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A Study on *Aphanamixis polystachya* for evaluation of phytochemical and pharmacological properties

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Abstract

The present study was conducted to detect possible chemicals (phytoconstituents), and investigate antioxidant, antimicrobial and thrombolytic activities of the extract of *Aphanamixis polystachya* (stem bark). Phytochemical screening was carried out using the standard test methods of different chemical group. For investigating the antioxidant activity, two complementary test methods namely DPPH free radical scavenging assay and total phenolic content determination were carried out. For the evaluation of *in Vitro* antimicrobial activity, disc diffusion method, and to determine the thrombolytic activity, the method of Prasad et al., 2007 with minor modifications were used. The bark extracts were a rich source of phytochemicals. In DPPH free radical scavenging test, the Carbon tetra chloride soluble fraction showed the highest free radical scavenging activity with IC_{50} value 19.86 µg/ml. while compared to that of the reference standards ascorbic acid. *Aphanamixis polystachya* was also found as a good source of total phenolic contents. Moreover, the extracts revealed broad spectrum antimicrobial activity at the concentration of 400 µg/disc. By comparing with the negative control the mean clot lysis % was significant (p value <0.0009). Therefore, further studies are suggested to determine the active compounds responsible for the biological activities of the plant extracts.

Keywords: Phytochemical; Antioxidant; Antimicrobial; Thrombolytic; *Aphanamixis polystachya*

1. Introduction

Nature acts as a nice source of salvation for human being by providing different remedies from its plants, animals and other sources to treat almost all ailments of mankind. Among the natural sources, medicinal plants are important contributors to the medicinal preparations as raw plant materials, refined crude extracts and mixtures etc. Several thousands of plants containing medicinal values have been identified for treating different ailments [1]. Even in this recent time, peoples are still depending on the traditional medicine for their primary health care.[1] New drugs of plant derivation are so much crucial because they are cheap and have little side effects [2]. So medicinal plants are essential. Report shows that more than 80% of the world's population still uses plants to maintain their health and to cure their ailments [3]. It has been studied that fruits and herbs containing phytochemicals and non-nutritive may protect human from a lot of diseases for their biological activities [4]. It is now established and fully believed that phytoconstituents

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obtained from the medicinal plants serve as major and active molecules in the modern medicines [3]. *Aphanamixis polystachya* (*A. polystachya*) belonging to the family Meliaceae is a traditional plant with potential medicinal uses [5]. It is a large handsome evergreen tree, with a dense spreading crown and a straight cylindrical bole up to 15m in height and 1.5-1.8 m in width. It is distributed in the sub-Himalayan tract from Gonda (Uttar Pradesh) to Bengal, Sikkim and Assam [6]. The local names of this plantinclude Roina, Pitraj (Bengali); Rohitak, Pithraj (English), etc [5]. The stem barks of *A. polystachya* is used traditionally for treating tumour, cancer, spleen diseases, rheumatism at Bengal, southeast and north region [6]. The fruits are applied as anthelmintic, laxative, refrigerant and is said to be useful against ulcers and rheumatism [7].

In this study, our main goal was to evaluate possible chemical groups, antioxidant, antimicrobial and thrombolytic activities of *A. polystachya* to validate its use in folkloric treatments. Plants contain various types of phytochemicals or chemical groups and these phytoconstituents are responsible for showing a variety of pharmacological actions in human body [8]. Medicinal plants containing antioxidant property are being considered for use in antioxidant formulations. Antioxidants inhibit free radicals and also minimize oxidative damage, thus decreasing the risk of free radical-induced diseases [9]. A proper balance between antioxidation and oxidation is believed to be critical for maintaining a healthy biological system [10]. Synthetic antioxidants may have adverse effects on human body; hence, much attention has been put toward natural antioxidants. And plants are nice sources of natural antioxidants [11]. Antimicrobial assay procedures for plant extracts provide a valid measure of antibiotic activity. Among the methods, disc diffusion is a simple and convenient method [12]. Thrombosis is a patho-physiological process which is characterized by the development of a blood clot (thrombus) in the circulatory system of the body due to the failure of homeostasis. This condition is responsible for the development of acute coronary disorders such as strokes and heart attacks which are the main causes of morbidity and mortality in developed countries [13]. Therefore, anticoagulation therapy is essential and the proper choice of thrombolytic agents to decrease platelet aggregation can be critical in the management of thrombosis patients [13]. Streptokinase and urokinase are widely used as thrombolytic agents because of their cost effectiveness as compared to other thrombolytic drugs. Recently, the thrombolytic activity of some traditional plants have been studied using an *in Vitro* model and significant thrombolytic activity were found for some plants [14].

