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ANTIOXIDANT, CYTOTOXICITY AND ANTIMICROBIAL ACTIVITIES OF *APHANAMIXIS POLYSTACHYA* (WALL.) R. N. PARKER

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Abstract

Antioxidant, cytotoxicity and antimicrobial activities of methanol extract and its different soluble partitionates of the bark and leaves of *Aphanamixis polystachya* (Wall.) R. N. Parker were evaluated. In the antioxidant assay by DPPH method, the crude methanolic extract of bark and chloroform soluble fraction of crude extract of leaves of *A. polystachya* revealed the highest free radical scavenging activity with IC_{50} values of 5.36 ± 0.85 and 8.41 ± 0.83 µg/ml, respectively. The highest amount of phenolics, i.e. 29.25 ± 0.75 mg of gallic acid equivalent (GAE)/g of extractives was observed in crude methanolic extract of bark, and 26.0 ± 0.54 mg of GAE/g of extractives in chloroform soluble fraction of methanolic extract of leaves. The chloroform soluble fraction of crude extract of bark, and petroleum ether soluble fraction of methanolic extract of *A. polystachya* displayed the highest cytotoxic potential having LC_{50} values of 0.67 ± 0.64 and 0.68 ± 0.25 µg/ml, respectively, as compared to standard vincristine sulphate (LC_{50} value of 0.41± 0.55 µg/ml). In case of antibacterial screening, the chloroform soluble fraction of bark and crude extract of leaves of *A. polystachya* revealed the highest zone of inhibition of 11.0 and 10.0 mm, respectively against *Bacillus subtilis*.

Introduction

World Health Organization (WHO) depicts that majority of the world's population nowadays depends on traditional medicines for their primary healthcare. Plants used frequently to treat common diseases in rural areas as conventional medicines are either too expensive or not available (Adamu *et al.* 2004). The demand for more drugs from plant sources is increasing and consequently there is a need to screen unexplored medicinal plants with promising biological activities in order to discover novel drug candidates (Chowdhury *et al.* 2009).

Aphanamixis polystachya (Wall.) R. N. Parker (Synonym: *Amoora rohituka*, English name: Amoora, Bengali name: *Pitraj, Royna*, Family: Meliaceae) is a medium-sized evergreen tree and distributed in India, Pakistan, Nepal, Bhutan, Myanmar and Sri Lanka (Ahmed *et al.* 2009). The plant is characterized by its compound leaves, more or less supra-axillary inflorescence, 5 reddish sepals and cream to yellow coloured petals. Anthers are 6 in number, stigmas are 3-lobed and fruits with 1-3 deep brown seeds. It grows in low lands as well as forests. In Bangladesh, this species occurs in the forests of Chittagong, Cox's Bazar, Gazipur, Mymensingh, Sherpur, Tangail and Sylhet districts, and the Chittagong Hill Tracts (Ahmed *et al*. 2009). *A. polystachya* is used in the treatment of liver disorders, tumor, ulcer, dyspepsia, intestinal worms, skin diseases, diabetes, eye diseases, jaundice, hemorrhoids, burning sensation, rheumatoid arthritis and leucorrhoea (Malviya *et al.* 2012).

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Previous phytochemical investigations on *A. polystachya* led to the isolation of fatty acids, glycosides, limonoid, flavonoid, sterol, saponin and triterpenoids (Mishra *et al.* 2014). Several studies were made on pharmacological activities of *A. polystachya*. Apu *et al.* (2013) carried out phytochemical screening and *in vitro* evaluation of pharmacological activities of fruit extracts of *A. polystachya*, while Leo *et al.* (2011) demonstrated cytotoxic effects of *Amoora rohituka* on breast and pancreatic cancer cells. Talukder and Miyata (2002) paid attention on *in vivo* and *in vitro* toxicities of seed extracts of Pithraj (*A. polystachya*) and Neem (*Azadirachta indica*) against rice green leaf hopper. Recently chemical composition and antimicrobial activity of leaf and fruit oils from *Amoora rohituka* have been investigated (Aboutabl *et al.* 2000, Chowdhury *et al.* 2003). However, no detailed study on antioxidant, cytotoxicity and antimicrobial potential of stem bark and leaves of *A. polystachya* was carried out so far. The objectives of the present study are to investigate antioxidant potential, cytotoxic, and antimicrobial activities of *Aphanamixis polystachya* occurring in Bangladesh.

