20 mg) and 2 mL methanol (99.8 % v/v) were taken in a test tube and incubated at room temperature for 48 h in the dark; 2 mL of the solution was transferred to a centrifuge tube and centrifuged at 5000 rpm for 5 min after which 300 µL of the supernatant was mixed with 600 µL of Folin-Ciocalteu reagent (10%) (Merck, Germany) and 2.4 mL of sodium carbonate (700 mM) in a test tube and the mixture was incubated for 2 h at room temperature and the absorbance of the mixture was measured at 765 nm using a UV-VIS spectrophotometer (Shimadzu, Japan). Total phenolic content of the extracts were determined (as gallic acid equivalent) in triplicate from a standard curve ( $y = 0.037x - 0.209$ ;  $r^2 = 0.984$ ) prepared with gallic acid.

**Total flavonoid assay:** Total flavonoid content of the extracts was determined according to colorimetric method described by Jothy *et al.* (2011), with some modifications. A 0.5 mL (1 mg mL<sup>-1</sup> in 99.8% v/v methanol) leaves extract was added in test tubes and mixed with 2 mL of distilled water. Subsequently 0.15 mL of 5% w/v sodium nitrite (NaNO<sub>2</sub>) was added into each test tubes and the reaction mixture was allowed to stand for 6 min. Then 0.15 mL 10% aluminium trichloride (AlCl<sub>3</sub>) was added and allowed to stand for 6 min, followed by addition of 2 mL of 4% w/v

sodium hydroxide (NaOH) to the reaction mixture. Then distilled water was added to the mixture to bring the final volume up to 5 mL. the reaction mixture was mixed thoroughly and allowed to stand for another 15 min. Then absorbance of pink color that developed was measured at 510 nm using spectrophotometer. Distilled water was used as blank. Total flavonoid content of the extracts were determined (as catechin equivalent) in triplicate from a standard curve ( $y = 0.0071x + 0.1139$  and  $r^2 = 0.9927$ ) prepared with catechin by Jothy *et al.* (2011).

**Determination of DPPH radical scavenging activity**

**(Jain *et al.*, 2010):** The free-radical scavenging activity of *A. polystachya* leaves extracts was measured by the decrease in the absorbance of the methanol solution of a stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl). A stock solution of DPPH (Sigma-Aldrich, USA, 400  $\mu\text{g mL}^{-1}$ ) was prepared in 99.8% (v/v) methanol and 100  $\mu\text{L}$  of this stock solution was added to 5 mL of a methanol solution of *A. polystachya* extracts of varying concentrations (20-100  $\mu\text{g mL}^{-1}$ ). The solutions were mixed properly, kept in the dark for 20 min at room temperature and the absorbance measured at 517 nm against blank (methanol). Scavenging activity was calculated as:

$$\text{Inhibition (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

where,  $A_c$  is the absorbance of control (DPPH in methanol;  $\sim 8 \mu\text{g mL}^{-1}$ ) and  $A_s$  is the absorbance of the test sample. For each concentration, a separate blank sample was used for background subtraction. Inhibition (%) was plotted against the respective extract concentrations used and  $\text{IC}_{50}$  values were extrapolated from the plot. Ascorbic acid (AA), an antioxidant, was used as positive control.

**Nitric oxide scavenging assay (Jain *et al.*, 2010):** Sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with various concentrations of the extracts (5-200  $\mu\text{g mL}^{-1}$ ) dissolved in 99.8% (v/v) ethanol and incubated in the dark at room temperature for 2 h. The solution (2 mL) was mixed with 1.2 mL of Griess reagent and the absorbance measured spectrophotometrically at 546 nm against blank (phosphate buffered saline). Ascorbic acid was used as a positive control and was treated in the same way as the extract. Nitric oxide (NO) scavenging activity was computed as:

$$\text{Inhibition (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

where,  $A_c$  is the absorbance of control (solution contained every reagent except extract) and  $A_s$  is the absorbance of

the test sample. For each concentration, a separate blank sample was used for background subtraction. Inhibition (%) was plotted against the respective extract concentrations used and  $\text{IC}_{50}$  values were extrapolated from the plot.