The present study was aimed to study antioxidant, antimicrobial and thrombolytic activities of the extract of *Aphanamixis polystachya* (stem bark) and it's fractionate.

2. Material and methods

2.1 Plant materials

For this present investigation, the *A. polystachya* barks were collected from Comilla in December 2015. After collection the taxonomic identification of the plant was carried out with the help of taxonomist of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh (Accession no. 38304). The voucher specimen was also deposited there for future reference.

2.2 Chemicals and Reference drug

For performing these experiments , 1,1-Diphenyl-2-picryl hydrazyl (DPPH), Trichloro acetic acid (TCA), L- Ascorbic acid, Butylated Hydroxy Anisole (BHA), Gallic acid, Folin-ciocalteuphenol reagent, phosphate buffer (pH 6.6), distilled water, streptokinase (30000 IU and 15000IU) of analytical grade (Merck, Darmstadt, Germany) were used. All the chemicals and reagents were purchased from Sigma Chemical Co. Ltd, (St. Louis, MO, USA) and E. Merck (Germany).

2.3 Extraction of plant materials

For methanolic extraction 400 gm of air dried and powdered sample were immersed in 2500 ml of 80% methanol (Merck KGaA, Darmstadt, Germany) in clean, sterilized and flat- bottomed glass container. The container with its contents was sealed and kept for maceration for 15 days accompanying occasional shaking and stirring. At the end of 15th day, the whole mixture was filtered using filter cloth and Whatman® filter paper (Sargent-Welch, USA).The resultant filtrate was then allowed to evaporate in water bath maintained 45oC to dryness and thus a greenish black colored semisolid mass of the extract was obtained (yield 24 gms). This gummy concentrate was designated as crude extract of methanol.

2.4 Solvent-solvent partitioning

Solvent-solvent partitioning was done using the protocol designed by Kupchan and here this protocol is slightly modified [15]. The crude extract (5 gm) was dissolved in 10% aqueous methanol. It was extracted with Petroleum Ether, then with carbon tetrachloride and finally with Chloroform.

2.5 Phytochemical screening

Small quantity of freshly prepared methanolic extract and different fractions of barks of *A. polystachya* were subjected to preliminary phytochemical analysis for the detection of phytochemicals such as alkaloids with Mayer's and Hager's reagent, Carbohydrates with Benedict's test and Fehling's test, glycosides with Legal's test and Modified Borntrager's test, phytosterols with Salkowski's test and Libermann Burchard's test, proteins with xanthoproteic test, flavonoids with alkaline reagent test and lead acetate test, tannins with gelatin test, saponins with Froth test and foam test, phenols with ferric chloride test [16].

2.6 Antioxidant activity

There are various well known methods, which are followed to determine the antioxidant properties. Among them, two complementary test methods namely total phenolic content determination and DPPH free radical scavenging assay methods were used for investigating the antioxidant activity of *A. polystachya.*

2.7 Total phenolic content determination

The amount of total phenolics in extracts was determined with the Folin-Ciocalteu reagent [3]. Here, gallic acid was used as a standard and the amount of total phenolics were expressed as mg/g of gallic acid equivalents (GAE). Concentration of 6.25, 12.5, 25, 50, and 100 µg/ml of gallic acid and concentration of 2 µg/ml of plant extract were prepared in methanol and 0.5 ml of each sample were placed into test tubes and mixed carefully with 2.5 ml of a 10- fold dilute Folin-Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The test tubes were covered with para-film and allowed to stand for almost 30 min at room temperature before the absorbance was read at 760 nm spectrophotometrically (UV-1800, Shimadzu, Japan). All determinations were performed nicely in triplicate [3]. Thus, total phenolic content was determined as mg of gallic acid equivalent per gram using the equation obtained from a standard gallic acid calibration curve.

