In vitro Membrane Stabilizing Activity, Total Phenolic Content, Free Radical Scavenging and Cytotoxic Properties of *Aphanamixis polystachya* (Wall.)

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Abstract

Different extractives of bark of *Aphanamixis polystachya* were evaluated by using free radical scavenging (DPPH) effect along with their membrane stabilizing and cytotoxic activities. The total phenolic content was also determined and expressed in gallic acid equivalent. Here, butylated hydroxytoluene (BHT) and ascorbic acid (ASA) were used as standard antioxidants while vincristine sulphate was utilized as standard cytotoxic agent. The membrane stabilizing activity was assessed by using erythrocyte in hypotonic solution and was compared with acetyl salicylic acid. A positive correlation was seen between total phenolic content and free radical scavenging activity of *A. polystachya* having correlation coefficient (R^2) of 0.80. In the present studies the pet-ether soluble partitionate of methanolic extract of stem bark of *A. polystachya* demonstrated strong membrane stabilizing activity and antioxidant potentials whereas the crude extract and its chloroform soluble fraction revealed moderate membrane stabilizing activity and significant antioxidant potentials.

Keywords: *Aphanamixis polystachya*, membrane stabilizing, antioxidant, free radical scavenging, brine shrimp lethality bioassay.

Introduction

The effect of synthetic and herbal anti-inflammatory agents on the stabilization of erythrocyte membrane exposed to hypotonic solution has been studied extensively. The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane (Omale and Okafor, 2008). Recently, attention is being focused on the protective biochemical functions of naturally occurring elements. On the other hand, a common theme which underlies etiology of several degenerative disorders is free radical stress. The production of free radicals is inextricably linked to the inflammatory process (Grimble, 1994; Halliwell, 1997). A potent scavenger of these free radical species may serve as a possible preventive intervention for free radical mediated diseases (Ames *et al.*, 1995). They are the vital substances that possess the ability to protect the body from damage caused by free radical induced oxidative stress (Yoshida *et al.*, 1993; Souri *et al.*, 2004). Although the body possesses such defense mechanisms, as enzymes and antioxidant nutrients, which arrest the damaging properties of ROS (Halliwell *et al.*, 1995; Sies 1993), continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them, and cause irreversible oxidative damage (Tseng *et al.*, 1997). Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention and therapeutics of diseases in which oxidants or free radicals are implicated (Soares *et al.*, 1997). In this respect, polyphenolic compounds, like flavonoids and phenolic acids, commonly found in plants have been reported to have multiple biological effects, including antioxidant activity (Brown and Rice-Evans, 1998; Gil *et al.*, 1999; Kahkonen *et al.*, 1999; Vinson *et*

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al., 1995). Currently, the possible toxicity of synthetic antioxidants has been criticized. Thus, interest in natural antioxidants, especially of plant origin, has greatly increased in recent years (Jayaprakash and Rao, 2000).

Aphanamixis polystachya (Wall.) (Bengali: Pithraj) is an evergreen tree that grows wild and planted in many districts of Bangladesh (Ghani 1998). The bark a has strong astringent and antimicrobial activities and is known to be used for the treatment of liver and spleen diseases, rheumatism and tumors (Chopra *et al.,* 1956; Graham *et al.,* 2000; Choudhury *et al.,* 2003)

In the present study, the organic soluble materials of a methanol extract of the bark and its different organic soluble partitionates were evaluated for the antioxidant activity in terms of total phenolic content, free radical scavenging activity and membrane stabilizing capability of *A. polystachya* for the first time. Attempt has been taken to establish a correlation between the free radical scavenging activity and total phenolics in the extractives.

Materials and Methods

Plant materials

The bark of *A. polystachya* was collected from Dhaka, Bangladesh, in July 2009. The sun dried and powdered bark (800 gm) of *A. polystachya* was macerated in 2.5 L of methanol for 7 days and then filtered through a cotton plug followed by Whatman filter paper number 1. The extract was concentrated with a rotary evaporator at low temperature (40-45ºC) and reduced pressure. The concentrated methanolic extract was partitioned by modified Kupchan method (Van Wagenen *et al.,* 1993) and the resultant partitionates i.e., pet-ether (PESF), carbon tetrachloride (CTCSF), chloroform (CSF), and aqueous (AQSF) soluble fractions were used for the experimental processes.