Materials and Methods

Both the bark and leaves of *Aphanamixis polystachya* were collected from Bhatira, Kaliganj under Gazipur district of Bangladesh in June 2012. The plant has been identified in Dhaka University Salar Khan Herbarium (DUSH). The voucher specimens for this plant have been maintained both in DUSH and Bangladesh National Herbarium (DACB), Dhaka, Bangladesh for future reference.

The collected plant materials were cleaned, sun dried and pulverized. The powdered materials (500 g each) from both bark and leaves were separately soaked in 2.5 litre of methanol at room temperature for 7 days. The extracts were filtered through fresh cotton bed and finally with Whatman filter paper No. 1. The filtrates were concentrated with a rotary evaporator at reduced temperature and pressure. An aliquot (5 g) of each of the concentrated methanol extracts was fractionated by the modified Kupchan partitioning protocol (Van Wagenen *et al.* 1993) and the resultant partitionates were evaporated for dryness to yield pet-ether soluble fraction (PESF), chloroform soluble fraction (CHSF), ethyl acetate soluble fraction (EASF) and aqueous soluble fraction (AQSF) (Table 1). The residues were then stored in a refrigerator until further use.

Table 1. Kupchan partitionates of bark and leaves of *Aphanamixis polystachya*.

Crude extract/ fractions	Bark (g)	Leaves (g)
Methanolic crude extract (ME)	5.00	5.00
Pet-ether soluble fraction (PESF)	0.75	0.85
Chloroform soluble fraction (CHSF)	0.55	0.65
Ethyl acetate soluble fraction (EASF)	0.40	0.30
Aqueous soluble fraction (AQSF)	2.50	2.65

Antioxidant activity of the test samples was assessed by evaluating the scavenging activities of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical by using synthetic antioxidants like butylated hydroxytoluene (BHT) and ascorbic acid as positive controls (Brand-Williams *et al.* 1995). An amount of 2.0 ml of methanol solution of the sample (extractives/ control) at different concentration (500 to 0.977 µg/ml) were mixed with 3.0 ml of a DPPH in methanol (20 µg/ml). After 30 min reaction period at room temperature in dark place the absorbance was measured at 517 nm against methanol as blank by UV spetrophotometer. Inhibition of free radical by DPPH in per cent (I%) was calculated as follows:

$$I\% = (1 - A_{sample}/A_{blank}) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test material), and A_{sample} is the absorbance by the test sample.

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted by percentage of inhibition against extract concentration.

The total phenolic content of the extractives was determined with Folin-Ciocalteau reagent following the method developed by Harbertson and Spayd (2006). An amount of 0.5 ml of extract solution (conc. 2 mg/ml), 2.5 ml of Folin-Ciocalteu reagent (diluted 10x with water) and 2.0 ml of Na_2CO_3 (7.5% w/v) solution was taken in a test tube. The mixture was incubated for 20 min at room temperature, and the absorbance was measured at 760 nm by UV-spectrophotometer. The total phenolic content of the sample was determined using the standard curve prepared from gallic acid solution of different concentration. It was expressed as mg of GAE (gallic acid equivalent)/g of the extractives.

Brine shrimp lethality technique was employed for determination of general toxic properties of the dimethylsulfoxide (DMSO) solution of plant extractives against *Artemia salina* in a single day assay using vincristine sulphate as positive control (Meyer *et al.* 1982). Brine shrimp eggs were hatched in simulated sea water to get nauplii. By addition of calculated amount of DMSO, the desired concentration of the test sample was prepared. The nauplii were counted by visual inspection and were taken in vials containing 5 ml of simulated sea water. Then samples of different concentrations were added to the pre-marked vials through micropipette. The vials were then left for 24 hrs and survivors were counted. The lethal concentration LC_{50} of the test samples after 24 hrs was obtained by a plot of percentage of the shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis.