2.8 DPPH scavenging activity

DPPH radical serves as the oxidizing radical to be reduced by the antioxidant (AH) and as theindicator for the reaction. The stable DPPH radical-scavenging activity was measured using the modified method described by Gupta [17]. In this assay, 2 ml of 0.2 mµ methanolic DPPH solutions was added to 2 ml of extract solution at different concentrations and the contents were stirred vigorously for 15 seconds. Then the solutions were allowed to stand at dark placeat room temperature for 30 min for reaction to occur. After 30 min, absorbance was measuredagainst a blank at 517 nm with a double beam UV/Visible spectrophotometer. The percentageof DPPH radical-scavenging activity of each plant extract was calculated as:

$$DPPH\ radical - scavenging\ activity\ (\%I): \{(A_0\ -\ A)/A_0\}\ \times 100$$

Where,

A_0 is the absorbance of the control solution (containing all reagents except plant extracts);A is the absorbance of the DPPH solution containing plant extract.

The DPPH radical-scavenging activity (%) was plotted against the plant extract concentration to determine the concentration of extract necessary to decrease DPPH radical-scavenging by 50% (called IC_{50}). The IC_{50} value of each extract was estimated by sigmoid non-linear regression.

These values were changed to antiradical activity, defined as $1/EC_{50}$, since this parameterincreases with antioxidant activity. All determinations were performed in triplicate.

2.9 Antimicrobial activity

Two strains of Gram-positive (Staphylococcus aureus, Bacillus subtilis), three strains of Gram-negative bacteria (Escherichia coli, Pseudomonas aeruginosa, Salmonella enteric), and two strains of fungi (Candida albicans, Aspergillus

alliaceus) were used to evaluate the antimicrobial activity. The organisms were subcultured properly in nutrient broth and nutrient agar. They were collected from the, Chittagong Veterinary and Animal Sciences University (Department of Microbiology), Bangladesh.

For the evaluation of antimicrobial activity, disc diffusion method is widely acceptable]18]. In this classical method, antibiotics were diffused from a reliable source through the nutrient agar and a concentration gradient was created. Dried, sterilized filter paper discs (6 mm diameter, HI-Media, China) containing the test samples of known amounts (400 µg/disc) were placed on nutrient agar medium consistently seeded with the test microorganisms. Standard antibiotic of ciprofloxacin (5 µg/disc) and blank discs were used as positive and negative control. For the maximum diffusion of the test materials to the surrounding media, these plates were kept at low temperature (4 °C) for 24 h**.** Then the plates were incubated at 37 °C for about 24 h to allow optimum growth of the organisms. The test materials with antimicrobial property inhibited microbial growth in plates and thereby yielded a clear, distinct zone defined as zone of inhibition. The antimicrobial activity of the test sample was then determined nicely by measuring the zone of inhibition expressed in millimeter [3].

2.10 Thrombolytic activity

In Vitro clot lysis activity of *A. polystachya* was carried out according to the method of Prasadet al., 2007 with minor modifications [19].

2.10.1 Streptokinase (SK)

To the commercially available lyophilized S-Kinase™ (Streptokinase) vial (Batch no: VEH 09, Popular Pharmaceuticals Ltd., Bangladesh) of 15, 00,000 I.U., 5 ml 0.9% sodium chloride(NaCl) was added and mixed properly. Then the solution was diluted up to 300000IU and 15000IU conc. which was used as the reference standard for thrombolytic activity [19].

2.10.2 Specimen

Venous blood (5 ml) was drawn from healthy human volunteers (n =10) without a history of oral contraceptive or anticoagulant therapy (using a protocol approved by Institutional Ethics Committee). A consent form was filled up for every volunteer before collecting the blood. 500 µl of blood was transferred to each of the previously weighed micro centrifuge tubes to form clots [19].

2.10.3 Preparation of sample

The prepared 0.9% NaCl solution was used to make different concentrations of plant extract.

2.10.4 Study design

Venous blood drawn from healthy volunteers (n = 10) was immediately citrated using 3.1% sodium citrate solution and then was transferred in different pre-weighed sterile micro centrifuge tube (500 µl/tube). Two hundred microlitre of 2% calcium chloride was then addedto each of these tubes, mixed well and incubated carefully at 37°C for 45 minutes for clotting to occur. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed) and each tube having clot was again weighed consciously to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). Each micro centrifuge tube containing clot was properly labeled and five hundred microlitre of different concentrations of the plant extract such as 5 mg/mL (n = 10), 10 mg/mL (n = 10) and 20 mg/mL (n = 10) or saline (negative control) (n =10) or 30,000 I.U. or 15000IU reference drug (n = 10)] was added to tubes with clots. All the tubes were incubated at 37ºC for almost 90 min. The fluid left was then carefully removed and the tubes were weighed again properly. The difference in weight before and after clot lysis was expressed as % clot lysis [19]. The result was expressed as percentage of clot lysis following the underneath equation.

$$\% \text{ of clot lysis } = \frac{\text{wt.of released clot}}{\text{clot wt.}} \times 100$$

2.11 Statistical Analysis

The results are expressed as mean ± SEM. Statistical comparisons were made using one-way ANOVA with Dunnett t test. Significance was set at $p < 0.05$. Dose dependencies were determined by the regression coefficient (r).