Membrane stabilizing activity

The membrane stabilizing activity of the extractives was assessed by using hypotonic solution induced mice erythrocyte hemolysis designed by Shinde *et al*., 1999. To prepare the erythrocyte suspension, whole blood was obtained using syringes (containing anticoagulant EDTA) from mice through cardiac puncture. The blood was centrifuged and blood cells were washed three times with solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min at 3000 g. The test sample consisted of stock erythrocyte (RBC) suspension (0.50 mL) mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extract (2.0 mg/mL) or acetyl salicylic acid (0.1 mg/mL). The control sample consisted of 0.5 mL of RBCs mixed with hypotonic-buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 *g* and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of either hemolysis or membrane stabilization was calculated using the following equation-% inhibition of hemolysis = 100 x (OD_1-OD_2/OD_1), Where, OD_1 = Optical density of hypotonic-buffered saline solution alone (control) and OD_2 = Optical density of test sample in hypotonic solution

Total phenolics analysis

Total phenolic content of *A. polystachya* extractives was measured by employing the method described by Skerget *et al*., 2005 involving Folin-Ciocalteu reagent as an oxidizing agent and gallic acid as a standard. To 0.5 ml of extract solution (2 mg/ml) in water, 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of sodium carbonate (7.5 % w/v) solution were added. After 20 minutes of incubation at room temperature the absorbance was measured at 760 nm using a UV-visible spectrophotometer. Total phenolics were quantified by calibration curve obtained from measuring the known concentrations of gallic acid (0-100 µg/ml) and were expressed as gm of GAE (gallic acid equivalent) / 100 gm of the dried extract.

Free radical scavenging activity

The free radical scavenging activity (antioxidant capacity) of the plant extractives on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was estimated by the method established by Brand-Williams *et al.,* 1995. Here, 2.0 ml of a methanol solution of the sample (extractive/ standard) at different concentration (500 µg/ml to 0.977 µg/ml) were mixed with 3.0 ml of a DPPH methanol

solution (20 µg/ml). After 30 min of reaction at room temperature in dark place the absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer. Inhibition of free radical DPPH in percent (I %) was calculated as follows:

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

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test material). Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted with inhibition percentage against extractive/standard concentration.

Brine shrimp lethality bioassay

Brine shrimp lethality bioassay (Meyer *et al.,* 1982 and McLaughlin et al., 1998) technique was applied for the determination of general toxic property of the plant extractives. DMSO solutions of the samples were applied against *Artemia salina* in a 1-day *in vivo* assay. For the experiment, 4 mg of each of the pet - ether, carbon tetrachloride and chloroform soluble fractions were dissolved in DMSO and solutions of varying concentrations (400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.781 µg/ml) were obtained by serial dilution technique using DMSO. Vincristine sulphate was used as positive control.

Statistical analysis

Three replicates of each sample were used for statistical analysis and the values are reported as mean ± SD. Correlation analysis of total phenolic content versus free radical scavenging activity was carried out using the correlation and regression program.

Results and Discussion

The present study was undertaken to evaluate the membrane stablizing activity of different organic soluble materials of the methanol extract of *A. polystachya*. The extractives of *A. polystachya* at concentration 2.0 mg/mL significantly protected the lysis of mice erythrocyte membrane induced by hypotonic solution, as compared to the standard acetyl salicylic acid (0.10 mg/mL) (Table 1). The pet-ether soluble extract (PESF) produced 57.51% inhibition of hemolysis of RBC as compared to 71.9% produced by acetyl salicylic acid (0.10 mg/mL). The methanol and carbon tetrachloride soluble extractives also revealed significant inhibition of hemolysis of RBC.

Table 1: Effect of extractives of *A. polystachya* on hypotonic solution-induced hemolysis of erythrocyte membrane

Sample code	Conc.	Absorbance	% inhibition of haemolysis
Hypotonic medium	50 mM	3.225±0.02	--
MEF	2 mg/mL	1.481±0.039	54.07
PESF	2 mg/mL	1.371±0.04	57.51
CTCSF	2 mg/mL	1.514±0.02	53.54
CSF	2 mg/mL	1.721±0.02	46.63
AQSF	2 mg/mL	2.26±0.01	29.92
Acetyl salicylic acid	0.10 mg/mL	0.906±0.004	71.9

The average values of three calculations are presented as mean ± S.D. (standard deviation)

The total phenolic content varied for different partitionates ranging from 39.29 gm to 157.26 gm of GAE/100 gm of dried extract (Table 2). The highest total phenolics was found in PESF (157.26 gm of GAE/100 gm of dried extract) and the lowest in AQSF (39.29 gm of GAE/100 gm of dried extract).