Antimicrobial activity was determined by the disc diffusion method (Bauer *et al.* 1966). Dried and sterilized filter paper discs (6 mm in diameter) containing the test samples of known amounts were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic (Ciprofloxacin) discs and blank discs were used as positive and negative control, respectively. These plates were kept at 4°C for 24 hrs to allow maximum diffusion of the test materials to the surrounding medium (Barry 1976). The plates were then inverted and incubated at 37°C for 24 hrs for optimum growth of the organisms. The test materials having antimicrobial property inhibited microbial growth in the medium surrounding the discs and thereby yielded a clear, distinct zone of inhibition. The antimicrobial activity of the test agent was then determined by measuring the diameter of zone of inhibition expressed in millimeter (Barry 1976, Bauer *et al.* 1966).

For all bioassays, three replicates of each sample were used for statistical analysis and the values are expressed as mean ± Sd.

Results and Discussion

The crude methanol extracts of bark and leaves of *Aphanamixis polystachya* and their Kupchan partitionates were evaluated for the total phenolic content, free radical scavenging, cytotoxic and antimicrobial activities. In the DPPH free radical scavenging assay, the crude methanol extract of bark of *A. polystachya* revealed maximum free radical scavenging activity (IC_{50} = 5.36 ± 0.85 µg/ml) when compared to ascorbic acid (IC_{50} = 2.06 ± 0.25 µg/ml). The total phenolic content of the extractives of bark of *A. polystachya* was found to be 4.12 - 29.25 mg of GAE/g of extractives, with the highest amount of phenolics (29.25 ± 0.75 mg of GAE/g of extractives) being observed in the crude methanol extract (Fig. 1).

Among the test samples of leaves of *A. polystachya*, the chloroform soluble fraction demonstrated the highest free radical scavenging activity (IC_{50} = 8.41 ± 0.83 µg/ml). Among the extracts of leaves of *A. polystachya*, the total phenolic content was found to be 12.87 - 26.0 mg of GAE/g. The maximum phenolic content (26.0 ± 0.54 mg of GAE/g) was observed in the chloroform soluble fraction (Fig. 2).

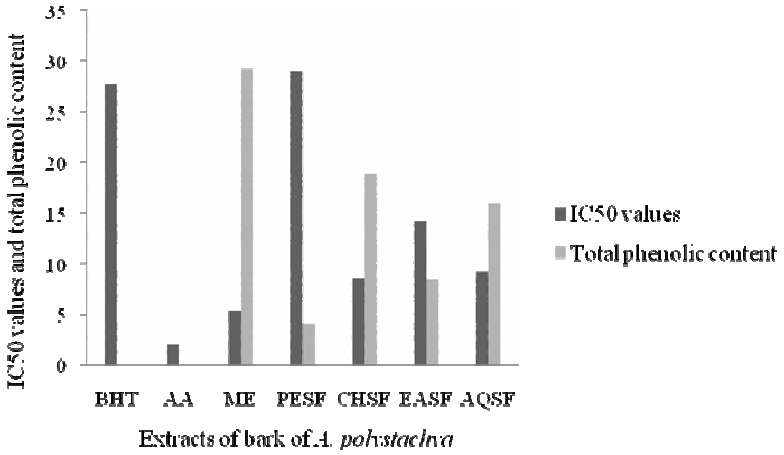

Fig. 1. IC_{50} values and total phenolic content of different extracts of bark of *A. polystachya*. BHT= Butylated hydroxytoluene; AA = Ascorbic acid; ME = Crude methanolic extract; PESF = Petroleum ether soluble fraction; CHSF = Chloroform soluble fraction; EASF = Ethyl acetate soluble fraction; AQSF = Aqueous soluble fraction.