3. Results

3.1 Phytochemical screening

The phytochemical screening of *Aphanamixis polystachya* bark extracts of methanol, pet ether, carbon tetra chloride and chloroform solvent showed varied results. The results are shown in Table-1.

Table 1 Preliminary Phytochemical screening of methanolic extract and different fractions of barks of *A. polystachya*

Serial	Test for	Crude methanolic extract	Pet ether extract	Carbon tetrachloride extract	Chloroform extract
1	Reducing sugar	+	-	+	+
2	Tannin	+	+	-	+
3	Flavonoids	+	+	+	+
4	Saponins	+	-	-	-
5	Tarpenoids	+	+	+	+
6	Alkaloids	+	-	-	+
7	Glycosides	-	-	-	+
8	Phytosterols	+	-	-	+

Key: (+) = Present and (−) = Absent

Here, in every extracts flavonoids and terpinoids were found. Glycosides were presented in chloroform extract only. Reducing sugar was not found in pet ether extracts but in other three extracts. Saponin was also found in methanolic crude extract only and alkaloids were not found pet ether and carbon tetra chloride extracts.

3.2 Total phenolic content determination

The methanolic crude extracts of bark of *A. polystachya* and their different soluble fractions i.e. pet ether, chloroform and carbon tetra chloride were subjected to total phenolic content determination. Based on the absorbance values of the extract solution, the colorimetric analysis of the total phenolic of the extracts were determined consciously and compared with the standard solutions of gallic acid equivalents (Table 2 and figure 1). Total phenolic content of the samples are expressed as mg of gallic acid equivalent (GAE)/ gm of dry extract. *A. polystachya* was found as a good source of total phenolic contents where crude methanolic, pet ether, carbon tetra chloride and chloroform extract showed total phenolic contents of 19.73µg of GAE / mg,16.075µg of GAE / mg, 25.75µg of GAE / mg and 7.65µg of GAE / mg extracts respectively.

Table 2 Results of Total Phenolic Content of *A. polystachya* barks extracts

***A. polystachya* Bark**	**Absorbanceat 760 nm**	**Total Phenolic Content (mg of GAE / gm) ofExtracts**
Methanolic Crude Extract (250 µg/ml)	0.831	19.73
Pet Ether Extract (250 µg/ml)	0.685	16.075
Chloroform Extract (250 µg/ml)	0.348	7.65
Carbon tetra Chloride Extract (250 µg/ml)	1.072	25.75

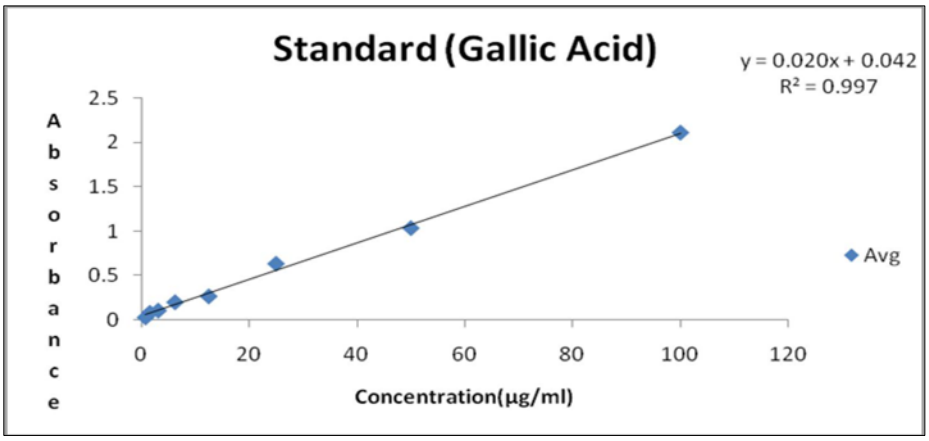

Figure 1 Total Phenolic Content of Gallic Acid (Standard)

3.3 DPPH scavenging activity

DPPH free radical scavenging activity of crude extracts of bark of *A. polystachya* and their different soluble fractions were found to be increased with the increase of concentration ofthe extract (Table 3). Different partitions of methanolic extract of *A. polystachya* were subjected to free radical scavenging activity. Here, Ascorbic acid was used as reference standard. In this investigation, the Carbon tetra chloride soluble fraction showed the highest free radical scavenging activity with IC_{50} value 19.86 µg/ml. At the same time the Pet ether soluble fractions, aqueous and chloroform fractions also exhibited moderate antioxidant potential having IC50 value 82.69µg/ml, 233.17µg/ml and 195.28µg/ml respectively. Also crude methanolic extracts showed IC_{50} value 44.43 µg/ml.