**Cytotoxic activity:** *A. polystachya* leaves extracts were screened for their cytotoxic activity using brine shrimp lethality bioassay (Apu *et al.*, 2013b). For the experiment, 200 mg each of the extracts was dissolved in dimethylsulfoxide (DMSO) and solutions of varying concentrations were obtained by serial dilution using simulated sea water. The solutions were taken in each pre-marked test tubes containing 5 mL simulated seawater and 10 shrimp nauplii (hatched in a tank at a temperature  $\sim 37^\circ\text{C}$  and pH at 8.4 with continuous oxygen supply). After 24 h, the numbers of survivors were counted and mortality (%) was calculated for each dilution as well as for control. Percent mortality was corrected and then converted to Probit (Apu *et al.*, 2013b). The  $\text{LC}_{50}$  values (concentration of sample required to kill 50% of brine shrimp) were calculated (at the confidence interval level of 95%) using Microsoft Excel 2007 by a plot of Probit (y) against the logarithm of the sample concentrations (x). Simulated seawater containing varying quantities of DMSO were used as control. Potassium permanganate (KMn) was used as the positive control.

**In vitro thrombolytic study:** Median cubital venous blood (3 mL) was drawn from 10 healthy volunteers (5 males and females each) who had not taken any oral contraceptives or anticoagulants in the preceding two weeks. The blood was transferred to 5 pre-weighed sterile Eppendorf tubes (500  $\mu\text{L tube}^{-1}$ ) and incubated at  $37^\circ\text{C}$  for 45 min. After clot formation, serum was completely removed without disturbing the clot formed. Each tube having clot was again weighed to determine clot weight. The extract (100  $\text{mg mL}^{-1}$ , 100  $\mu\text{L}$ ) was added to the tube (Prasad *et al.*, 2006). As positive control, 100  $\mu\text{L}$  of streptokinase (CSL Behring GmbH, Germany, 3000,000  $\text{IU mL}^{-1}$ ) was used, whereas, 100  $\mu\text{L}$  of normal saline served as control. The tubes were then incubated at  $37^\circ\text{C}$  for 90 min and observed for clot lysis. The released fluid was removed and the tubes again weighed to compute the difference in weight after clot disruption. The percentage difference in weight before and after clot lysis was expressed as clot lysis (%).

**Statistical analysis:** Statistical comparisons were performed with Student's 't' tests using Microsoft Excel 2007. A p-value of 0.05 and 0.001 or less was considered to be significant and highly significant, respectively. Mean  $\pm$  SEM was calculated for the parameters where applicable.

**RESULTS**

Phytochemical screening of the extracts of leaves of *A. polystachya* revealed the presence of various bioactive components of which alkaloids, anthraquinones, cardiac glycosides, flavonoids and terpenoids were the most prominent (Table 1).

On the basis of zone of inhibition (ZOI) (Table 2), all the extracts were inactive at the lower dose against the tested microorganisms but showed partial activity against *Staphylococcus aureus* at the higher dose (ranges of ZOI: 9-10 mm). Against the fungus, the leaves extracts were inactive (ZOI: <9 mm) or unable to create clear zone around the discs at both doses. At the higher dose, APEA and APME showed partial activity (ZOI: 10 mm) against *Shigella dysenteriae*.

The crude extracts possess promising antioxidant activity which can be concluded from the results (Table 3)

of the total phenolic content, total flavonoid assay and free radical scavenging activity (DPPH and NO). Among the extracts, ethyl acetate extract (APEA) exhibited the highest total phenolic content as well as high radical scavenging activity (low IC<sub>50</sub> values), comparable to that of positive control used in the tests. Flavonoid content was higher in non-polar solvent extract (APHE) than the polar solvent extracts (APEA and APME).

In brine shrimp lethality bioassay, % mortality increased gradually with the increased in concentration of the test samples. The cytotoxic potentiality exhibited (Table 3) by *A. polystachya* leaves extracts were very promising, because Peteros and Uy (2010) reported that the crude extracts that show LC<sub>50</sub> values < 100 µg mL<sup>-1</sup> are considered significantly active.