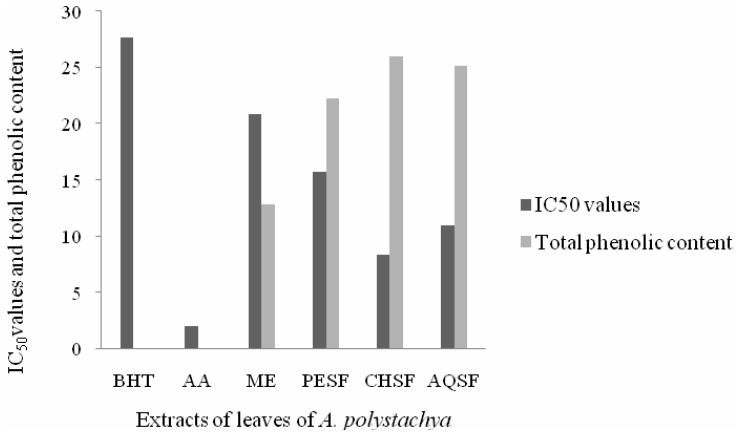

Fig. 2. IC_{50} values and total phenolic content of different extracts of leaves of *A. polystachya*. BHT= Butylated hydroxytoluene; AA= Ascorbic acid; ME= Crude methanolic extract; PESF= Petroleum ether soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction.

The present study revealed significant cytotoxic activities of crude methanolic extract, pet-ether, chloroform, ethyl acetate and aqueous soluble fraction of bark of *A. polystachya* with LC_{50} values 2.46, 1.39, 0.67, 2.03, 2.11 µg/ml, respectively. In the brine shrimp lethality bioassay, the chloroform soluble fraction of bark of *A. polystachya* displayed the highest cytotoxic potential

with LC_{50} value 0.67 ± 0.64 µg/ml compared to 0.41± 0.55 µg/ml for vincristine sulphate. In the case of leaf the crude methanolic extract, pet-ether, chloroform and aqueous soluble fraction showed cytotoxic activities with LC_{50} values 1.33, 0.68, 1.21, 1.66 µg/ml, respectively (Table 2). The highest cytotoxic potential with LC_{50} value 0.68 ± 0.25 µg/ml was found in pet-ether soluble fraction of leaves of *A. polystachya*. Previous study showed the cytotoxic activities of crude methanolic extract, pet-ether and chloroform soluble fraction of *A. polystachya* with LC_{50} values 11.0, 10.36 and 16.45 µg/ml by brine shrimp lethality bioassay (Majumder *et al.* 2014). In the present study, the mass potent cytotoxic activity of the extractives could be explained by the variable nature and amount of phyto-constituents with change of geographical location, condition of soil etc. Rabi *et al.* (2002) showed cytotoxicity of amooranin and its derivatives isolated from stem bark against MCF-7 and HeLa cells, whereas present study was limited to the cytotoxic activities of bark and leaves of *A. polystachya* only in brine shrimp lethality bioassay.

Table 2. Cytotoxic activities of bark and leaves of *Aphanamixis polystachya.*

Plant parts	Sample/ Standard	Cytotoxicity (LC_{50}) (µg/ml)
Bark	Methanolic crude extract (ME)	2.46 ± 0.14
	Pet-ether soluble fraction (PESF)	1.39 ± 0.21
	Chloroform soluble fraction (CHSF)	0.67 ± 0.64
	Ethyl acetate soluble fraction (EASF)	2.03 ± 0.41
	Aqueous soluble fraction (AQSF)	2.11 ± 0.59
Leaves	Methanolic crude extract (ME)	1.33 ± 0.91
	Pet-ether soluble fraction (PESF)	0.68 ± 0.25
	Chloroform soluble fraction (CHSF)	1.21 ± 0.62
	Aqueous soluble fraction (AQSF)	1.66 ± 0.07
	Vincristine sulphate (VS)	0.41± 0.55