Table 3 Comparative DPPH radical scavenging activity of different extracts of the bark of *A. polystachya* and standards of Ascorbic Acid (AA)

% Inhibition of different solvent extract and Standard						
Concentration (µg/ml)	**Methanolic Crude Extract**	**Carbon tetra Chloride Extract**	**Pet Ether Extract**	**Chloroform Extract**	**Aquous Extract**	**Ascorbic Acid (Standard)**
5	7.31	30.02	20.03	2.03	1.96	41.66
10	14.71	38.20	25.63	4.31	5.61	49.46
20	18.02	45.89	29.14	13.25	12.21	54.29
40	50.06	48.69	32.61	17.42	18.06	56.85
60	59.31	65.20	39.25	22.34	21.64	59.03
80	69.96	85.45	46.18	30.24	29.99	65.44
100	81.26	87.31	52.36	36.25	36.25	72.29
250	83.21	95.81	65.31	51.64	51.64	83.96
500	92.99	97.30	82.54	80.32	70.56	97.40
IC_{50} (µg/ml)	44.43	19.86	82.69	193.28	233.17	14.52

3.4 Antimicrobial activity

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms (at concentrations of 400 µg/disc.) surrounding the discs whichgives clear zone of inhibition. After incubation, the antimicrobial activities of the test materials such as methanolic crude extract of *A. polystachya* (MEAP), pet ether fraction of *A. polystachya* crude extract (PEAP), chloroform fraction of *A. polystachya* crude extract (CAP), carbon tetra chloride fraction of *A. polystachya* crude extract (CTAP) and aqueous fraction of *A. polystachya* crude extract (AEAP)

were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale (Table 4). Here, Standard antibiotic disc of ciprofloxacin (5 µg/disc) was used for the comparison.

Table 4 Results of *in Vitro* Antimicrobial Screening of *Aphanamixis polystachya*

Types of micro organism		Samples of *A. polystachya* inhibition zone diameter (mm /sample)					Ciprofloxacin
		MEAP	PEAP	CLAP	CTAP	AEAP	
Gram PositiveBacteria	*Bacillus subtilis*	16	8	12	9	13	34
	Staphylococcus aureus	12	7	11	8	12	30
Gram Negative Bacteria	*Pseudomonas aeruginosa*	13	8	9	11	8	36
	Salmonella enterica	17	6	8	7	6	25
	Escherichia coli	10	9	12	6	10	28
Fungi	*Candida albicans*	15	7	10	8	11	32
	Aspergillusallia ceus	14	8	11	7	7	30

The methanolic extract exhibited significant activity against the growth of the most of the test organisms (Table 4), while the carbon tetra chloride extracts, aqueuous extract and chloroform soluble extracts exhibited moderate inhibitory activity against most of the microorganisms and pet ether extract exhibited less than moderate inhibitory activity against most of the microorganisms (Table 4). The zones of inhibition produced by crude methanolic extract were ranged from 10-17 mm, while the carbon tetra chloride soluble fractions showed 7.0-11 mm, aqueous fractions showed 6-13 mm and chloroform soluble fractions showed 8-12 mm at a concentration of 400 µg/disc. The pet ether soluble fraction of the methanolic extract exhibited lowest inhibitory activity against tested bacteria and fungi.