Among the *A. polystachya* leaves extracts, statistically highly significant (p<0.001) clot lysis activity was showed by n-hexane (APHE) and methanol (APME)

Table 1: Preliminary phytochemical screening of leaves extracts of *A. polystachya*

Phytoconstituents	Tests	Observations	APHE	APEA	APME
Alkaloids	Hager's test	Yellow color precipitation	+	+	++
Anthraquinones	Chloroform layer test	Rose pink color	+++	+	+
Cardiac glycosides	Killer-Killani's test	Brown ring at the interface	++	+	+
Flavonoids	Ammonia test (modified)	Yellow coloration	++	+	++
Reducing sugars	Fehling's test	Red precipitation	-	-	-
Saponins	Frothing test	Emulsion formation	-	+	-
Steroids	Salkowski test	Yellow green fluorescent color	-	-	-
Tannins	FeCl <sub>3</sub> test	Brownish green or blue black color	-	-	+
Terpenoids	Salkowski test (modified)	Red violet color	+++	+++	+++

+++; Highly present, ++: Moderately present, +: Slightly present, -: Absent; APHE, APEA and APME denote n-hexane, ethyl acetate and methanol extracts, respectively

Table 2: Antimicrobial activity of n-hexane (APHE), ethyl acetate (APEA), methanol (APME) extracts of *A. polystachya* leaves, positive control ciprofloxacin (CP) and negative control (respective solvents)

Name of microorganisms	Types	Negative control	Zone of inhibition (mm): Mean±SEM (n=3)						CP (5 µg disc <sup>-1</sup> )
			APHE		APEA		APME		
			500 µg disc <sup>-1</sup>	1000 µg disc <sup>-1</sup>	500 µg disc <sup>-1</sup>	1000 µg disc <sup>-1</sup>	500 µg disc <sup>-1</sup>	1000 µg disc <sup>-1</sup>	
<i>Staphylococcus aureus</i>	Gram(+ve) bacteria	-	-	9.00±0.12	-	9.13±0.15	8.53±0.09	10.27±0.15	42.67±1.33
<i>Shigella dysenteriae</i>	Gram(-)ve bacteria	-	-	-	7.20±0.21	10.30±0.15	7.60±0.21	10.07±0.12	38.67±1.45
<i>Candida albicans</i>	Fungus	-	-	-	-	-	-	8.10±0.21	49.33±0.88

Zone of inhibition (ZOI, mm): <9, inactive; 9-12, partially active; 13-18, active; >18, very active (Smania *et al.*, 1995), -: No zone of inhibition

Table 3: Antioxidant and cytotoxic activities of extracts of *A. polystachya* leaves

Extracts	LC <sub>50</sub> ±SEM (µg mL <sup>-1</sup> ) (n=3)	R <sup>2</sup>	95% CI		Extracts	Total phenols	Total flavonoids	DPPH radical scavenging activity IC <sub>50</sub> (µg mL <sup>-1</sup> )	Nitric oxide scavenging assay IC <sub>50</sub> (µg mL <sup>-1</sup> )
			Upper limit	Lower limit		Mean±SEM (n=3) (in mg g <sup>-1</sup> , Gallic acid equivalents)	Mean±SEM (n=3) (in mg g <sup>-1</sup> , Catechin equivalents)		
APHE	20.09±1.44	0.955	22.91	17.27	APHE	92.09±2.58	504.84±3.84	74.84	76.15
APEA	36.33±0.33	0.960	36.98	35.68	APEA	107.77±1.97	376.20±15.06	50.07	39.90
APME	60.12±0.65	0.877	61.39	58.84	APME	59.15±2.41	240.52±1.88	12.54	57.58
KMN*	11.27±0.90	0.790	13.03	9.51	AA*			76.11	34.06