The extracts of bark of *A. polystachya* when screened for antibacterial activity against five Gram positive and eight Gram negative bacteria at a concentration of 400 µg/ disc, the test samples revealed mild to moderate inhibitory activity against the pathogens having zone of inhibition ranging from 7.0 - 11.0 mm, with the highest inhibition of bacterial growth by the chloroform soluble fraction (11.0 mm) against *Bacillus subtilis* (Table 3). Likewise, the extracts of leaves of *A. polystachya* revealed similar inhibitory activity against the test pathogens having zone of inhibition ranging from 7.0 - 10.0 mm, with the highest inhibition of bacterial growth of the crude methanol extract (10.0 mm) in *B. subtilis* (Table 4). On the other hand, the aqueous soluble fraction exhibited no antimicrobial activity and its other fraction demonstrated antimicrobial activity. The inhibitory activity of the extracts was compared with ciprofloxacin as standard in both cases.

Our results on antibacterial properties of *A. polystachya* have been found consistent with that of Chowdhury *et al.* (2003) where they showed that stem bark of *A. polystachya* exhibited the antibacterial activity. Aboutabl *et al.* (2000) showed potential antibacterial activity in leaf and fruit oil of *Amoora rohituka*, and Mishra *et al.* (2014) exhibited significant antimicrobial activity in stem bark of *A. polystachya*, whereas, our present study revealed mild to moderate antibacterial activity in bark and leaves of *A. polystachya*.

Table 3. Antibacterial activity of bark of *Aphanamixis polystachya*.

Test microorganisms	Diameter of zone of inhibition (mm)					
	ME	PESF	CHSF	EASF	AQSF	Ciprofloxacin
Gram positive bacteria						
Bacillus cereus	-	-	-	8.0	-	42.0
B. megaterium	-	-	-	7.0	-	45.0
B. subtilis	7.0	10.0	11.0	8.0	8.0	40.0
Staphylococcus aureus	8.0	8.0	9.0	7.0	7.0	39.0
Sarcina lutea	-	-	-	-	-	43.0
Gram negative bacteria						
Escherichia coli	-	-	-	8.0	-	45.0
Pseudomonas aeruginosa	-	-	-	-	-	43.0
Salmonella paratyphi	-	8.0	7.0	7.0	-	41.0
S. *typhi*	8.0	8.0	7.0	8.0	-	43.0
Shigella boydii	8.0	8.0	7.0	8.0	-	40.0
Sh. dysenteriae	8.0	7.0	8.0	7.0	-	40.0
Vibrio mimicus	7.0	-	9.0	8.0	-	40.0
V. parahemolyticus	7.0	7.0	7.0	7.0	-	42.0

Table 4. Antibacterial activity of leaves of *Aphanamixis polystachya*.

Test microorganisms	Diameter of zone of inhibition (mm)				
	ME	PESF	CHSF	AQSF	Ciprofloxacin
Gram positive bacteria					
Bacillus cereus	8.0	8.0	8.0	-	42.0
B. megaterium	8.0	9.0	9.0	-	45.0
B. subtilis	10.0	-	-	-	40.0
Staphylococcus aureus	-	-	9.0	-	39.0
Sarcina lutea	8.0	7.0	8.0	-	43.0
Gram negative bacteria					
Escherichia coli	9.0	8.0	8.0	-	45.0
Pseudomonas aeruginosa	7.0	-	-	-	43.0
Salmonella paratyphi	8.0	7.0	9.0	-	41.0
S. *typhi*	-	-	8.0	-	43.0
Shigella boydii	8.0	8.0	7.0	-	40.0
Sh. dysenteriae	-	-	7.0	-	40.0
Vibrio mimicus	8.0	7.0	8.0	-	40.0
V. parahemolyticus	8.0	-	-	-	42.0

Finally, it is clearly evident from the present study that the bark and leaves of *A. polystachya* have significant free radical scavenging, cytotoxic and mild to moderate antibacterial properties. *A. polystachya* is used in the treatment of cancer. Our findings justify the traditional use of this plant. Therefore, the plant is good candidate for further chemical investigation to isolate the active constituents.

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