3.5 Thrombolytic potential

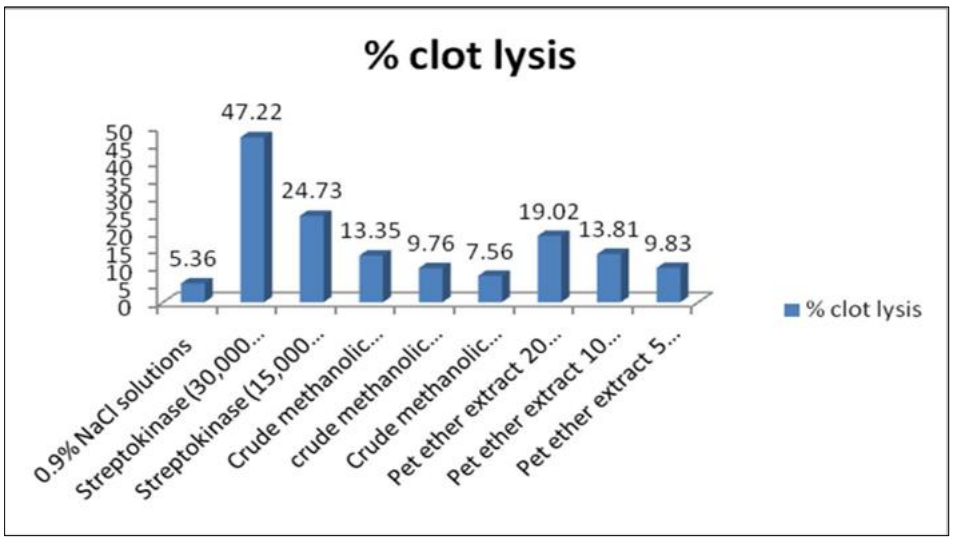

Figure 2 Clot lysis of streptokinase, Nacl and various concentrations of *A. polystachya*. Maximum clot lysis was observed in a clot treated with streptokinase. Crude methanolic extract and pet ether extract of *A. polystachya* at 20,10 and 5 mg/ml showed 13.35, 9.76, 7.56, 19.02, 13.81, 9.83 % respectively

Streptokinase (30000 and 15000 I.U.) as a positive control to the clots along with 90 minutes of incubation at 37 degree centigrade, showed 47.22 and 24.73% clot lysis respectively. Clotswhen treated with 100 microlitre 0.9% NaCl solutions (negative control) showed only 5.35% blood clot lysis. The mean difference in clot lysis percentage between positive and negative control was very significant (p value <0.0009). After treatments of clot with 100 microlitre ofcrude

methanolic extract, petroleum ether extract of *A. polystachya* in the concentration of 20, 10, 5 mg/ml showed varying clot lysis i.e., 13.35, 9.76, 7.56% and 19.01, 13.81, 9.83 % respectively. By comparing with the negative control the mean clot lysis % was significant (p value <0.0009). Percent clot lysis obtained after treating clots with different concentration of *A. polystachya* and appropriate control are shown in figure 2.

4. Discussion

Medicinal plants containing phytochemicals are well known because they show a variety of pharmacological actions in human body and in our study preliminary phytochemical screening showed the presence of various phytochemicals [20].

The presence of polyphenolic compounds such as flavonoids and tannins are responsible for antioxidant activity of the extracts [9,10]. In this investigation the extract of the plant showed moderate antioxidant activities with an IC_{50} which were compared with the values of standarddrugs used. Antioxidant activities of different extracts of the bark of *A. polystachya* was foundto increase with the increasing concentration. This plant is a good source of total phenolic content. It also contains moderate DPPH scavenging activity although carbon tetra chloride soluble fraction showed highest free radical scavenging activity. The effect of antioxidants of plant on DPPH is thought to be due to their hydrogen donating ability [18]. Therefore, the extracts of this plant could be used potentially for the prevention of free radical-mediated diseases. Since the present study showed the presence of various bioactive secondary metabolites such as tannins, flavonoids, saponin and alkaloids, that singly or in combination may be responsible for the defense mechanism against microorganisms and insects [3]. For this reason, methanolic extract contains significant antimicrobial activity.

Platelets play an important role in the process of formation of thrombus on the endothelial surface [13]. Several thrombolytic agents are used to dissolve the clots that have already formed in the blood vessels; but these drugs have some limitations which can lead to serious and sometimes fatal consequences [21]. In the present study, the plant extracts showed significant thrombolytic activity compared to negative control. This thrombolytic activity may be due to the fact that the extracts are good sources of alkaloids, flavonoids, tannins and terpenoids which are said to exert clot lysis [21].

5. Conclusion

In the context of the above discussion, it can be revealed that the extracts of bark of *A. polystachya* showed nice antioxidant activity. These extracts also possess significant antimicrobial and clot lysis activity. However, further investigations, based on these preliminary studies are required to explore the bioactive molecules which are responsible for the extracts' activities as well as their mechanisms of action.

Compliance with ethical standards

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Disclosure of conflict of interest

There is not any conflict of interest.

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