Table 2: The total phenolic content, free radical scavenging and cytotoxic activities of different partitionates of *A. polystachya*

Sample	**Total Phenolic Content** (gm of GAE/100 gm of dried extract)	**Free Radical Scavenging Activity** (IC_{50} µg/ml)	**Cytotoxic activity** (LC_{50} µg/ml)
VS	-	-	0.451±0.004
BHT	-	27.5±0.54	-
ASA	-	5.8±0.21	-
MEF	145.09±1.35	72.16±0.65	4.305±0.012
PESF	157.16±1.56	51.28±1.38	6.966±0.004
CTCSF	102.07±0.71	118.43±1.2	8.59±0.11
CSF	141.11±1.26	108.26±1.0	3.899±0.10
AQSF	39.29±0.24	149.84±1.5	2.193±0.09

The average values of three calculations are presented as mean ± S.D. (standard deviation); VS = Vincristine sulphate; BHT = Butylated Hydroxy Toluene; ASA = Acetyl Salicylic Acid; MEF = Methanolic extract; PESF = Pet-ether soluble fraction; CTCSF = Carbon tetrachloride soluble fraction; CSF= chloroform soluble fraction; AQSF = Aqueous soluble fraction of the methanolic extract of *A. polystachya.*

In the DPPH assay for free radical scavenging property the most potent fraction was found to be pet-ether soluble extract (PESF) (Table 2). Free radical scavenging activity of the PESF was highest having IC_{50} value of 51.28 µg/ml. On the other hand MEF and CSF demonstrated moderate free radical scavenging activity with the IC_{50} value of 72.16 µg/ml and 108.26 µg/ml, respectively (Table 2).

The correlation analysis revealed that correlation exists between total phenolic content and free radical scavenging activity. The correlation coefficient (R^2) for the total phenolics and free radical scavenging (Figure 1) was 0.80 indicating a positive relationship between the total phenolics and free radical scavenging activity. This result suggests that 80% of the free radical scavenging activity resulted from the contribution of the phenolic compounds (Hajimahmoodi *et al.*, 2008). Different secondary metabolites, such as volatile oils, carotenoids and vitamins may also contribute to the antioxidant capacity, which in this case contributed to approximately 20% of the antioxidant activity (Odabasoglu *et al.*, 2005).

In case of brine shrimp lethality bioassay, the lethality of the methanol extract (MEF) and its pet-ether (PESF), carbon tetrachloride (CTCSF), chloroform (CSF) and aqueous (AQSF) soluble fractions were evaluated against *A. salina*. Table 2 shows the results of the brine shrimp lethality testing after 24 hours of exposure to the samples and the positive control, vincristine sulphate (VS). The aqueous soluble fraction (AQSF) showed potent cytotoxic activity having LC_{50} of 2.193 µg/ml as compared to 0.451 µg/ml for vincristine sulphate.

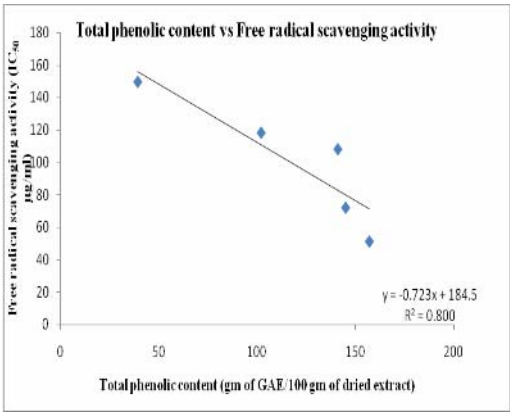

Figure 1: Correlation between the total phenolic content and free radical scavenging activity

In our preliminary studies, the extracts of *A. polystachya* demonstrated strong membrane stabilizing activity, moderate antioxidant activity, reducing power and free radical scavenging activity.

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