\*Positive control, CI: Confidence interval

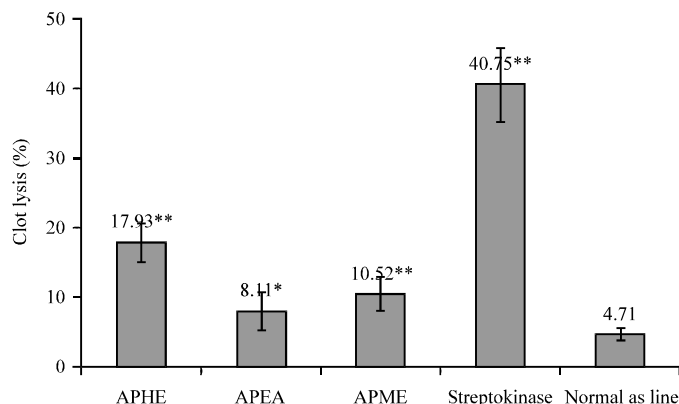


Fig. 1: Clot lysis of blood samples of normal subjects by APHE, APEA and APME extracts of *A. polystachya* leaves, streptokinase and normal saline (n =10, \*\*p<0.001, \*p<0.05 compared with control)

extracts whereas, ethyl acetate (APEA) extract showed significant (p<0.05) clot lysis activity compared to control (Fig. 1). % clot lysis exhibited by APHE and APME were 17.93 and 10.52, respectively.

## DISCUSSION

Krishnaraju *et al.* (2009) and Chowdhury *et al.* (2003a) have reported the presence of alkaloids, tannins, steroids, saponins, glycosides, flavonoids, anthraquinones and terpenoids in *A. polystachya*. As the previous results, current phytochemical screening (Table 1) reveals the presence of different secondary metabolite categories. These are best-known to possess therapeutic activities against several diseases and therefore could counsel its ancient uses for the treatment of varied sickness.

At the 1000 µg disc<sup>-1</sup>, only methanol extract of *A. polystachya* leaves (APME) showed activity against all the tested microorganisms (Table 2). This weak antimicrobial activity may be due to the presence of insufficient quantity of antimicrobial agents in the leaves which were primarily polar in nature. Alkaloids, flavonoids and terpenoids were abundantly present in the solvent extracts (Table 1) reported to have antimicrobial activity (Paiva *et al.*, 2010). These molecules are associated to defense mechanisms of plants by their repellent or attractive properties. The polar solvent extracts of bark showed higher antibacterial property compared to non-polar solvent extracts (Chowdhury *et al.*, 2003a; Yadav *et al.*, 2010).

It is known that most polyphenolic compounds (phenolic acids, flavonoids) are extremely reactive scavengers of free radical due to their ability to produce stable free radical intermediates (Govindappa *et al.*, 2011).

The extracts, therefore, might act as effective antioxidants (Table 3). The *in vitro* antioxidant assay using extracts (APHE, APEA and APME) showed IC<sub>50</sub> values less than positive control, vitamin C in *in vitro* DPPH radical scavenging activity; whereas, IC<sub>50</sub> values were high in comparison to positive control in nitric oxide scavenging activity (Table 3). Therefore, for the prevention of free radical-mediated diseases, the extracts could be used potentially. Krishnaraju *et al.* (2009) reported similar pattern in DPPH free radical scavenging activity by the bark extracts of *A. polystachya*.

From the results of brine shrimp lethality bioassay (Table 3), it can be concluded that the n-hexane (APHE), ethyl acetate (APEA) and methanol (APME) extracts of *A. polystachya* leaves showed potent *in vitro* toxicity compared to positive control as the extracts showed lower LC<sub>50</sub> values than 100 µg mL<sup>-1</sup> (Peteros and Uy, 2010). Some of the phytochemicals present in the plant are basically carcinogenic in nature. Potent antioxidant and cytotoxic activities of bark were also reported by Sikder *et al.* (2010).

The presence of alkaloids, anthraquinones, flavonoids and terpenoids in higher quantities could be responsible for the clot lysis activity (Dwivedi, 2007) of APHE and APME extracts (Fig. 1). The compounds that are present in the extracts could be isolated and used as potent thrombolytic agent against thrombosis disorders.

## CONCLUSION

Significant antioxidant, cytotoxic and thrombolytic activities were demonstrated by the leaves extracts of *Aphanamixis polystachya*. These extracts also possess partial antimicrobial activity at higher concentration. Based on these preliminary studies, further investigations

can be carried out to explore the bioactive molecules which are responsible for numerous activities with mechanism of actions.

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